



Honey authenticity testing by *LC-Orbitrap-HRMS*

Dr. Christof Kunert
Eurofins Food Integrity Control Services GmbH

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Introduction

Over the past five years, tackling food fraud has become a major global issue. Honey is among the top ten foods susceptible to economically motivated adulteration. For the consumer, honey is a high-quality natural product with an excellent reputation. Therefore, it is essential to safeguard honey as a product with a rigid quality control using state-of-the-art methods to verify purity and authenticity. Over the last ten years, there has been a steady increase in the number of analytical methods used to detect various types of honey adulteration, including stable-isotope methods, specific methods for individual adulteration marker compounds and foreign enzymes. Since several years, the $^1\text{H-NMR}$ profiling technology has been established to provide a rapid and comprehensive authenticity screening of honey, including quality parameters, geographical and botanical origin verification and adulteration detection. However, the recent experience in routine testing of honeys from international markets indicates that there is still no universal method to detect all currently occurring sugar syrup adulterations with sufficient sensitivity. Even when all existing methods, including NMR, are applied, adulterations with “honey-tailored” sugar syrups offered on the global market can pass the current testing systems. Therefore, there is a need for improved detection of these emerging adulterants. Eurofins has achieved this by method development and application of the LC-Orbitrap-HRMS (**H**igh **R**esolution **M**ass **S**pectrometry) technology for which sensitive robust and affordable equipment is now commercially available. This technique not only allows the detection of the common and emerging adulterants. LC-Orbitrap-HRMS can also replace several single adulteration marker methods (e.g. **S**pecific **M**arker **R**ice/**B**eet Syrup (SM-R, SM-B), E150d), so that one multi-method can be created that detects several hundred different adulteration markers simultaneously. In addition, the combined non-targeted/targeted approach makes it possible to identify yet unknown adulterants and the emergence of new sugars syrup species. Should new adulterants be detected in the future, the recorded data of previously measured samples can be re-evaluated retrospectively in order to trace their initial occurrence and origin. Moreover, a major advantage over other profiling methods such as NMR is that no extensive database of authentic honey reference samples is required for result interpretation. By using a sugar syrup database instead, we are able to directly detect foreign sugar addition in honeys through the detection of a certain sugar syrup profile, which represents a specific sugar type. Since the sugar syrup profile consist of a certain amount of markers, we can exclude the natural occurrence in authentic honeys such as it is the case by single marker methods.

In summary, LC-Orbitrap-HRMS significantly improves the analytical means to detect honey adulteration with foreign sugars. The combination of LC-Orbitrap-HRMS, stable-isotope testing ($^{13}\text{C-EA/LC-IRMS}$) and $^1\text{H-NMR}$ profiling is considered as the current gold standard for honey authenticity testing.

High-resolution mass spectrometry

High-resolution mass spectrometry (HRMS) has experienced an exciting phase of technological development. Starting with Time-of-flight (TOF) instruments, which have significantly higher resolution (40k) and wider dynamic range than conventional MS/MS instruments, followed by a new type of mass spectrometer, the Orbitrap-HRMS, which gave a significant boost to high-resolution mass spectrometry due to the even higher resolving power of up to 480k. With the development of highly sensitive and, for the first time, affordable instruments for routine analysis, Orbitrap-HRMS is becoming increasingly popular. In addition, the acquisition of high-resolution full scan data enables the combination of target analysis with screening of non-target compounds, identification of novel compounds, and retrospective data analysis. Furthermore, a wide range of mass-to-charge (m/z) ratios can be acquired simultaneously without the need for a list of target compounds and individual optimization as required for MS/MS instruments. All of this made it possible for the first time to develop non-targeted evaluation methods for a wide variety of issues such as authenticity screenings in food and feed products.

Workflow of the LC-Orbitrap-HRMS analysis at Eurofins

Briefly, the honey sample is diluted with extraction buffer and then a certain amount of the sample is injected by the autosampler into the Liquid Chromatography (LC) hyphenated to an *Orbitrap*-HRMS. In contrast to the HRMS methods currently available on the market, the separation of the compounds at Eurofins were performed on two different separation columns and in two analytical runs. The separation columns used are a reversed phase column for the separation of the non-polar compounds and a second separation by an HILIC-column for the polar compounds (e.g. sugars, organic acids) (**Figure 1**). These steps are essential from our point of view, since some tailor-made sugar syrups cannot be detected by a single chromatographic reversed phase separation.

