

Long-term stability study on a *Perna perna* mussel candidate reference material

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Abstract The long-term stability study, one of the steps in the characterization of new reference materials, is performed on freeze-dried biological reference materials to verify if they may be stored at room temperature without modification of the property values of interest. In this study, instrumental neutron activation analysis was used in a 12-month long isochronous long-term stability study performed on a *Perna perna* mussel candidate reference material. No trend in the results was observed in respect to measurement order and exposition period to room temperature. The assessment of the analysis of variance test as well as the normalized results to the control temperature showed no systematic changes in the mass fractions of Ag, As, Br, Co, Cr, Cs, Fe, Eu, La, Na, Rb, Sc, Se, Th and Zn during the test period. The result showed that the candidate reference material may be stored at room temperature without significant changes in composition for the determined elements.

Keywords Reference material · Stability study · Mussel · *Perna perna* · Neutron activation analysis · Trace elements

Abbreviations

ANOVA	Analysis of variance
a	Angular coefficient
b	Linear coefficient
CI	Confidence interval
F	F test statistic
F_c	F distribution critical value
H_0	Analysis of variance null hypothesis
INAA	Instrumental neutron activation analysis
MS	Mean square of the ANOVA test
n	Number of determinations
p value	Probability value
Q_T	Ratio of mass fractions
R^2	Linear fit determination coefficient
s_T	Standard deviation
t	Student's t distribution upper critical value
U	Expanded uncertainty
u_a	Uncertainty in the angular coefficient a
u_{ts}	Long-term stability contribution to the uncertainty of the certified value
u_T	Measurement uncertainty
w	Mass fraction
w_T	Mass fraction at temperature T
α	Level of significance
ν	Degree of freedom

Introduction

Due to their intimate contact with sea water, mussels may accumulate toxic trace elements which are a concern from a nutritional point of view. However, mussels are resistant to deleterious effects and remain alive in polluted environments. This fact is used in water quality monitoring studies as toxic elements bioaccumulate in the tissues of the animals [1, 2].

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To improve the confidence on biomonitoring measurement results and to make it possible to compare results from different locations in time, various quality assurance procedures such as the use of certified reference materials are needed [3]. Stability study is one of the steps in the characterization of new reference materials and it is composed of short-term and long-term stability studies [4]. The short-term stability study is related to the definition of transport conditions in which the material will not suffer degradation. On the other hand, the long-term stability study is used to verify if the new reference material may be stored at defined conditions such as room temperature, as temperature is a key factor in biological material degradation. The output of the stability studies are also used for estimating the contribution of residual instability to the uncertainty of the certified value of reference materials.

The short-term stability study of a new Brazilian mussel candidate reference material has been previously presented [5]. In this study, the mussel candidate reference material was submitted to an isochronous long-term stability study after bottling and gamma ray sterilization, following the premises of ISO Guide 35 [4]. In the isochronous design, the analyses are performed only after all samples are submitted to the conditions of the test. In this case, the measurements may be accomplished in a situation closer to repeatability conditions than in a traditional design, resulting in more precise conclusions from the measurements [6].

In order to implement the long-term stability study, bottles of the material are kept at 20 °C for various time periods up to 12 months. After the test periods, the mass fractions (*w*) of Ag, As, Br, Co, Cr, Cs, Fe, Eu, La, Na, Rb, Sc, Se, Th and Zn are determined by instrumental neutron activation analysis (INAA). To verify if the material may be stored at room temperature, a comparison of element mass fractions obtained for the bottles to the ones obtained for a bottle kept at the control temperature (−20 °C) is performed. This temperature was chosen as the control temperature for the test, since biological reference materials are usually stable for element content at this temperature.

To exclude the possibility of trends in the results during the measurement campaign, a trend analysis is performed by ordering the results as they are obtained. A linear regression is fitted to the results using the method of least squares and a significance test is performed for the linear regression coefficients. From the regression statistics, it is possible to test the hypothesis that none of the coefficients are significant, i.e., $a = 0$ and $b = \bar{w}$, where a is the angular coefficient (slope), b is the linear coefficient (intercept) and \bar{w} is the mean value of the measured mass fractions [7]. If any trend is detected, the analytical method is not suitable for stability testing [4]. In the case that no trend in the measurement campaign is detected, it is necessary to check for trends according to the periods of exposition at the testing temperature. If any trend is observed, then the material may not be considered stable for the test temperature. For this test, bottle results are ordered according to test temperature exposition periods prior to testing for significance of the linear fit.

