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# Hollow Fiber Based Liquid Phase Microextraction Combined High Performance Liquid Chromatography for the Determination Trace Amounts of Zopiclone in Biological Fluids

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**ABSTRACT**

Zopiclone is used to treat sleeping problems (insomnia). In the current work, for the first time, a three-phase hollow-fiber liquid-phase microextraction combined with HPLC-UV was proposed for the determination of zopiclone in urine samples. Different factors that can affect the extraction process such as extraction solvent, acceptor and donor phase composition, salt addition, stirring rate, extraction time and extraction temperature were optimized. Under the optimum conditions, detection and quantitation limits obtained53.9 and 161.7ng $mL^{-1}$, respectively, and enrichment factors 116 were obtained. The calibration curves were linear within the range of 161.7-2000 ng $mL^{-1}$ with estimation of coefficient higher than 0.9990. Within a day and between day RSDs were 5.51% and 5.34%, respectively. Finally, the proposed method was applied to the detection and determination of zopiclone in human urine sample.

**Key words***:* Zopiclone; High performance liquid chromatography; Hollow ûber based liquid phase microextraction; Microextraction.

## INTRODUCTION

Zopiclone is used to treat sleeping problems (insomnia)[1-5]. The chemical structure and phisico-chemical properties of the drug are summrized in Table 1[6]. Zopiclone belongs to a class of medicines commonly called Z drugs. It works by acting in the way messages are sent to your brain, which help you to sleep. It reduces the time it takes for you to fall asleep and increases the length of time you spend sleeping[7]. Along with their useful effects, most medicines can cause unwanted side-effects, although not everyone experiences them. Daytime drowsiness, dizziness, lightheadedness, bitter taste, dry mouth, headache or stomach upset may occur the first few days as you took the medication[8]. Several methods have been presented for detection and determination of zopiclone until now. There are some methods for determination of zopicloneconcentration in biological fluids including liguid chromatography[9-15], Liquid chromatography–mass spectrometry[16], HPLC using fluorescence detection [17], capillary electrophoresis[18-20], gas chromatography[21, 22], spectrophotometric techniques[23] and voltametrics methods[24]. In all of reported methods, for determination of the zopiclone in biological samples, sample preparation steps are necessary. Additionally, in some cases protein precipitation and derivatization steps should be used[19]. Therefore, it is necessary to introduce a new, fast, simple and sensitive method that improves detection and determination of zopiclone in biological fluid samples.

Tothe best of our knowledge, microextraction technique has not been introduced for extraction and preconcentration of zopiclone from biological fluids. for the first time, in this work, three phasehollow ûber based liquid phase

microextraction (HF-LPME) followed by HPLC with ultraviolet (UV) detection was optimized and validated for detection and determination of zopiclone in biological samples.

For the first time, LPME was reported by Dasgupta[25] and Cantwell[26] in 1996, which is a novel miniaturized sample preparation technique taken from traditionalliqid-liquid extraction. Hollow fiber liquid phase microextraction (HF-LPME) as one of LPME methods was introduced in 1999[27, 28]. HF-LPME is easy, cheap and environmentally friendly which allows multiple sample processing steps, for example, extraction, clean up, and analyte enrichment to be performed in a single step[29]. HF-LPME divided into two-phase HF-LPME and three-phase HF-LPME. In three-phase hollow fiber extraction, analytes are extracted from an aqueous sample into an organic phase, and then back extracted into a separate aqueous phase[30-37].

In the current work, the efficacy of different variables on HF-LPME efficiency were investigated and optimized. After optimization, the method followed by HPLC-UV was used for extraction and determination of zopiclone in urine sample.

## EXPERIMENTAL

### Chemicals, standard and stock solutions

Zopiclone standard wasobtained from Drug and Food Administration (Tehran, Iran).All of chemicals used were of analytical-reagent grades or better. 1-Octanol, *decanol*, isobutyl*methyl ketone,* n-heptan all from Merck (Darmstadt, Germany), were used as organic membrane solvents.HPLC grade acetonitrile and methanolwere purchased from merck.Sodium hydroxide and sodium chloride were obtained from Sigma–Aldrich (St. Louis, MO, USA).

