FoodQS GmbH

food analytics & quality control

Authenticity of honey

Detection and Identification of honey-foreign substance „Ectoine“ by means of LC-HRMS screening and assessment of its potential as a new adulteration marker for honey analytics

Authenticity of honey

Honey as a natural product is highly valued by the population due to its taste and valuable ingredients such as enzymes, minerals and vitamins. Because of the high price, the natural, fluctuating availability as well as the global trade, honey represents a potential target for economically motivated adulteration. For years, honey has consistently been listed in the top 10 most frequently adulterated foods, along with milk and olive oil [1,2]. In addition to the declaration of false geographical and botanical origin and unauthorized treatment, e.g. with resins to remove ingredients, the stretching of honey with cheap sugar syrups from a wide variety of sources is a major problem for the global honey trade. In the European Union, Directive 2001/110/EC Annex II prohibits any addition of sugar syrups [3]:

“When placed on the market as honey or used in any product intended for human consumption, honey shall not have added to it any food ingredient, including food additives, nor shall any other additions be made other than honey.”

The issue of the authenticity of honey is comparable to that of the doping industry - currently modified doping substances can only be detected with difficulty using common analytical methods. This requires further or new development of analytical methods by laboratories. Syrups used to adulterate honeys are also constantly being modified to make them undetectable by current analytical methods. Online platforms such as Alibaba offer syrups optimized for stretching honeys:

Fructose Syrup for Honey Food Grade (BS SMR TMR C3 C4 C13 test pass F55)

This so-called tailor-made syrup cannot, for example, be detected by the usual analysis of rice syrup markers and isotopes. In order to be able to detect such customized adulterations, a high level of investment in latest technology and qualified employees is required.

Screening methods

For years, screening methods for the authenticity analysis of foods, including honeys, have been increasingly developed and applied in the scientific community and in commercial laboratories. These methods are mainly based on spectroscopic and spectrometric techniques such as infrared spectroscopy (IR), Raman spectroscopy, nuclear magnetic resonance spectroscopy (NMR) and high-resolution mass spectrometry (HRMS). NMR screening in particular has gained increased importance and acceptance since about 2017, due in part to its commercial offering as Bruker's NMR Honey Profiling™. Recently, syrups of the latest generation are available, which cannot be detected by NMR screening. The honey experts at FoodQS GmbH have reacted to this and developed a screening method using LC-HRMS to sensitively detect the latest adulterations.

When developing a screening method to check the authenticity of honey, regardless of the analysis technology, it is essential to build up a database with honeys that are as authentic as possible (honey database). Then, many different syrups (syrup database) are compared with authentic honeys to determine differences. The differences, in the field of LC-HRMS so-called markers, consisting of pairs of retention time (Rt) and exact mass-to-charge ratio (m/z), are checked with the honey database. Chemical compounds are searched for that are present in syrups but not in authentic

honeys. This is the only way to exclude the possibility that a putative marker occurs naturally in honeys of specific geographic or botanical origin. The risk of false-positive results is thus reduced to a minimum. ,

Despite the advantages of modern screening methods such as sensitivity and turn-around time of a laboratory sample, the informative value of indicating the results with "positive/negative" or also "corresponds/not corresponds" is unfortunately very limited. The customer only receives the information that his sample is atypical or that syrup markers have been detected. The company FoodQS GmbH has recognized this deficiency and has continuously developed its LC-HRMS screening, introduced in 2019 in version 1.0. Currently, the result of the screening version 3.0 differentiates between detection of syrup markers, detection of invert syrup and detection of foreign oligosaccharides. Thus, the customer receives additional information on whether the syrup is an inverted syrup, e.g. from sugar beet, or a starch-based syrup, e.g. from rice or cereals. Furthermore, these indications are divided into 2 levels, which allows an estimation of the amount of syrup. Level 1 indicates small amounts of syrup, which most likely result from feeding residues. Larger amounts of sugar syrup are assigned to level 2 and are due to an active addition or large residues of feeding, which no longer corresponds to good beekeeping practice. However, a distinction between the different input routes is not possible from an analytical point of view.

To increase the comparability, plausibility and also reliability of an authenticity analysis, it is always advantageous to additionally identify determined marker compounds. The identity of a marker allows the entry path into the syrup and thus into the adulterated honey to be traceable. Furthermore, comparable analysis between laboratories are possible. In the past, FoodQS has already implemented this procedure several times (Psicose [4], 3-Methoxytyramine (in cooperation with QSI-Quality Services International GmbH, Bremen) [5] and Phosphatidylcholines [6]).

