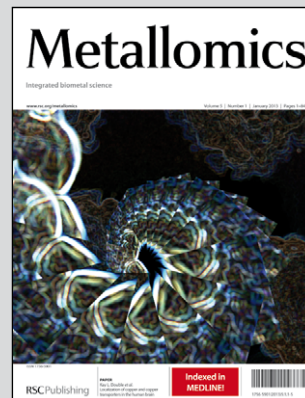


Showcasing research by Dr Marco Arruda and colleagues in the Spectrometry, Sample Preparation and Mechanization Group (GEPAM) and National Institute of Science and Technology for Bioanalytics, Institute of Chemistry, University of Campinas, Campinas, São Paulo, Brazil.

Laser ablation (imaging) for mapping and determining Se and S in sunflower leaves

Mapping and distribution of selenium and sulphur is visible in sunflower leaves using laser ablation imaging mass spectrometry. The challenges involved in finding a matrix that exactly matches the characteristics of the sample is explored.

As featured in:



See Marcelo da Silva and Marco Arruda *Metallomics*, 2013, 5, 62–67.

Laser ablation (imaging) for mapping and determining Se and S in sunflower leaves

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Qualitative and quantitative methods were developed for selenium and sulphur mapping/quantification in sunflower (*Helianthus annuus* L.) leaves. The plants were grown for 50 days in a greenhouse, being divided into two groups, one irrigated with only deionized water and the other treated with a daily dose of 7.58 mg of Na₂SeO₃. Leaves were collected during the growth period for directly evaluating the distribution of Se and S in these structures. For quantification analysis, pellets were produced from both CRM (100 and 1575a for Se and S, respectively) and the sunflower materials. The pellets were doped with 25 and 1200 µg g⁻¹ Se and 5 and 20 mg g⁻¹ S and analyzed by LA-ICP-MS. For accuracy purposes all the samples were also decomposed *via* microwave and analyzed by ICP-MS. To avoid polyatomic interferences Se and S were monitored as SeO⁺ and SO⁺ at *m/z* 96 and 48, respectively, ¹²C⁺ was used as an internal standard, and the ratios between SeO⁺/C⁺ and SO⁺/C⁺ were used for measurements. Statistic tests (*t* test at 95% confidence level) confirming good agreement between LA-ICP-MS and ICP-MS indicated the accuracy of this technique.

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Introduction

Studies seeking to understand plant metal(oid) metabolism are important for nutrition (both plant and human), physiology, and toxicology, and have grown exponentially in number in the last decade. This can be attributed to new areas in science, such as metabolomics,¹ and metallomics,^{2–4} and, in part, due to the development of new technologies, including those based on imaging.^{5–7} Depending on the imaging resolution, a tissue,⁸ organelle⁹ or cellular system¹⁰ can be mapped, enlarging the understanding of a studied system, and making *in situ* analysis possible.

Imaging techniques applied to inorganic species include X-ray emission spectrometry,¹¹ secondary ion mass spectrometry,¹² autoradiography,¹³ and laser ablation imaging mass spectrometry – LA(i)-ICP-MS, the latter being the most recently developed technique, as demonstrated in a diversity of applications.^{14–20}

Although great efforts are made to maximize the resolution as well as the quality of the images obtained through these systems, there are few applications in the literature focusing on

quantitative analysis. Not least, the calibration and accuracy are challenging, such that it is often not possible to compare analysed samples with certified reference materials because of matrix differences, preventing conclusive *in situ* analysis.²¹ Then, strategies for maximizing the use of quantitative imaging techniques should be encouraged for solving these drawbacks, in order to fully explore the potential of these techniques.

This work reports a method for evaluating the distribution of selenium and sulphur in sunflower leaves through LA(i)-ICP-MS while quantitative analyses were also successfully carried out using this technique. For this task, different certified materials were used for validation of the method, including the use of a sunflower sample as a matrix, once the calibration curve was measured using doped Se or S pellets from these materials. Thus, imaging and direct determination of Se and S in sunflower leaves *via* LA-ICP-MS were possible.