A PPQ3/2 Accurel polypropylene hollow fiber from Membrana (Wuppertal, Germany) with a pore size of 0.2 µm, the inner diameter of 0.6 mm and wall thickness of 200 µm was used for the extraction process.Ultrapure water was obtained from a Milli-Q water purification system from Millipore (Madrid, Spain).

A 1000 mg $L^{-1}$ stock solution of zopiclone was prepared in methanol and standard working solutions were prepared by spiking the proper amount of the stock solution in pure water.

### Instruments and equipment

AYounglin YL9100 HPLC (Seoul, Korea) containing a Quaternary9110 HPLC pump (Seoul, Korea), a 4-channel mixing valve with a 20 µL sample loop, YL9101 vacuum degasser and a YL 9120 UV-Vis detectorwas usedfor separation and detection of the target analyte. Chromatography data were recorded and analyzed using Younglin Auto Chro 3000 software.The separations were performed on an ODS-3 column (150 mm × 4.6 mm, with particle size of 5 µm) from MZ-Analysentechnik (Mainz, Germany).The mobile phase consisted of a mixture of methanol and acetonitrile (35:65), under isocratic condition. The flow rate of the mobile phase was set at 1.2 mL $min^{-1}$. Total analysis time was 8 min.The injection volume was 20 µL for all of the samples and detection was performed at a wavelength of 304 nm.

A MR Hei-standard magnetic stirrer from Heidolph (Schwabach, Germany) was used for agitation of sample solution. GPHR 1400 digital pH meter from Greisinger (Regenstauf, Germany) was used for pH measurements.

### Extraction procedure

A fresh 8.0 cm length of hollow ûber was cut and washed with acetone in an ultrasonic bath for 10 min and dried at room temperature. Fifteen milliliter of sample solution was ûlled into a 20 mL vial which was placed on a magnetic stirrer plate for agitation of sample solution during the extractions. Extraction process was shown in Fig. 1. The hollow ûber was placed at the end of 100 µL Hamilton syringe needle that filled with acceptor phase, and subsequently dipped for a 10 s period into the organic solvent (1-Octanol) used for impregnation. After impregnation, excess amount of organic solvent washed with distilled water for 30 s, and 20 µL of acceptor solution with pH=3.0 was injected into the hollow ûber. First, 80 ìL of acceptor solution was flushed out of the fiber in order to remove any organic solvent remaining inside the lumen of hollow fiber, and then 20 ìL

acceptor solution was remained in the lumen of hollow fiber, and the lower end of the hollow fiber was mechanically sealed by means of a piece of aluminum foil. Subsequently, the ûber was placed in the sample solution. During extraction, the solution was stirred at 1000 rpm. After extraction, the acceptor solution was collected into a micro-vial by Hamilton syringe and ûnally, 20 µL of acceptor solution was injected into the HPLC instrument for analysis.

**Real sample analysis**

A urine sample was collected from healthy young volunteer. The sample was stored at "4ÚCin the dark until analysis without further sample pretreatment and thawed and shaken before extraction

**Calculation of preconcentration factor**

The preconcentration factor (*PF*) was defined as the ratio of the final analyte concentration in the acceptor phase ($C_{f,a}$) and the initial concentration of analyte ($C_{i,s}$) in the sample solution:

$$PF = \frac{C_{f,a}}{C_{i,s}} \quad ...(1)$$

where $C_{f,a}$ was calculated from a calibration graph obtained by direct injection of analytes standard solutions.

## RESULTS AND DISCUSSION

**Selection of the extraction solvent**

The selection of a suitable extraction solvent is a important step in HF-LPME for achieving high extraction efficiency. The composition of chosen solvent should be compatible with the used fiber, immiscible with acceptor and donor phases, have low volatilityin order to prevent loss of solvent during the extraction, and should also have good affinity for

**Table 1: Chemical structures, p$K_a$ and log*P* of zopiclone**

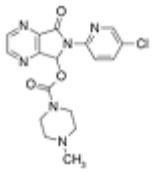
| Name | Chemical structure | IUPAC name | p$K_a$ | log*P* |
|---|---|---|---|---|
| zopiclone |  | (*RS*)-6-(5-chloropyridin-2-yl)-7-oxo-6, 7-dihydro-5H-pyrrolo[3,4-b]pyrazin-5-yl 4-methylpiperazine-1-carboxylate | 6.89 | 0.81 |

**Table 2: Figures of merit of HF-LPME in drug distilled water sample**

| LOD (µgmL$^{-1}$) | LOQ (µg mL$^{-1}$) | Linearity (µgmL$^{-1}$) | $R^2$ | PF[a] | RSD% [b] | | |
|---|---|---|---|---|---|---|---|
| | | | | | Within day | Between day | R% |
| 5.39*10$^{-6}$ | 16.17*10$^{-6}$ | 0.01-2 | 0.9990 | 116 | 5.51 | 5.34 | 15 |

[a] Drugs were present at 1.0µg mL$^{-1}$.