After trend analyses, an analysis of variance (ANOVA) approach is used to test each element, taking into account the variability within each bottle (four subsamples) and between bottles. The null hypothesis of the ANOVA test, H_0 , is that there is no difference among the mean mass fraction of the bottles. If F , the calculated statistic of the test, is lower than the critical F_c value, there is no evidence to reject H_0 . Hence, the reference material may be considered stable for the test conditions.

To supplement the ANOVA test, the mean results obtained at 20 °C are normalized for every element to the results obtained at the control temperature (−20 °C), according to Eq. 1:

$$Q_T = \frac{w_{+20^\circ\text{C}}}{w_{-20^\circ\text{C}}} \tag{1}$$

In the case of stability, the ratio Q_T should be equal to 1, considered the combined measurements uncertainty, u_T , which may be estimated from mass fraction, w_T , and its standard deviation, s_T , for measurements obtained at each temperature [8], as given by Eq. 2:

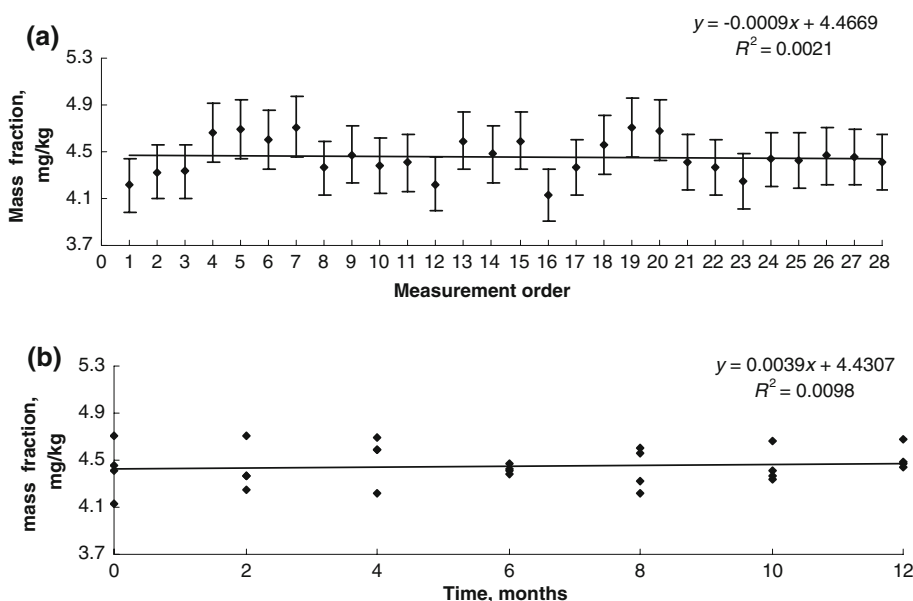
Fig. 1 Isochronous design for the long-term stability study

Temperature/Time	12 months	10 months	08 months	06 months	04 months	02 months	0 months
−20°C	X						
20°C	X						
		X					
			X				
				X			
					X		
						X	
							X

Table 1 Mass fraction in mg kg⁻¹ (mean result and confidence interval at 95% confidence level for n = 4) obtained by INAA for the long-term stability study of the mussel candidate reference material (wet mass basis)