Experimental

The reagents for pellet preparation and plant cultivation were from Merck (Darmstadt, Germany). All solutions were prepared in polypropylene flasks with deionized water (≥ 18.2 MΩ cm) from a Milli-Q water purification system (Millipore, Bedford, USA).

Sunflower cultivation

The sunflower plants (*Helianthus annuus* L.) used in this study were cultivated in a greenhouse, and the protocols adopted for

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cultivation were previously described in the literature.²² Seeds were germinated in 1 L plastic pots, containing autoclaved-sterilized soil (400 mg per pot) with pH (5.5) and phosphate concentration adjusted to promote adequate growth of the sunflower plants.²² The germination took about 3 days and during the first seven days, the plants were irrigated using only deionized water. After this period, they were divided into two groups, one irrigated with deionized water only and the other treated with a daily dose of 7.58 mg of selenium (2200 mg L⁻¹ Na₂SeO₃ solution was used as a selenium source). The daily volume of water added to the plants, and then the concentration of selenium solution, varied along the cultivation period and the needs of the sunflowers.

Leaves were collected from the upper region of the plants throughout the cultivation period (after 29, 45 and 50 days of cultivation) for directly evaluating the distribution of selenium and sulfur in these structures as well as to validate the methodology using the cultivated vegetal material as a matrix source for preparation of standards. Considering the sunflower variety used in this work, flowering begins about 50 days after germination.

Methodology of validation

The proposed procedure for quantification of S and Se was carried out using pellets produced in our laboratory from certified reference materials as well as vegetal materials obtained during the cultivation of sunflowers.

A certified reference material CRM 100 – minor and trace elements in beech leaves produced by the Institute for Reference Materials and Measurements – was used to prepare a calibration curve, allowing quantification. In this case, the CRM was spiked with 5.0, 10.0, 15.0 and 20.0 mg g⁻¹ of S. The proposed procedure for pellet preparation consisted of the addition of 500 µL of NH₄SO₄ solution with different concentrations to 200 mg of CRM. The material was homogenized for 10 min and allowed to stand for 2 days. Then, the spiked portions of the solids were mixed using a mortar and pestle and pressed at 7 psi, producing pellets with different concentrations. Pellets were also prepared using the reference material 1575a, tomato leaves, produced by National Institute of Science and Technology, for validating the proposed analytical procedure for S.

Leaves of sunflowers obtained from plants treated with Se were dried, ground into fine powder in a mortar using liquid nitrogen, and dried at 40 °C until constant weight. The material was pressed under the same conditions as the standards.

For Se quantification, the certified reference material 1575a – tomato leaves – was selected for the calibration curve. Pellets with Se concentrations varying between 25 and 1200 µg g⁻¹ were produced using the same analytical procedure described for S. Pellets were also prepared using the certified reference material CRM 281, trace elements in rye grass, produced by the Institute for Reference Materials and Measurements, in order to validate the analytical procedure for Se.

Sunflower leaves, obtained from control plants, were also used for preparing the pellets. In this case, the material was spiked with 100 µg g⁻¹ of Se following the same procedure used to prepare the standards.

Based on the results obtained the pellets prepared using the standard reference materials CRM 100 – minor and trace elements in beech leaves – and 281 – trace elements in rye grass – were used for synthetic laboratory standard calibration of S and Se, respectively, during the direct elemental analysis of sunflower leaves.

After the analysis *via* LA-ICP-MS, the pellets prepared with sunflower leaf material as well as those with certified materials used for validating the analytical procedure were decomposed. For this, a mixture of 6.0 mL of sub boiling concentrated nitric acid and 0.5 mL of hydrogen peroxide was employed. A microwave oven (DGT, ProvectoAnalítica) with a nominal power of 1200 W was used to perform the procedure,²³ comprised of three steps: 400 W at 5 min, 790 W at 8 min and 320 W at 4 min. Then, the samples were gently heated to evaporate the excess of nitric acid, and the volumes adjusted to 10 mL using 1.0% (v/v) nitric acid. Se and S determinations were performed with the ICP-MS operating with the standard sample introduction system, consisting of a cyclonic spray chamber and a Meinhard[®] nebulizer, according to the conditions shown in Table 1.