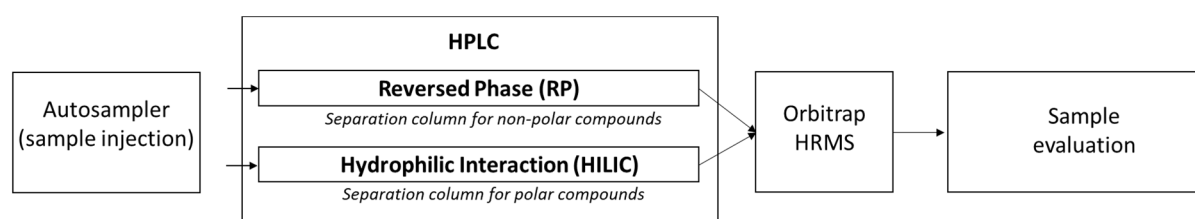


Figure 1: Workflow for the LC-Orbitrap-HRMS analysis of honey.

Sugar syrups for honey adulteration

Sugar syrups are thick and viscous liquids consisting mainly of the major sugars glucose and/or fructose. Sugar syrup can be made by dissolving sugar in water or by reducing naturally sweet juices such as sugar cane. Other syrups (e.g. corn) are made from starch that is hydrolyzed by an enzymatic process to release the sugar compounds from the starch. Due to the low price of sugar syrups and the very similar sugar composition, sugar syrups are perfectly suitable for an economical motivated adulteration of honey.

However, in addition to the major sugar compounds, each sugar syrup contains hundreds of minor compounds which were either formed during the sugar syrup manufacturing process or already present as by-products derived directly from the botanical source (e.g. beet, rice, corn) of the sugar syrup. Due to the high sensitivity of the *Orbitrap*-HRMS instruments, these major and minor compounds can be easily used for a sugar maker profile. During an untargeted full-scan these compounds can be determined and finally be used to create a sugar syrup database based on the sugar syrup profiles. In short, a sugar-syrup database entry is a compilation of markers specific for a single sugar syrup in the database. However, by increasing the sugar syrup database entries, some sugar syrup profiles may (partially) overlap with profiles of other types of sugar syrups from a different variety or source, making it difficult to identify and quantify retrospectively the sugar syrup actually employed. Therefore, the validated result is primarily of qualitative nature. A positive result is only reported, if the detected markers exceed the given threshold level corresponding to a 5% addition of syrup to honey (predefined practical detection limit).

Generally, using a sugar syrup marker profile is a major advantage over the previous approach of using only a single marker compound to detect the addition of a particular sugar syrup (e.g. SM-R, SM-B). False positives as sometimes observed with the single marker methods are greatly reduced with LC-*Orbitrap*-HRMS due to the multi factor assessment applied. A cross-validation study of the three methods ^{13}C EA/LC-IRMS, ^1H -NMR profiling and LC-HRMS was performed on a set of 1'000 samples. As the outcome, less than 0.7% of all adulterated samples were not detectable by LC-HRMS, whereas nearly 13% of all adulterated samples were not detectable by either ^{13}C EA/LC-IRMS or ^1H -NMR. (Eurofins 2020 Annual Report Honey Testing).

Non-target marker identification of sugar syrup compounds

A single analytical run results in several thousand features (approx. 18k) which can be detected by LC-Orbitrap-HRMS in an untargeted full-scan approach (**Figure 2**). Each feature represents a pair of retention time (RT) and a mass-to-charge ratio (m/z) and represents a potential marker compound. However, due to the ionization behavior, a single compound may consist of more than one feature. The majority of the features (approx. 10k) are in the molecular weight range between m/z 100 and 600, with the smaller portion in the range of m/z 600-1500. The low molecular range is one of the strengths of the Orbitrap-HRMS instrument (in contrast to TOF-MS) as the resolution is even higher at lower molecular weights.