Time:	2 months	4 months	6 months	8 months	10 months	12 months	12 months
Temp.:	20 °C	20 °C	20 °C	20 °C	20 °C	20 °C	-20 °C
Bottle:	62	14	128	42	99	147	115
Ag	2.36 ± 0.25	2.31 ± 0.10	2.23 ± 0.29	2.25 ± 0.07	2.25 ± 0.21	2.34 ± 0.24	2.21 ± 0.23
As	13.76 ± 0.76	13.50 ± 0.92	14.19 ± 1.27	14.51 ± 0.88	13.63 ± 0.87	13.66 ± 1.17	13.88 ± 1.27
Br	207 ± 38	212 ± 17	205 ± 33	211 ± 19	214 ± 17	222 ± 20	199 ± 33
Co	0.858 ± 0.049	0.849 ± 0.063	0.833 ± 0.055	0.839 ± 0.054	0.830 ± 0.015	0.837 ± 0.046	0.837 ± 0.058
Cr	1.06 ± 0.10	1.06 ± 0.20	1.25 ± 0.04	1.11 ± 0.11	1.04 ± 0.20	1.20 ± 0.05	1.15 ± 0.15
Cs	0.119 ± 0.017	0.114 ± 0.022	0.107 ± 0.014	0.107 ± 0.019	0.101 ± 0.010	0.116 ± 0.009	0.101 ± 0.012
Eu	0.0551 ± 0.0039	0.0505 ± 0.0036	0.0529 ± 0.0075	0.0520 ± 0.0050	0.0535 ± 0.0050	0.0530 ± 0.0026	0.0516 ± 0.0041
Fe	591 ± 25	595 ± 25	608 ± 29	604 ± 18	590 ± 48	604 ± 42	600 ± 29
La	0.695 ± 0.062	0.684 ± 0.065	0.710 ± 0.142	0.742 ± 0.071	0.666 ± 0.048	0.722 ± 0.092	0.719 ± 0.042
Na (%)	1.80 ± 0.24	1.80 ± 0.06	1.94 ± 0.32	2.00 ± 0.23	1.96 ± 0.04	1.93 ± 0.27	1.86 ± 0.30
Rb	4.63 ± 0.23	4.64 ± 0.30	4.84 ± 0.83	4.26 ± 0.65	4.62 ± 0.70	4.70 ± 0.48	4.53 ± 0.79
Sc	0.192 ± 0.020	0.188 ± 0.009	0.183 ± 0.014	0.182 ± 0.006	0.185 ± 0.020	0.194 ± 0.017	0.185 ± 0.016
Se	4.42 ± 0.32	4.53 ± 0.33	4.42 ± 0.06	4.43 ± 0.30	4.44 ± 0.24	4.52 ± 0.18	4.42 ± 0.38
Th	0.264 ± 0.035	0.250 ± 0.013	0.261 ± 0.037	0.247 ± 0.022	0.252 ± 0.030	0.257 ± 0.026	0.257 ± 0.019
Zn	114.5 ± 4.1	115.6 ± 7.6	114.5 ± 4.5	115.5 ± 4.8	112.2 ± 6.1	115.3 ± 7.6	113.2 ± 5.7

Fig. 2 Trend analysis for the stability study of Se: **a** linear fit for mass fraction results (mg kg⁻¹) in measurement order, **b** linear fit for mass fraction results (mg kg⁻¹) ordered by time period. Reported uncertainties are expanded uncertainties, k = 2



$$u_T = Q_T \sqrt{\left(\frac{s_{+20^\circ\text{C}}}{w_{+20^\circ\text{C}}}\right)^2 + \left(\frac{s_{-20^\circ\text{C}}}{w_{-20^\circ\text{C}}}\right)^2} \quad (2)$$

Experimental

Isochronous layout

The preparation of the candidate reference material has been described elsewhere [9]. Figure 1 presents the 12-month long isochronous design used in this study.

Shaded spaces represent the testing period for each bottle until time 0, i.e., the time for analyses. A stratified random scheme was used for selection of the seven bottles used. Bottles were kept at (-20 ± 2) °C (Continental FC 26 Freezer) and at (20 ± 2) °C (climatized room), for periods that varied from 0 to 12 months. After the test periods, all bottles were stored at -20 °C till analysis.

Samples and elemental standards preparation

Four subsamples of approximately 0.150 g from each bottle were weighed in properly cleaned polyethylene bags

Table 2 ANOVA table for the long-term stability test (F_c is the F distribution critical value for the level of significance $\alpha = 0.05$; and degrees of freedom $\nu_1 = 6$; $\nu_2 = 21$)