Determination and identification of a marker

Especially highly purified syrups, consisting only of Glucose and Fructose, are ideal for stretching of honeys. Due to its few minor compounds besides Glucose and Fructose, these syrups are extremely difficult to detect using standard adulteration methods. For this reason, a fructose-glucose syrup was prepared in the laboratory from pure glucose using the enzyme glucose isomerase at elevated temperature (**Figure 1**). This syrup was used for the determination of new markers by LC-HRMS screening.

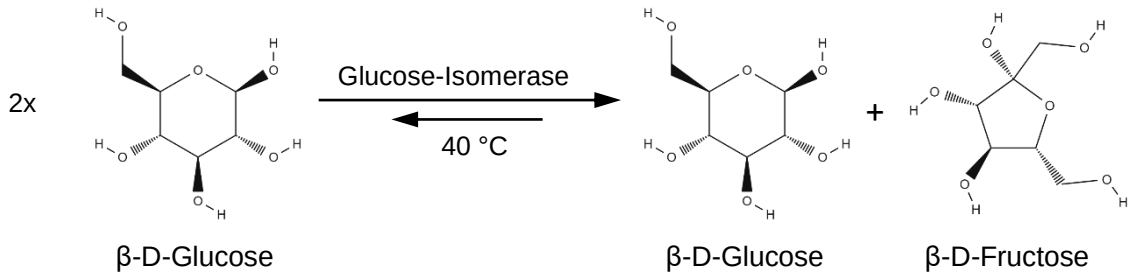

Figure 1: Isomerization of Glucose to Fructose

Authentic honeys as well as the self-produced Fructose-Glucose syrup were extracted in a mixture of double-destilled water and acetonitrile, centrifuged and then analysed by means of LC-HRMS with the parameters listed in **table 1**.

Table 1: Setup of the LC-HRMS Screening

Chromatography	
Sciex Exion AC	
Column	polar HPLC column
Eluents	Gradient of Ammonium-formiat buffer and Acetonitrile
Flow	0,4 ml/min
Temperature	40 ºC
Injection volume	8 µL
Mass spectrometry	
Sciex X500R QTOF	
Ionization	ESI positive
Modus	Full Scan (100-2000 Da) IDA MS/MS
Mass Error	≤ 5 ppm

This untargeted screening is used to collect as much data as possible in the form of pairs of m/z and Rt. These data were processed using statistical software and assigned to the two groups "authentic honeys" and "Fructose-Glucose syrup". Statistical procedures such as a t-test are used to compare the two groups. The aim is to use algorithms to find the smallest differences in chemical composition. In this way, it is possible to find markers for authentic honeys or for the Fructose-Glucose syrup. To display the results of the marker search, a so-called volcano plot is useful. Each point is defined by a combination of m/z and Rt and represents a chemical compound. The data points highlighted in

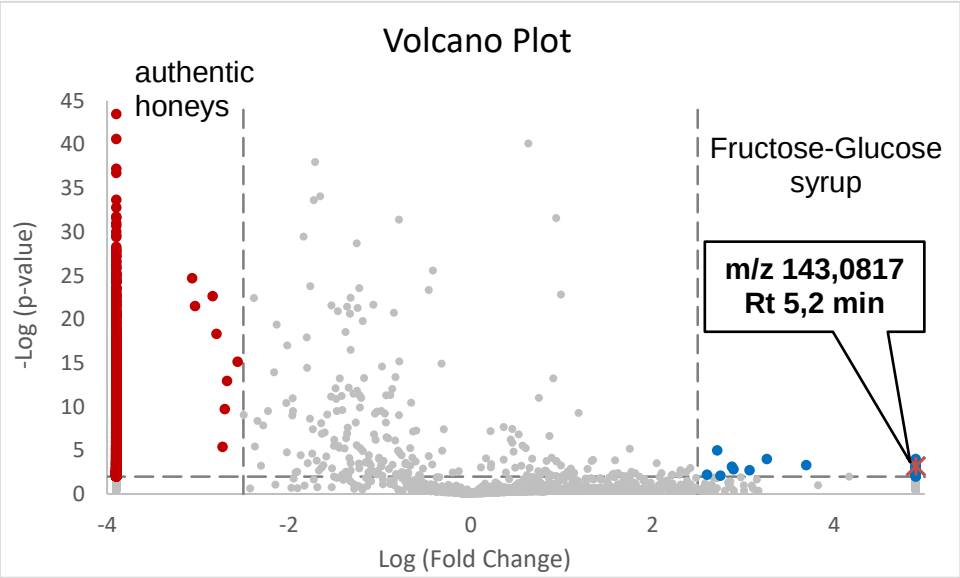

Figure 2: Results of t-Test as -Log (p-value) vs. Log (fold Change), visualised as Volcano Plot: authentic honeys (left panel), Fructose-Glucose syrup (right panel)