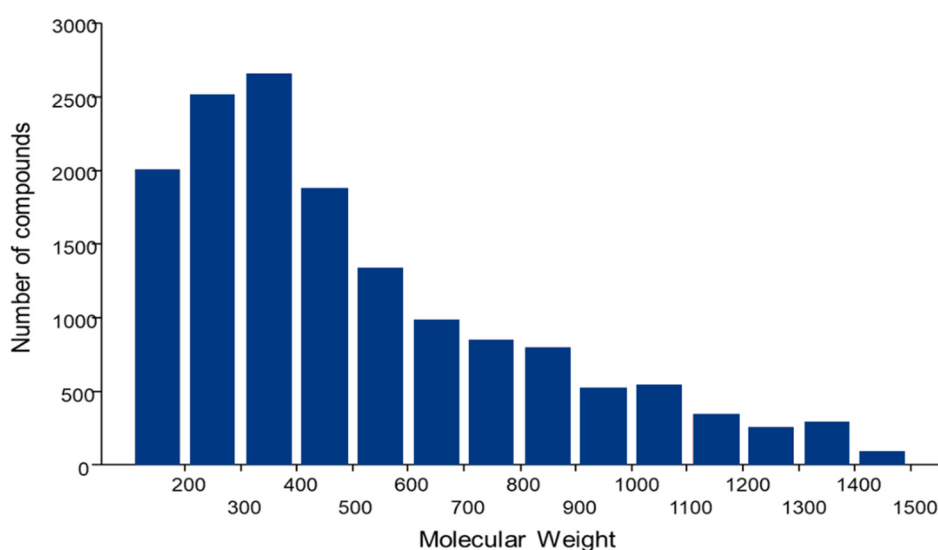


Figure 2: Distribution of compounds by their molecular weight (m/z) in a single analytical run of a honey.

The large amount of identified features can be used for the detection of suitable marker compounds by advanced mathematical approaches such as principle component analysis (PCA) or differential analysis. A spiking experiment can be performed by adding a sugar syrup of interest to honeys to define two groups, a honey group without sugar addition and a syrup group where sugar syrups are added to honeys. To check whether the “honey” and “syrup” can be separated into the former mentioned groups, a principal component analysis can be performed. If there is a good separation between the two groups, the responsible features for the separation of the groups can be filtered out. These identified characteristics form the basis for the syrup profile in the syrup database.

Since PCA (**Figure 3**) shows a good separation between the groups, implying that there is already a detectable difference between honeys and syrups, the next important step is to identify the marker compounds that are mainly responsible for separating the two groups.

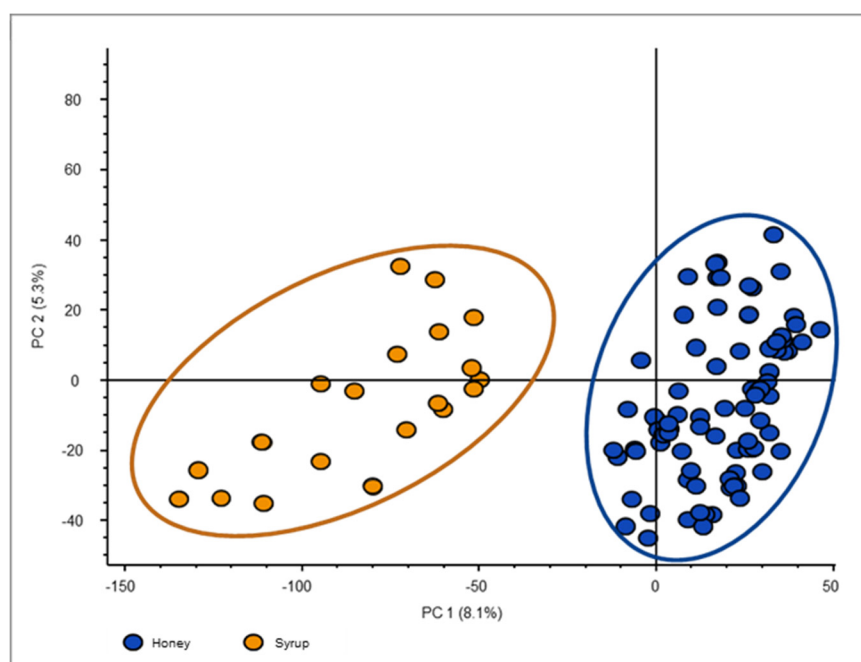


Figure 3: Principle Component Analysis (PCA) of syrup and authentic honeys

Identification of the marker compounds for a sugar syrup can be done by a differentiation analysis, which is shown as a Volcano plot in **Figure 4**. The Volcano plot shows all detected features as small dots measured during the full-scan with the *Orbitrap*-HRMS. Features detected in syrups are shown on the left side (green area). Features representing the honey group are shown on the right side (red area). The detected marker compounds on the upper left have the greatest impact on separating the groups and are therefore the most suitable compounds for the sugar syrup database.