Sample preparation and analysis of sunflower leaves

Sunflower leaves from Se treated plants were harvested after 29 and 45 days of cultivation. For the control plants the leaves were harvested after 50 days of cultivation. The leaves were selected randomly and measured by LA-ICP-MS.

To minimize the possibility of signal variations during the analyses, due to changes in water content, and also to increase the amount of ablated material introduced into the mass spectrometer with time, sunflower leaves were selected randomly, harvested and immediately dried at 40 °C until constant weight. After the drying process, the leaves were fixed onto acetate double-sided adhesive tape (3 M, Brazil) and placed into the ablation chamber for analysis.

Table 1 Optimized instrumental parameters for the ICP-MS. The integration time for each point is determined by multiplying the dwell time by the number of sweeps

	LA-ICP-MS	ICP-MS (digested samples)
RF power (W)	1000	1200
Carrier gas flow rate – argon (L min ⁻¹)	1.1	0.91
Auxiliary gas flow rate – argon (L min ⁻¹)	1.8	1.2
Reading mode	Peak hopping	Peak hopping
Dwell time (ms)	63	60
Integration time for each point (ms)	315	1000
Detector dead time (ns)	60	60
Sweeps	5	20

Conditions for the operation of the dynamic reaction cell

Element	Monitored species	Oxygen flow rate (L min ⁻¹)	RPq (V)	Rpa (V)
Selenium	⁸⁰ Se ¹⁶ O ⁺	0.70	0.50	0
Sulfur	³² S ¹⁶ O ⁺	0.70	0.50	0
Carbon	¹² C ⁺	0.70	0.50	0

Table 2 Instrumental parameters for the laser ablation system. The resolution on the Y axis is given by the distance between two successive lines, while on the X axis it is determined by multiplying the scan speed by the integration time for each point

Wavelength of Nd:YAG laser (nm)	213
Frequency (Hz)	20
Laser intensity (%)	80
Average energy output (mJ)	1.9
Average fluence (J cm ²)	22
Scan speed (μm s ⁻¹)	70
Laser beam diameter (μm)	110
Resolution – X axis (μm)	22.05
Resolution – Y axis (μm)	300

A quadrupole-based ICP-MS (PerkinElmer ELAN DRC-e) coupled with a laser ablation system (New Wave UP 213) was used for imaging Se and S distribution in sunflower leaves. The analytical strategy adopted for carrying out the determination of Se and S free of interferences was based on the reaction of Se⁺ and S⁺ species formed in the plasma with oxygen in the reaction cell of the instrument. In this case, the monitored species consisted of the product ions SeO⁺ and SO⁺ at *m/z* 96 and 48, respectively. The parameters for the operation of the ICP-MS are also shown in Table 1. These conditions were previously optimized for monitoring S and Se using this hyphenation.^{22,24}

To compensate for possible variations in the ablation process,²⁵ which can be related to changes in the ablation process itself or to the heterogeneity of the prepared standards, ¹²C⁺ was chosen as the internal standard to normalize the analytical signals. Then, ¹²C⁺ is an interesting alternative for signal normalization since the reaction cell of the instrument is pressurized and optimized for the determination of Se and S. In this situation, the obtained signal for carbon was attenuated and ratios between SeO⁺/C⁺ and SO⁺/C⁺ were typically 0.001 and 1.0, respectively.

The conditions adopted for the analysis are described in Table 2, and adjusted for achieving the maximum material removal while avoiding cutting of the leaves by the laser during the analysis. Regarding resolution, the distance between successive lines was set to 300 μm.

The pellets prepared for the calibration of the instrument and for validating the method were scanned in five randomly selected regions, and for each one, 100 points were collected for each monitored ion. The average value was calculated and the ratios between SeO⁺/C⁺ and SO⁺/C⁺ were determined.

The software MatLab version 6.5 was used to construct the images of the scanned leaves and correlate the obtained signals with the concentrations of the elements in the leaves.