[b] Within day and between day RSDs% were obtained by four replications.

**Table 3: Determination of zopiclone in urine sample**

| Sample | $C_{real}$(µg mL$^{-1}$) | $C_{added}$(µg mL$^{-1}$) | $C_{found}$(µg mL$^{-1}$) | RSD% (n = 4) |
|---|---|---|---|---|
| Urine | nd[a] | 1 | 0.4 | 3.4 |

[a] Not detected

the analytes[38]. In the current work, four different organic solvent,1-Octanol, n-*decanol*, isobutyl *methyl ketone*and n-heptanewere tested as extraction solvent. The corresponding results obtained for the extraction solvents studied are shown in Fig. 2A. As shown in Fig. 2A, the target analyte exhibited the highest peak area when 1-octanol was used. Therefore, 1-octanol was chosen as the extraction solvent for subsequent analysis.

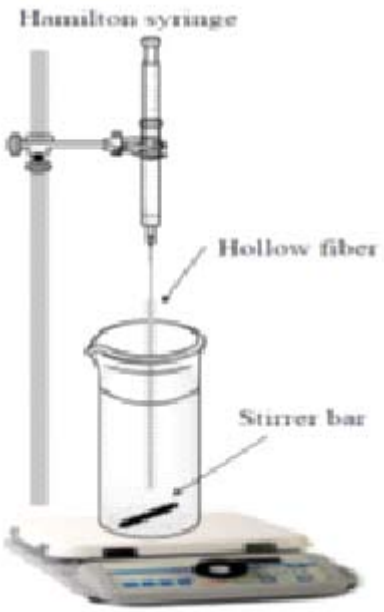



**Fig. 1: Schematic diagram of proposed HF-LPME setup**

**The pH in sample solution and acceptor phase**

Zopiclone is a weak basic drug with pKa value about 6.89. In order to obtain high extraction efficiency of the target analytes by the HP-LPME, they should be first transformed to their neutral molecular forms, which could be done by the addition of sodium hydroxide to the samples. This step dramatically facilitates the diffusion of the zopiclone through the liquid membrane and then permeation to the acidic acceptor solution, where they will be irreversibly trapped because of the formation of the resulting ionic species. In order to investigate the effect of the donor phase pH, donor pH was tested in the basic pH from 7.0 to 12.0. As shown in Fig. 2B, the highest extraction efficiency for zopiclone was obtained using pH=9.0. Therefore, pH=9.0 was used for subsequent experiments.

To extract basic compounds, the acceptor phase should be acidic to provide high solubility for basic analyte and to ionize them to prevent reentry into the organic phase. For this purpose, pH of acceptor phase was studied from two to four. Results in Fig. 2C show that highest extraction efficiency was obtained at pH=3.0. Therefore, pH=3.0 was used for subsequent experiments.

**Table 4: Comparison of the HF-LPME with other analytical techniques for determination of zopiclone**

| Analytical method | Sample preparation method | Sample | LOD (µg mL-1) | Linearity (µg mL-1) | RSD% | Ref. |
|---|---|---|---|---|---|---|
| HPLC | HF-LPME | Urine | 5.39*10-6 | 10-2000 | 5.34 | This work |
| GC-MS | LLE | Plasma | 0.4 | 0.002 -1.3 | 4.6 | [39] |
| CE | LLE | Urine | R(-)0.509 | | | |
| R(+)0.501 | 0.57-30 | R(-)5.5 | | | | |
| R(+)6.6 | [40] | | | | | |
| HPLC-UV | LLE | Tablet | 0.02 | 1-6 | 6.7 | [40] |
| ADSV | - | Urine | 2.78*10-7 | 6*10-7-2*10-5 | 1.0 | [41] |
| HPLC-ESI-MS-MS | SPE | Plasma | 0.98*10-3 | 0.5*10-3-0.15 | 9.7 | [42] |
| HPTLC | - | Serum | 52.8*10-2 | 0.1-0.4 | 1.49 | [43] |