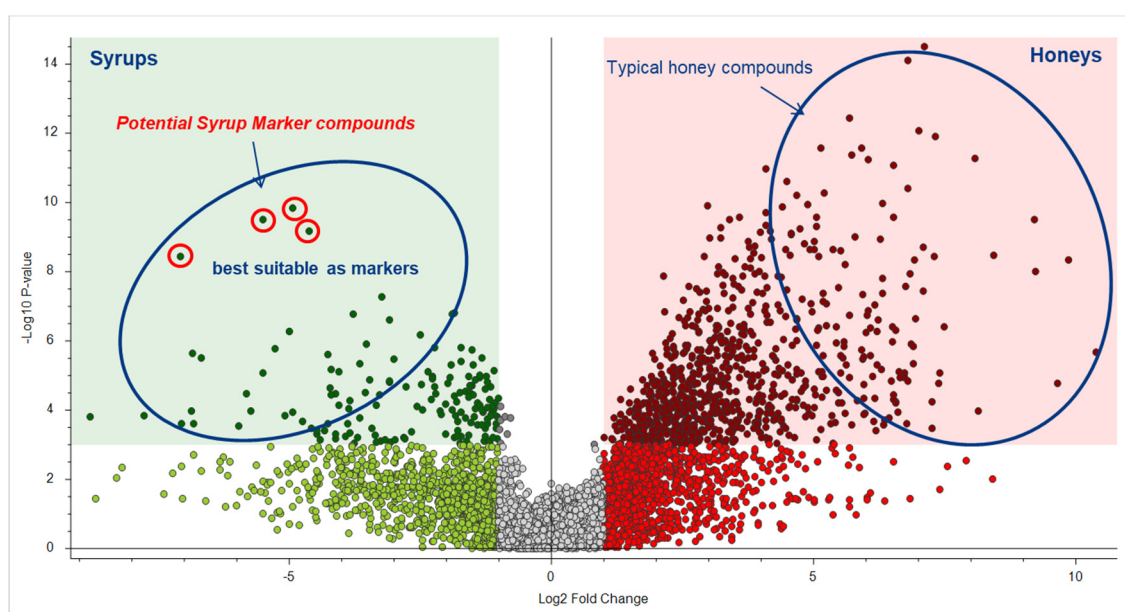


Figure 4: Volcano Plot for the detection of best suitable marker compounds

Using these mathematical tools, the marker identification process is much simpler, as compounds of no interest can be easily sorted out and only compounds of interest can be considered as syrup marker compounds. In a subsequent validation process, it must be checked whether some of the identified marker compounds can be used in routine analysis.

In addition, for each newly detected marker compound, the fragment ions are also determined using MS/MS and used as an additional determination criterion in the routine analysis. For the detection of the sugar syrup, the exact mass, the fragment ions, the retention time and the sugar syrup profile (presence of at least 3 marker compounds) are used, so that the possibility of false-positive results can be almost completely excluded, since it is very unlikely that these compounds all occur "naturally" at the same time in honey.

Before a new syrup is added to the database, the syrup is analyzed by LC-Orbitrap-HRMS to check whether the syrup is already detectable by the existing sugar syrup database with a sufficient detection limit. If it is not the case, the syrup is added to the database to ensure the detection of the new sugar syrup in future authenticity testing.