Element	Variation source	Mean square	F test statistic	p value	F_c
Ag	Between	0.0134	0.747	0.619	2.573
	Within	0.0180			
As	Between	0.507	1.196	0.346	2.573
	Within	0.424			
Br	Between	223	0.791	0.587	2.573
	Within	282			
Co	Between	0.00038	0.376	0.886	2.573
	Within	0.00102			
Cr	Between	0.0246	3.422	0.016	2.573
	Within	0.0072			
Cs	Between	0.000212	2.293	0.074	2.573
	Within	0.000092			
Eu	Between	0.00000874	1.081	0.405	2.573
	Within	0.00000809			
Fe	Between	186	0.451	0.836	2.573
	Within	413			
La	Between	0.00261	1.009	0.446	2.573
	Within	0.00259			
Na	Between	0.0244	1.119	0.385	2.573
	Within	0.0218			
Rb	Between	0.129	0.883	0.524	2.573
	Within	0.146			
Sc	Between	0.0000734	0.784	0.592	2.573
	Within	0.0000936			
Se	Between	0.0086	0.286	0.937	2.573
	Within	0.0301			
Th	Between	0.000145	0.496	0.804	2.573
	Within	0.000292			
Zn	Between	6.43	0.467	0.825	2.573
	Within	13.78			

using a Shimadzu AEM-5200 analytical balance. Elemental standards were prepared by pipetting Spex standard element solutions onto Whatman paper filters, using variable volume pipettes (Eppendorf). After drying, paper filters were kept in polyethylene bags with the same geometry as for the subsamples.

Irradiation and element mass fraction determination

After the test periods, subsamples and elemental standards were simultaneously irradiated for 8 h at 10^{12} n cm⁻² s⁻¹ thermal neutron flux of the IEA-R1 Nuclear Research Reactor at IPEN-CNEN/SP. ⁷⁶As, ⁸²Br, ¹⁴⁰La and ²⁴Na radionuclides were measured for 1.5 h, after a 7-day decay period, while ^{110m}Ag, ⁶⁰Co, ⁵¹Cr, ¹³⁴Cs, ⁵⁹Fe, ¹⁵²Eu, ⁸⁶Rb,

⁴⁶Sc, ⁷⁵Se, ²³³Pa (for Th) and ⁶⁵Zn radionuclides were measured for 10 h, after a 22-day decay period [5]. Gamma ray measurements were performed using a Canberra GC2018 HP Ge detector coupled to a Canberra DSA-1000 multichannel analyzer. Gamma ray spectra were collected and processed using a Canberra Genie 2000 version 3.1 spectroscopy software. Element mass fraction calculations were carried out using a Microsoft Excel spreadsheet. Subsamples were irradiated and measured in random in order to avoid interferences from any possible trends that might arise in the results during the measurement campaign.

Results and discussion

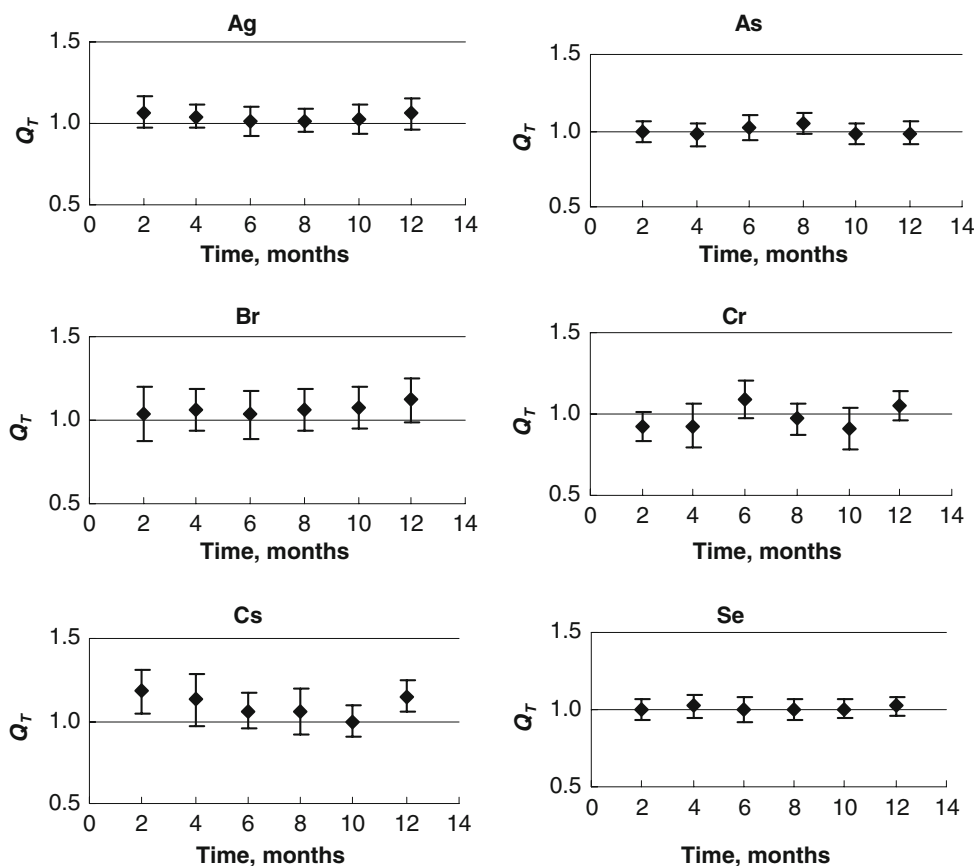
Table 1 presents the INAA mass fraction results obtained for the mussel candidate reference material bottles submitted to the test temperatures and various time periods of the long-term stability study. Results are presented on a wet mass basis, as no correction for residual moisture was performed. Confidence intervals were calculated as $CI = t(s/\sqrt{n})$, where t is the upper critical value for the Student's t distribution for $\alpha/2 = 0.025$.