**Effect of stirring rate**

The stirring rate is another significant parameter that can greatly influence the extraction efficiency. Agitation of the sample makes the mass transfer equilibrium between the phases faster and hence reduces extraction time. Stirring facilitates analyte diffusion from donor phase into the acceptor phase. In this work, the effect of the stirring rate

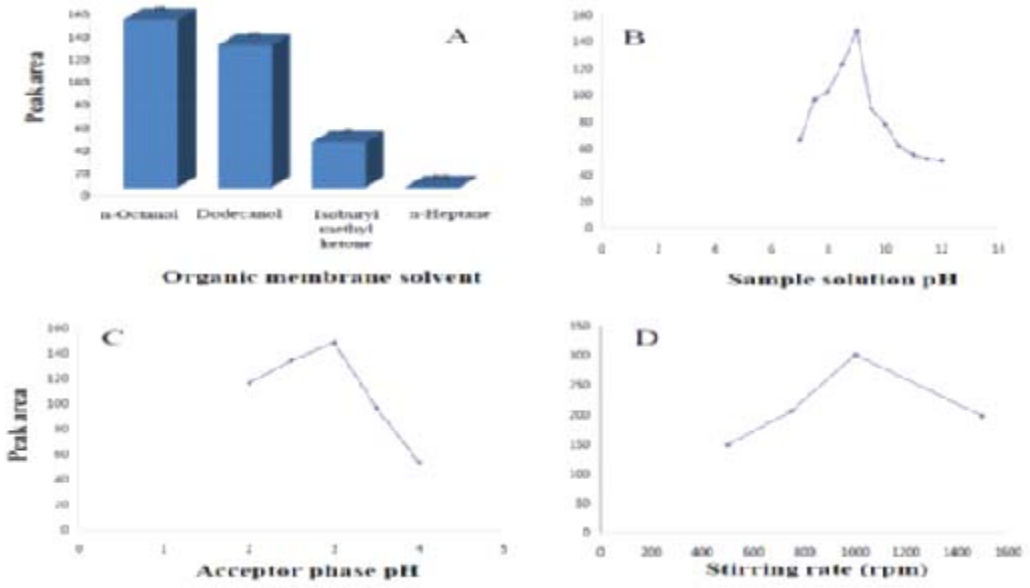



**Fig. 2: Optimization of (A) organic membrane solvent, (B) sample solution pH, (C) acceptor phase pH and (D) stirring rate for extraction of zopiclone**