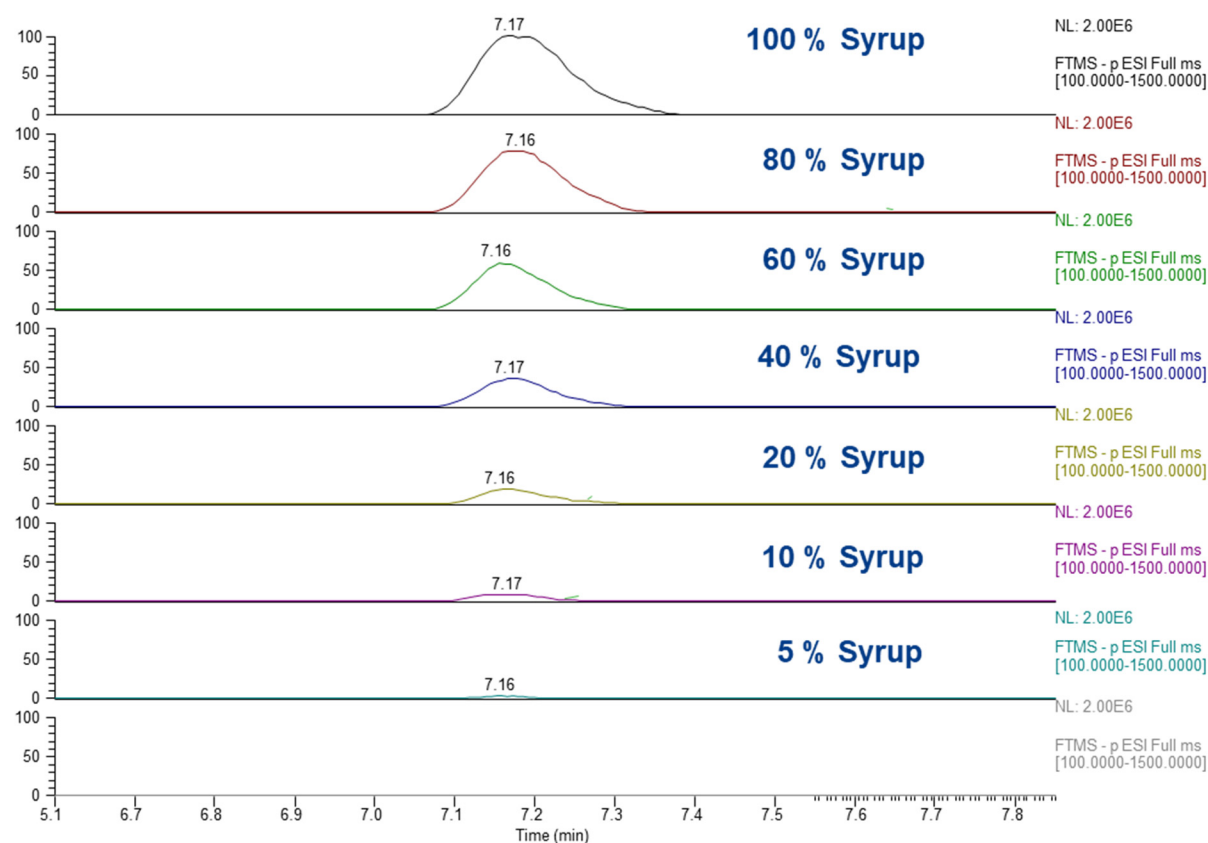


Figure 5: LC-HRMS Chromatogram of a spiking experiment with beet sugar syrup based on an exemplary single marker compound detected in beet syrup.

Generally, the detection limit of these novel marker compounds is 5% sugar syrup addition at best (**Figure 5**). Unfortunately, it is not possible to distinguish between direct addition of sugar syrup to the honeys and the indirect addition of sugar syrup by overfeeding of the bees to increase harvest. This feature is common to all honey adulteration detection methods. In experiments with beekeepers, feeding according to good beekeeping practice and removing of the sugar syrup combs before the nectar flow does not result in any sugar syrup findings in honey by LC-Orbitrap-HRMS, since the expected technically unavoidable contamination (1-3%) is below the predefined detection limit.

Frequently asked questions (FAQ)

► **Do you need an authentic honey database for LC-Orbitrap-HRMS?**

For the detection of added sugar syrup to honey, we use a sugar syrup database and not an authentic reference database of honeys. The major advantage is that we have a direct indication of a sugar syrup. In case of an authentic honeys database (NMR), it is only possible to observe a mismatch of the profile, but the reason of the mismatching profile cannot be indicated.

However, for the formation of the sugar syrup database we use also authentic honeys for validation purposes of the sugar syrup database.

A honey database for LC-Orbitrap-HRMS could be interesting for other purposes, such as the botanical and geographical origin determination of honey complementary to microscopic pollen analysis and NMR. This is currently subject to further research.