light grey represent uninteresting compounds that are not distinctively characteristic of either group (**Figure 2**). The red highlighted data in the left part of the plot represent molecules which were detected in large amounts in authentic honeys and are typical for them. The substances represented by the blue dots on the right of the diagram show high signal intensities in the fructose-glucose syrup and can be regarded as characteristic markers for it, which also includes the data pair m/z 143.0817 / Rt 5.2 min.

This marker was matched against the honey database in a retrospective search to rule out natural occurrence in honeys of specific geographic or botanical origin. The subsequent search of the Pubchem database yielded the molecular formula C6H10N2O2 for the monoisotopic mass 142.0744 (143.0817- mass [proton]) with a mass error of 1.4 ppm. The neutral loss of 46 Da in the MS/MS spectrum suggests the presence of a carboxylic acid function in the molecular structure (**Figure 3**, left spectrum). After cross-checking with further databases and comparing MS/MS spectra of potential molecular candidates, the substance was suspected to be Ectoine. To confirm this assumption, Ectoin was purchased commercially as an analytical standard. The MS/MS spectrum of the marker in the Fructose-Glucose syrup agrees well with that of Ectoine (**Figure 3**). The retention time in the syrup matches to that of the standard. The so-called co-chromatography, a chromatogray of a mixture of syrup and standard substance, shows only one peak without shoulder or splitting, conclusively proving the identity of the marker as ectoine (**Figure 4**). Consequently, the molecule represents an identified marker compound for the detection of Fructose-Glucose syrup in honeys.

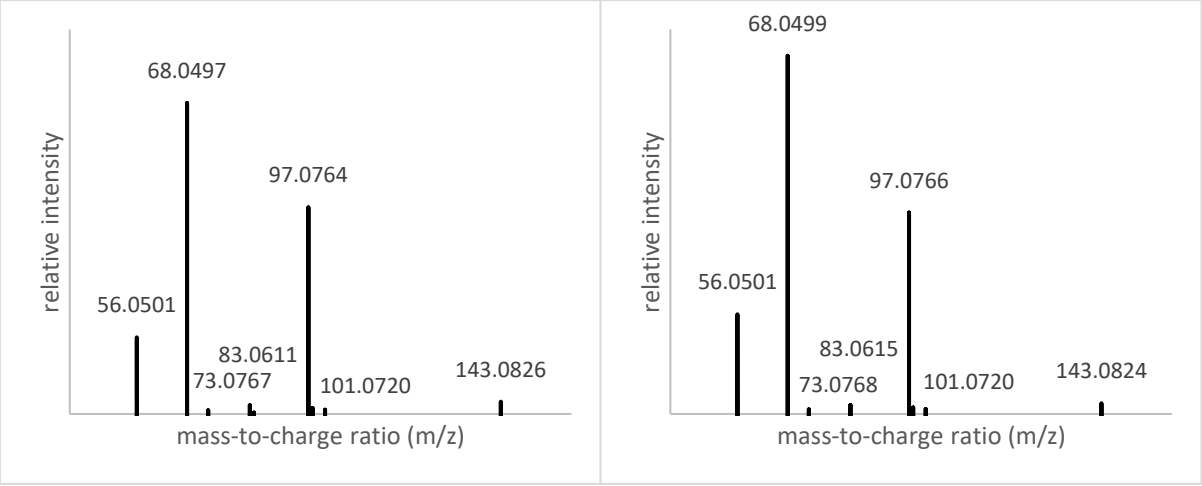

Figure 3: MS/MS spectra of the marker to be identified in Fructose-Glucose syrup (left) and analytical standard of Ectoine (right)

Occurrence of Ectoine

The heterocyclic amino acid L-Ectoine (**Figure 5**) is naturally produced by mainly aerobic, chemoheterotrophic and halophilic bacteria. Due to its water-binding properties, the molecule stabilizes cell membranes and macromolecules such as enzymes and DNA as a compatible solute, protecting the cell from extreme environmental conditions such as heat and high salt concentrations [8,9]. The presence of Ectoine in syrup is due to the enzymatic preparation Glucose Isomerase used,