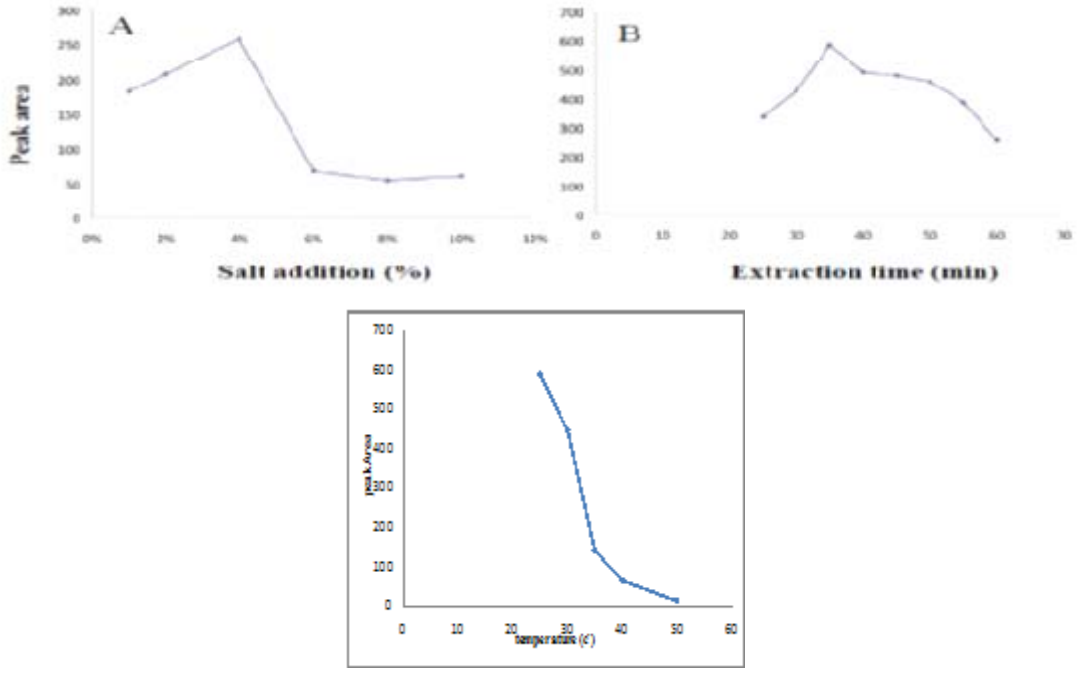



**Fig. 3: Optimization of (A) salt addition effect, (B) extraction time and (C) temperature for extraction of zopiclone**

onthe extraction efficiency was tested in the range of 500 to 1500 rpm. It can be seen that when the stirring ratewas increased from 500 to 1000 rpm, the extraction efficiency oftarget analytewas enhanced. However, another increasing in stirring rate over 1000 rpm decrease extraction efficiecye due to the loss of organic phase and air bubble formation around the hollow fiber under the higher stirring rate.. Hence,according to Fig. 2D, astirring rate of 1000 rpm was selected as the optimum stirring rate for nextexperiments.

**Effect of salting-out**

Commonly, the addition of a small amount of salt to the sample solution produces a salting-out effect that decrease the solubility of analytes in the aqueoussolution. However, by the addition of salt, the aqueous solution viscosity would increase, which lead to difficult mass transfer and decrease extraction efficiency. In current work, the effect of different concentrations m/v) of NaCl in the range of 0% to 10% to extract target analytewas tested. As shown inFig. 3A, Theextraction efficiency increased when the NaCl concentration was increased from 0 to 4% (w/v). Over4% salt addition, decreased extraction efficiency. Thus, 4% (w/v) addition of salt was selected for the subsequent experiments.

**Effect of extraction time**

The extraction time is an important parameter in HF-LPME procedure. HF-LPME is a non-exhaustive extraction method as the analytes are partitioned between the donor and the acceptor phases until the equilibrium is established. In current work, the efficiency of extraction time in zopiclone extraction was tested in the range of 20 to 60 min.as shown in Fig. 3B,the extraction efficiency increased dramatically with the increasing of the extraction time up to 35 min. Another increasing in the extraction time to 60 min,lead to rapidly decrease in the extraction recovery due to organic membrane dissolution in the sample solution. Therefore, 35 min was selected as the best extraction time in the subsequent experiments.

**Effect of temperature**

The effect of temperature on the extraction efficiency of zopiclone by HF-LPME was investigated over a temperature range of room temperature to 50 °C.Increasing of temperature from 4 to 25 °C increase extraction efficiency, butwhen the extraction temperature was higher than 25 °C, decreased the extraction efficiency seriously due