Results and discussion

Validation of the analytical procedure

The development of quantitative procedures for the evaluation of the elemental distribution in different materials, in special biological tissues, still presents a challenge because of the difficulty in finding a matrix that exactly matches the characteristics of the sample. In the present case, two other points hamper even more the determinations. The first consists of the fact that Se is considered a micronutrient and it is present at low concentrations in biological tissues,²⁶ making difficult its determination. The second refers to the content of S in the certified reference material used for preparing the calibration curve. In this situation, the lowest limit for the calibration curve depends on the concentration of S present in the certified material.

Taking into account these two points, the calibration curves obtained with the prepared pellets are shown in Fig. 1. The

Table 3 Results obtained for validation of the methodology for semi-quantitative determinations of selenium via LA-ICP-MS. The certified reference material 1575a – tomato leaves – was used to build the calibration curve used for quantification. Values represent the average and the standard deviation for the measurements (*n* = 3). The Student *t* value (95% confidence level) must be ≤ 2.776

	Spiked conc. (mg kg ⁻¹)	MW ICP-MS ^a (mg kg ⁻¹)	LA-ICP-MS (mg kg ⁻¹)	Statistic test
Pellet CRM 281	800	865 ± 16	886 ± 26	1.191
Pellet sunflower Se treated group	0	703 ± 12	707 ± 36	0.182
Pellet sunflower control group	100	97 ± 9	121 ± 15	2.376

^a Pellets decomposed using a microwave oven.

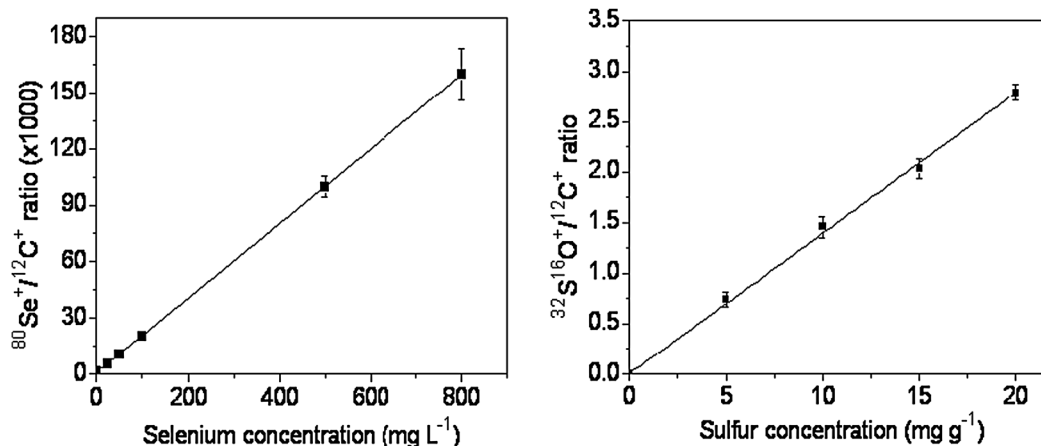


Fig. 1 Calibration curves for Se and S, obtained after the analysis of certified reference materials pellets.

Table 4 Results obtained for validation of the methodology for semi-quantitative determinations of sulphur via LA-ICP-MS. The certified reference material CRM-100 – minor and trace elements in beech leaves – was used to build the calibration curve used for quantification. Values represent the average and the standard deviation for the measurements ($n = 3$). The Student t value (95% confidence level) for CRM 281 must be ≤ 2.160 and for the sunflower Se treated group must be ≤ 2.776 . CRM 1575a is a reference material for sulphur

	Certified value (mg g^{-1})	MW ICP-MS ^a (mg g^{-1})	LA-ICP-MS (mg g^{-1})	Statistical test
Standard addition CRM 100	2.69 ± 0.04	—	2.7 ± 0.1	0.126
Pellets CRM 1575a	(0.96)	—	0.99 ± 0.2	—
Pellets sunflower Se treated group	—	7.6 ± 0.2	6.8 ± 0.6	2.191

^a Pellets decomposed using a microwave oven.

correlation coefficients obtained for Se and S were 0.9999 and 0.9992, respectively, indicating that the proposed procedure for pellet preparation as well as the strategy adopted for data collection and treatment were adequate.