Figure 2 presents mass fraction results for Se arranged in the order they were obtained in the randomized measurement campaign (Fig. 2a) and according to the periods of exposition at the testing temperature of the stability study (Fig. 2b). Uncertainty bars in Fig. 2a represent expanded uncertainties, U , in Se determination via INAA with a coverage factor $k = 2$. The considered sources of uncertainty were: mass of sample and standards, decay constants and sample and standards activities (which include irradiation and gamma ray spectrometry sources) [10]. In Fig. 2b, uncertainty bars are not presented for clarity. It was observed that the linear coefficients (intercepts) of the equations obtained from both linear regression fits correspond to the mean value of the 28 mass fraction results used to plot the linear regression curves. In addition, the angular coefficients (slopes) of the equations tend to zero in Fig. 2a as well as in Fig. 2b. These findings indicate, along with the determination coefficients R^2 , close to zero, that there is no correlation and thus, no trend among the variables. Similar results were obtained for the mass fractions for the other elements.

Table 2 summarizes the ANOVA test output obtained for the mean mass fraction of four subsamples for the seven bottles for each element. As a general trend, it was observed that the within bottle variability (represented by its mean square, MS) was greater or of the same order of magnitude than the between bottle variability. This was an indication that no significant differences were observed among the bottles other than the resulting from the

Table 3 Normalized results of the long-term stability study obtained by INAA (Q_T is the ratio of mass fractions and u_T is the estimated measurement uncertainty, calculated according to Eqs. 1 and 2)

Element	$Q_T \pm u_T$					
	2 months	4 months	6 months	8 months	10 months	12 months
Ag	1.068 ± 0.099	1.045 ± 0.074	1.011 ± 0.093	1.018 ± 0.070	1.021 ± 0.090	1.062 ± 0.098
As	0.991 ± 0.066	0.973 ± 0.070	1.022 ± 0.081	1.045 ± 0.072	0.982 ± 0.069	0.984 ± 0.078
Br	1.04 ± 0.16	1.07 ± 0.12	1.03 ± 0.15	1.06 ± 0.13	1.08 ± 0.13	1.12 ± 0.13
Co	1.025 ± 0.058	1.014 ± 0.065	0.995 ± 0.062	1.002 ± 0.060	0.992 ± 0.045	1.000 ± 0.056
Cr	0.93 ± 0.09	0.92 ± 0.13	1.09 ± 0.12	0.97 ± 0.10	0.91 ± 0.13	1.05 ± 0.09
Cs	1.18 ± 0.14	1.13 ± 0.16	1.06 ± 0.10	1.06 ± 0.14	1.00 ± 0.10	1.15 ± 0.10
Eu	1.067 ± 0.071	0.978 ± 0.066	1.025 ± 0.071	1.008 ± 0.063	1.037 ± 0.080	1.027 ± 0.061
Fe	0.986 ± 0.040	0.991 ± 0.040	1.013 ± 0.043	1.007 ± 0.036	0.984 ± 0.059	1.006 ± 0.054
La	0.966 ± 0.065	0.950 ± 0.067	0.987 ± 0.052	1.031 ± 0.073	0.926 ± 0.054	1.004 ± 0.089
Na	0.97 ± 0.13	0.97 ± 0.10	1.04 ± 0.15	1.08 ± 0.14	1.05 ± 0.11	1.04 ± 0.14
Rb	1.02 ± 0.12	1.02 ± 0.12	1.07 ± 0.15	0.94 ± 0.14	1.02 ± 0.15	1.04 ± 0.13
Sc	1.039 ± 0.088	1.018 ± 0.062	1.017 ± 0.075	0.982 ± 0.055	1.002 ± 0.086	1.048 ± 0.081
Se	0.999 ± 0.070	1.023 ± 0.073	0.999 ± 0.077	1.001 ± 0.069	1.004 ± 0.064	1.021 ± 0.061
Th	1.028 ± 0.098	0.971 ± 0.055	1.015 ± 0.065	0.962 ± 0.070	0.981 ± 0.085	0.999 ± 0.078
Zn	1.012 ± 0.039	1.021 ± 0.053	1.012 ± 0.044	1.021 ± 0.042	0.992 ± 0.046	1.018 ± 0.053