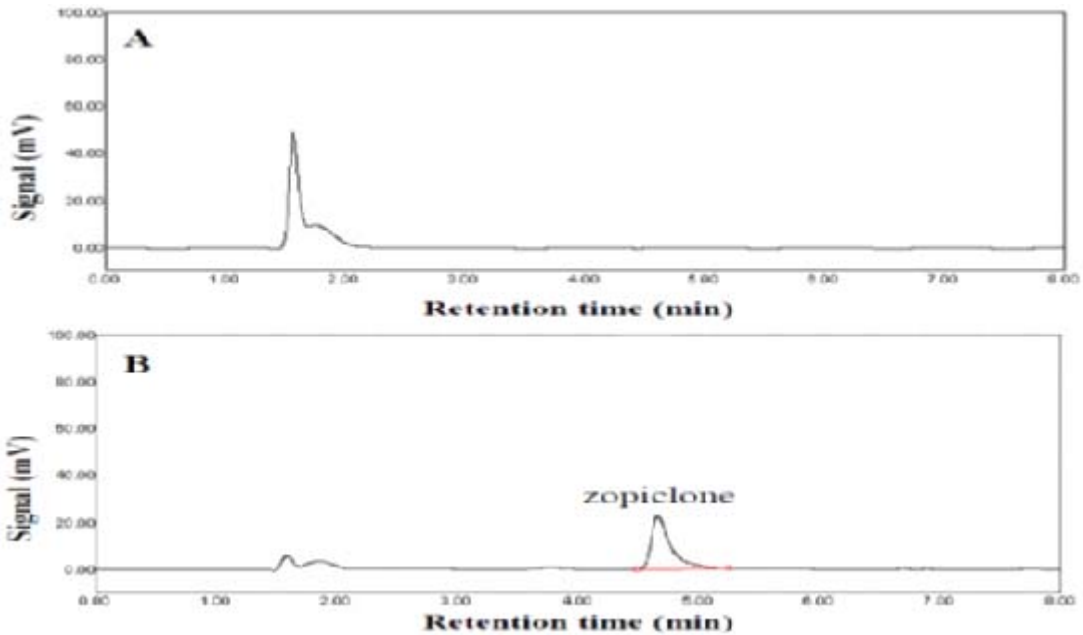



**Fig. 4: Chromatograms obtained after HF-LPME extraction of urinesample ((A) non-spiked sample and (B) spiked sample at a concentration level of 1.0 mg $L^{-1}$)**

to air bubble formation around the hollow ûber. Therefore, the temperature of sample temperature of 25 ºC was selected as the optimum temperature in current work.As shown in Fig. 3B,

As a consequence, the optimal conditions were attained by using pH=3.0 and pH=9.0 as theacceptor and donor phases composition, respectively and using 1000 rpm for agitation for 35 min. Additionally, the organic membrane composition was 1-Octanol. Fifteen milliliter of sample solution, 4% addition of salt and sample temperature of 20 °C was selected as the optimal condition for zopiclone extraction.

**Validation of the proposed method**

To evaluate the practical applicability of the proposed HF-LPME method, figures of merit were studied using standard solutions of the analyte in a drug-free urine samples. Optimal condition was applied to find out linearity, repeatability, and LODs of this method. The figures of merit for the optimized method are summarized in Table 2.The calibration curvewas linear in the range of 161.7–0000 µg $L^{-1}$ with coefûcient of determination ($r^2$)more than 0.9990. The relative standard deviations (RSD %) for extraction and determination of zopiclone were less than 5.51% and 5.34% for within day and between day experiment, respectively. LODs less than 53.9 µg $L^{-1}$ wasviewed for target analyte. PF values higher than 116-fold were obtained for the extraction of zopiclone at the concentration level of 1.0mg $L^{-1}$. The LOD and LOQ were calculated as 3× S/N and 10× S/N, respectively.

**Analysis of real sample**

HF-LPME is a powerful method for detection and determination of zopiclone from untreated biological fluids. Therefore, the optimal conditions of HF-LPME were used for extraction of zopiclone from human urine sample. To reduce matrix effects calibration curve wasplotted in drug free urine sample.

Drug-free human urine sample was spiked with the target drug and extraction was accomplished after dilution of urine samples (1:3) and the addition of proper amount of NaOH solution to achieve pH 9.0. The results are summarized in Table3. RSD% values less than 3.4% confirm the acceptable precision of proposed HF-LPME method. Fig. 4 shows typical chromatograms of blank and spiked urine sample after applying the proposed HF-LPME method.

In comparison to other alternative methods recently reported for the determination of zopiclone in biological samples, the proposed method has in general, lower LOD, higher PF, good precision, suitable linear dynamic range and sensitivity. Comparison results of the proposed method with different existing methods is provided in Table 4.

## CONCLUSIONS

In the current work, for the first time, we have developed a three-phase HF-LPME method combined with HPLC–UV for the sample preparation and determination of zopiclone in urine sample. The optimized three phase HF-LPME method provides enrichment of the analyte and cleanup processes in a single step, and the final extract being compatible with direct injection into the HPLC instrument. Acceptable LODs and RSDs, high preconcentration factor and extraction efficiency, and good linearity ranges were obtained with the proposed method. The developed method is a simple alternative that can be successfully applied for detection and determination of zopiclone in biological samples.

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