The results related to the validation of the procedure can be seen in Tables 3 and 4 for Se and S, respectively. The precision of measurements using the hyphenation LA-ICP-MS is lower and explained in terms of the analysis of solid samples.²⁷

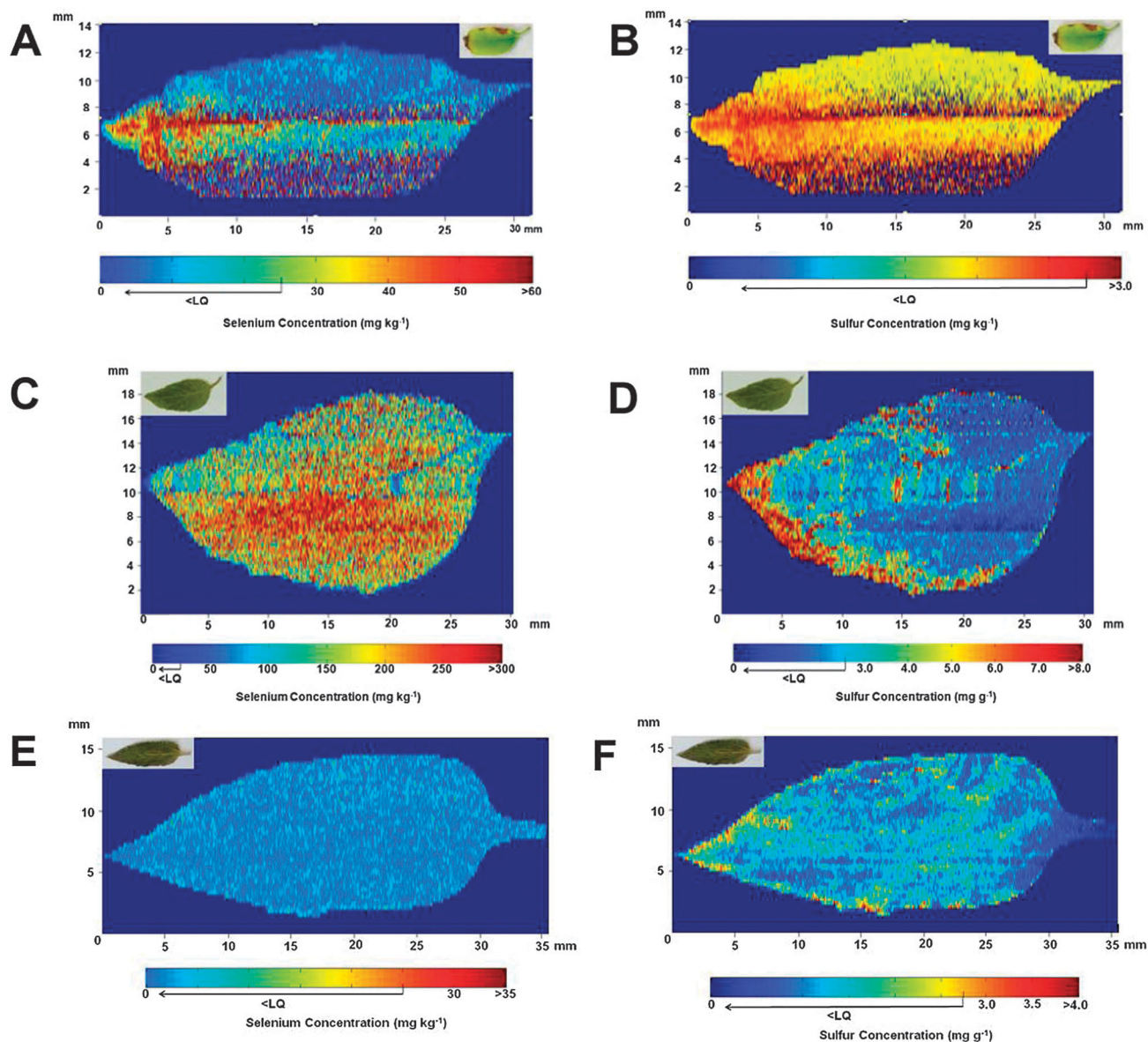


Fig. 2 Se (images A, C and E) and S (images B, D and F) distributions observed in sunflower leaves after 29 (A and B), 45 (C and D) and 50 (E and F) days of cultivation. Leaves in A and B were irrigated with 106 mg of Se (sodium selenite), and in C and D with 174 mg of Se. Both Se and S distributions are shown in the sunflower leaves (E and F) collected from the control group after 50 days of cultivation. The picture of each leaf analyzed is shown on the upper left side of the image.

In fact, all other data were obtained through techniques using analysis of solutions, which present homogeneity at the molecular level, thus explaining the higher precisions. The application of the *t*-test at 95% confidence level indicates good agreement between each group of the results.

Analysis of the images

Fig. 2 shows the results obtained for the distribution of Se and S in the leaves. For the leaves corresponding to plants treated with Se after 29 days of cultivation, Fig. 2A shows that Se is present mainly at the tip of the leaf. Additionally, the leaf itself (shown in the upper right corner) presents chlorosis regions caused by the excess of Se added during cultivation. However, a direct correlation between the accumulation of Se in a specific region of the leaf and morphological problems in the leaf was not observed. When the Se distribution is compared with the profile obtained for S (see Fig. 2B), a correlation between both species is noted. In a previous work,²² the authors reported that sunflower plants irrigated with Se preferentially promoted the translocation of this element to the leaves. In addition, the concentration of S in this region of the plant also increased, probably due to activation of the S uptake pathway. The literature reports that Se can replace S in amino acids,^{23,28} and this result indicates that the metabolism of S can change in the presence of high concentration of Se.^{21,29}