Fig. 3 $Q_T \pm u_T$ ratios for the long-term stability study of selected elements by INAA (Q_T is the ratio of mass fractions and u_T is the estimated measurement uncertainty, calculated according to Eqs. 1 and 2)

precision of the INAA method used. Except for Cr, $F < F_c$ at the 95% confidence level for all elements, indicating that there were no significant differences among the mean mass

fractions obtained, regardless of the period and temperature at which the bottles were kept. In the case of Cr, if a 99% confidence level is considered, the results may be

considered equal as $F < F_c$ ($F_c = 3.811$) when the less restrictive level is used. These conclusions are corroborated by the obtained p values which, with the exception of Cr, were all greater than the level of significance $\alpha = 0.05$.

Table 3 presents the normalized results of the long-term stability study as defined in “Introduction”. The obtained Q_T ratios were comprised between the respective $Q_T - u_T$ and $Q_T + u_T$ ranges, and hence, no instability may be concluded. To illustrate, Fig. 3 shows Q_T values for selected elements. Even though Cr failed the ANOVA test at the 95% confidence level, no significant degradation was observed from its plot, and it was concluded that this element was also stable in this study. The same conclusion may be drawn for the Cs plot, which although passing the ANOVA test for equality of mean values presented an F value close to the critical value F_c . The observed variations for these elements seem to be related to poor precision and not to any degradation during the test period.

For the estimation of the shelf life period from the long-term stability data, ISO Guide 35 suggests that u_a , the uncertainty in the angular coefficient a , of the linear regression fit multiplied by a suitable shelf life period in months be used as the long-term stability contribution of uncertainty, u_{lts} , to the combined uncertainty of the certified value [4]. The quantity u_a is promptly available from the output of the ANOVA test for linear regression. However, for the expected shelf life for this kind of matrix material, obtained u_{lts} results were high when compared to the combined uncertainty of the INAA mass fraction results. The ISO Guide 35 approach seems to be very conservative. Possibly, the limitation is that it assumes the degradation as occurring at a regular pace over time following a linear model. This usually is not the case for element mass fraction in biological reference materials. In this study, an intermediate approach is intended for the certification of the mussel reference material. First, u_{lts} estimation for shorter shelf life periods will be performed, in order to obtain acceptable values (in comparison to certified values). Then, a scheme for checking the stability after certification will be proposed in order to extend the shelf life of the reference material.

A collaborative study, proposed for the certification of the reference material, is still in process. Hence, it is not possible at the moment to verify the impact of the stability contribution to the overall uncertainty of assigned certified values.

Conclusion

With the application of the INAA method to the isochronous long-term stability study, it was possible to confirm, on statistical grounds, that there were no systematic changes in mass fraction for 15 elements during the time period of this study. Thus, it may be concluded that the candidate mussel reference material is stable enough to be stored at room temperature, without any significant changes in mass fraction for the determined elements. The results from this study will be subsequently used in the assessment of the long-term stability uncertainty contribution to the expanded uncertainties of certified values of the mussel reference material under characterization and in the definition of a suitable shelf life for the material.

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