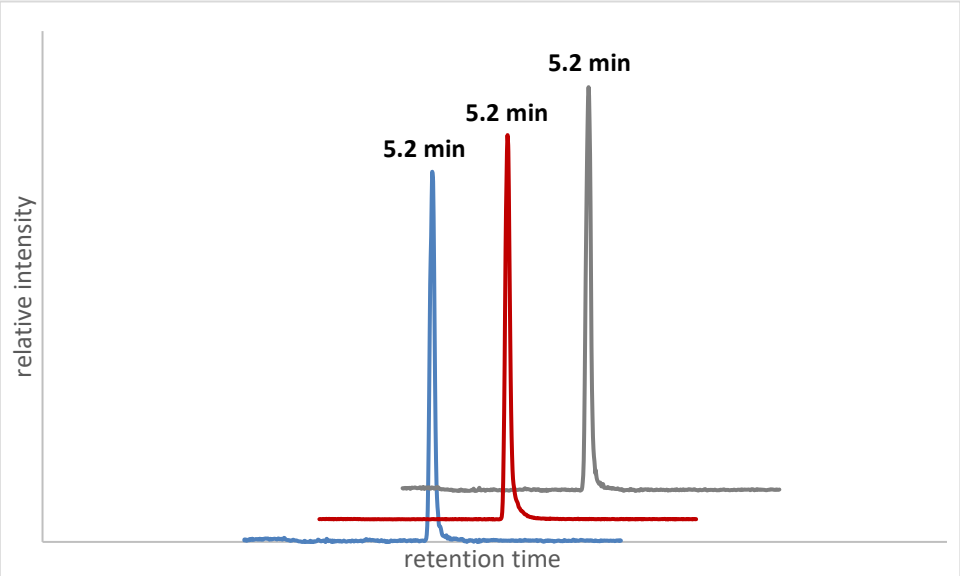

Figure 4: Chromatograms of the extracted mass trace m/z 143,0817 Fructose-Glucose syrup (blue), analytical standard (red) and in a mixture of Fructose-Glucose syrup and analytical standard of Ectoine [co-chromatography] (grey)

which contains Ectoine to maintain its enzymatic activity. This was confirmed by the analysis of an aqueous extract of the enzyme. This enzyme preparation is also used in the industrial production of syrups, which means that the molecule Ectoine can serve as a marker for this. By means of further measurements, the substance was also detected in high amounts in authentic South American bee feeds based on soybean and jatoba flour. Small amounts were detected in a German feed dough whose ingredient list includes soy. It is possible that Ectoine is used in the production or processing of soy flours. In the course of routine measurements, Ectoine was detected in 151 honey samples, with about a quarter of them originating from India. Just under 20 % each of the honeys are of Mexican and Argentinian origin. Other positive samples are assigned to the countries of Cuba (9 %), Vietnam (8 %) and Brazil (3 %). The remaining honeys are from Guatemala, Greece and Thailand. 13 % of the samples have no indication of their geographical origin (blue bars, **Figure 6**). The positive samples are mainly from Central and South America as well as South and Southeast Asia. If we now look at the distribution of global soy production, correlations can be found in some cases (red bars, **Figure 6**). Especially in honeys from Argentina and Brazil, which together are responsible for almost 50 % of the global soy production, Ectoine was detected relatively frequently. Possibly, as in Mexico, Guatemala and Cuba, this is due to the feeding of bees with soy-containing products. Soy is also widely grown in Asia, mainly India and China, which may be related to the Ectoine-positive honey samples from India, Thailand, and Vietnam. Nevertheless, an entry of Ectoine also via syrup must always be considered.

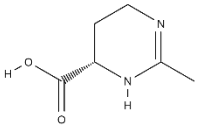

Abbildung 5: Strukturformel L-Ectoin

Evaluation of the results of the HRMS screening

Based on the investigations carried out, it can be stated that Ectoine does not occur naturally in honeys. In the course of an HRMS screening for the authenticity of honey, in addition to the result "HRMS screening positive/negative", a statement can now be made regarding the detection of Ectoine. The detection of the marker indicates an entry of foreign material into honey. Here, unfortunately, it cannot be differentiated analytically whether it is the residue of a feeding of soy-containing material or the addition of Fructose-Glucose syrup. This issue can be explained in a text for the evaluation of the result. By identifying the marker Ectoine and stating its analytical result, the content of information and thus the informative value of the HRMS screening for assessing the authenticity of honey is significantly increased. The customer receives more information for the evaluation of the honey sample and thus a better basis for deciding how to proceed with the corresponding honey batch. Without the identification and subsequent studies on the occurrence of the marker, its analytical detection results only in the result "HRMS screening positive", which corresponds to the assessment "An addition of sugar syrup could be detected". These explanations illustrate that the identification of a marker of a screening method allows a more precise and differentiated assessment of the laboratory sample, thus avoiding false-positive results.