The evaluation of a leaf after 45 days of cultivation reveals that the levels of Se increased *ca.* 5 times when compared with the leaf studied after 29 days, as can be seen in Fig. 2C. In this case, the distribution of Se was essentially homogeneous in all regions of the leaf. It is necessary to emphasize that under both studied conditions, part of the Se may have been lost during the sample preparation procedure since some volatile Se compounds, such as dimethylselenide and dimethyldiselenide, are supposed to be present in the leaves³⁰ and could have been eliminated during the heating process. Related to the S distribution shown in Fig. 2D, although its level has been increased when compared with the leaf after 29 days of cultivation, this element is mainly concentrated at the tip of the structure and it is not homogeneously distributed in the leaf.

The comparison between Se treated plants and the control group reveals that both Se and S levels are lower in the control plants. The concentration of Se for the reference plants shown in Fig. 2E is lower than 25 $\mu\text{g g}^{-1}$. The same profile is observed for S, as can be seen in Fig. 2F, but, in this case, the level of this element is close to the level found in the certified reference material, which is in the expected concentration of S in leaves for most of the plants.

Conclusions

The initial purpose of mapping and determining Se in leaves after adding sodium selenite to the sunflower plants was successfully attained. Besides good accuracy obtained by quantitative laser ablation, which is due not only to the optimization for SeO^+ formation inside the reaction cell of the equipment for eliminating/avoiding polyatomic interferences, but also to the

use of $^{12}\text{C}^+$ as the internal standard for compensating signal fluctuations during ablation, this technique was shown to be useful for mapping Se and S in sunflower leaves, demonstrating different behavior in terms of Se and S distributions in the leaves. Additionally, through the LA(i) it was also possible to conclude that Se is not present in the oxidized regions of the leaves. Another important point in the work was the lower than usual plasma radiofrequency used (1000 W), making possible good sensitivity even when working with a different mass-charge ratio from 80 (most abundant Se isotope).

Finally, this work demonstrates that both LA(i)-ICP-MS and LA-ICP-MS are excellent techniques for macro or local analyses, since optimization conditions can be carefully evaluated.

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