► **Can you detect sugar syrup adulteration, when the sugar syrup is not in the database?**

Many sugar syrups share the same marker compounds; therefore, there is no need to include every sugar syrup to the sugar syrup database. If the syrup can already be detected by the existing database with a sufficient detection level (by generic markers or unique marker compounds), there is no need to add the syrup to the database. However, if the syrup cannot be detected yet, we can easily add the syrup to the database to detect the novel syrup in future authenticity screenings by LC-Orbitrap-HRMS (database update). This makes the LC-Orbitrap-HRMS method also “future-proof” due to the regular updates of the database.

The Eurofins syrup database currently includes over 100 different syrups and over 700 marker compounds and is constantly updated.

► **Can you determine the geographical or botanical source of the syrup?**

As we often have marker compounds that occur in many syrups, it cannot be guaranteed that origin or source of the syrup can be unequivocally identified. Moreover, we often do not know the true origin or source of the syrups we get into our laboratory, except that there is a strong indication that the syrup is used in economically motivated adulteration of honey.

On the other hand, also typical syrups used in beekeeping can be added to the syrup database which should help to distinguish improper beekeeping practice from economically motivated adulteration of honey.

► **Can you quantify the amount of syrup in the honey?**

As the concentration of the markers varies in the different syrups, we do not have an analytical standard to quantify the amount of sugar syrups exactly. Rough estimations in the order of magnitude can sometimes be done upon special request.

► **What is the detection limit of the LC-Orbitrap-HRMS**

The predefined detection limit is at 5% sugar addition in case the sugar syrup is well represented in the database; otherwise, the detection limit might be higher until the sugar syrup is added to the database.

► Are the LC-Orbitrap-HRMS methods harmonized between the labs?

In 2017, Eurofins introduced the authenticity testing of honey by LC-Orbitrap-HRMS, in 2018 the method became accredited for the first time. In the meantime, more laboratories started testing of honey authenticity by LC-Orbitrap-HRMS. However, as LC-Orbitrap-HRMS is only a term for a technique and not a certain methodology or instrumentation, there is currently no harmonized procedure or method.

The main differences between the existing LC-HRMS methods that are currently available on the market are:

- The used instrument device (TOF-MS vs. Orbitrap-MS) and the acquisition mode
- Measurement of routine samples only by LC-Orbitrap-HRMS vs. routine measurement by LC-MS/MS (LC-HRMS is used in the latter case for database creation only, often displayed as LC-HRMS/LC-MS/MS)
- Number of analytical runs per sample: double (polar and non-polar) separation vs. single separation
- Use of a syrup database vs. the use of an authentic honeys database
- Different number and types of syrups, different profiles and/or marker compounds in the database

It is expected that harmonization will take place over time as honey samples will be regularly cross-checked by different laboratories offering the LC-HRMS methodology (Orbitrap/TOF-MS), so that detection levels and scope of markers can be adjusted between labs.

► Does LC-HRMS replace the other adulteration detection methods?

In addition to the new detection features previously not covered by other adulteration methods, the LC-Orbitrap-HRMS method can replace some single marker methods directly (e.g. SM-B, SM-R, E150d). Other adulterants, such as those that can be detected by mannose/psicose, foreign oligosaccharides, Trace-Marker Rice syrup (TM-R), and foreign enzymes, are covered by LC-Orbitrap-HRMS using alternative (complementary) marker substances.

In our experience to date, it is the case that in the vast majority of cases, samples that are conspicuous in the afore mentioned analyses are also conspicuous in LC-Orbitrap-HRMS. In contrast, it is rather rare that LC-Orbitrap-HRMS positive results are automatically confirmed by positive results in other adulteration methods (because this type of adulteration is not detectable by other methods). We recommend using a triple assessment by combining the most advanced adulteration analysis methods LC-Orbitrap-HRMS, ¹H NMR and ¹³C EA/LC-IRMS for the best possible risk assessment. Eurofins is able to offer the triple assessment as test package for an affordable price in-line with the market requirements.

Contact:
Dr. Christof Kunert
Eurofins Food Integrity Control Services GmbH
Berliner Str. 2, 27721 Ritterhude, Germany
E-mail: ChristofKunert@eurofins.de

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