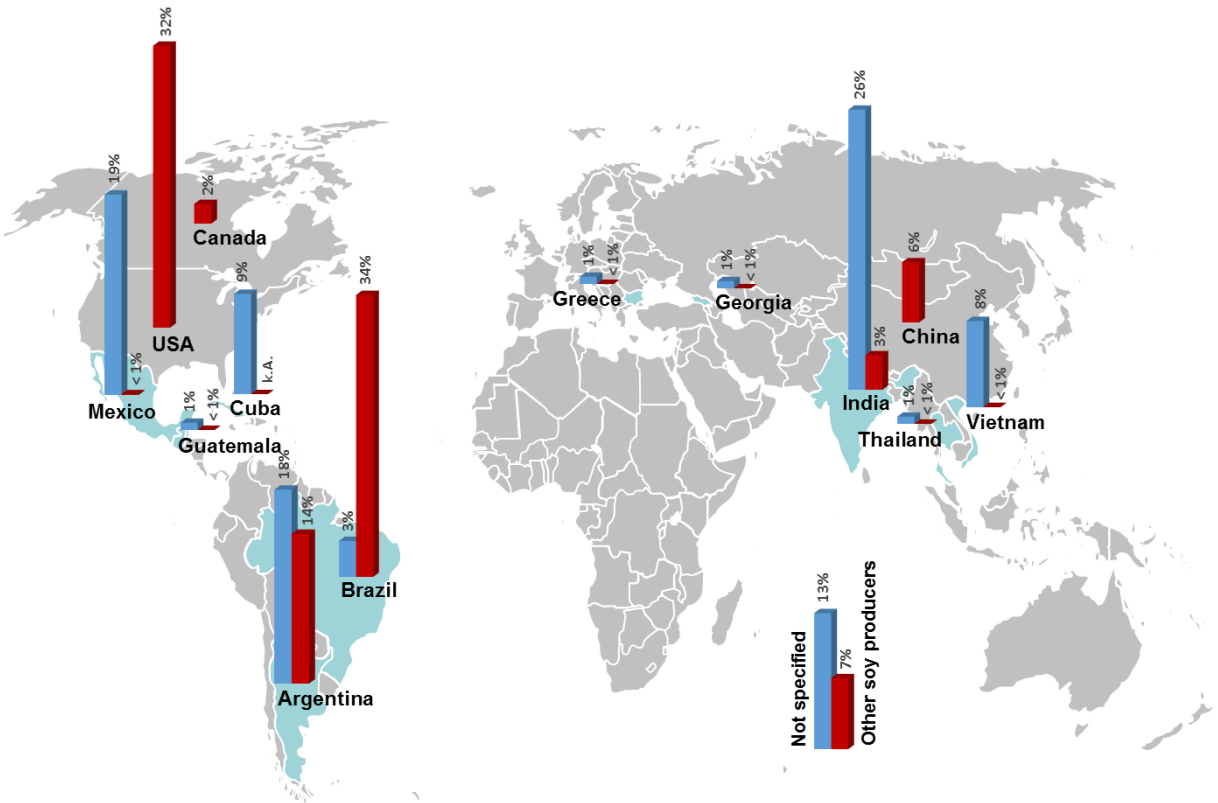

Figure 6: Worldwide distribution of honey samples with positive Ectoine finding (countries highlighted in turquoise). The blue bars show the percentage of positive samples. The red bars represent the percentage of global soy production in 2020 [10].

Conclusion

In the quality assurance of honeys, modern screening methods by means of NMR and LC-HRMS are increasingly used to assess authenticity. In the present article, the characteristics of screening methods are considered. LC-HRMS screening was used to determine a marker for the addition of Fructose-Glucose syrup in honeys and identified Ectoine as the chemical molecule. Further investigations revealed that the honeys with positive detection originated mainly from the regions of Central and South America as well as South and Southeast Asia. In addition, Ectoine was detected in soy-based bee feed. The described identification of the marker Ectoine as well as the results of the further investigations allow to state the result of the HRMS screening in a more differentiated way. Instead of a positive result of the screening, which confirms the addition of sugar syrups, the detection of Ectoine can now be reported separately. In order to be able to guarantee transparent and also comparable analytics, the procedure described in the article for identifying a marker should always be the goal in the context of a screening method.

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