Proteomics: A new method for the detection of honey adulteration

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Introduction

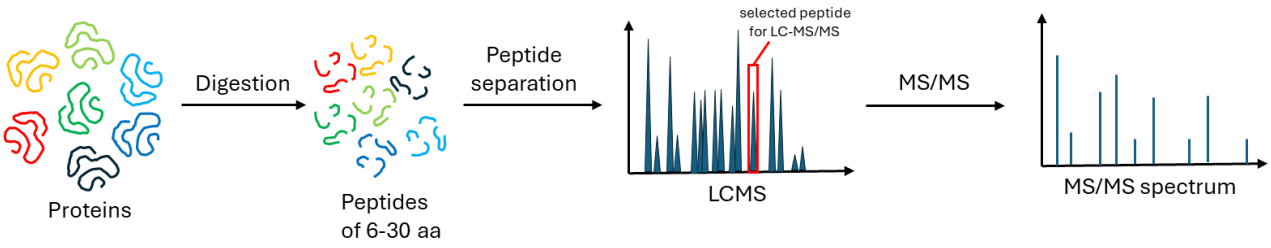
Nowadays, food fraud is a hot topic in the daily news. Especially high-priced food, such as honey, have provided significant incentives for adulteration (e.g. addition of inexpensive sugar products such as rice, corn or beet syrup). The determination of honey authenticity is important for quality and safety reasons. Not only customers have a high interest of high quality in food, also the food industry needs quality assurance to meet quality criteria of their products. Due to its antioxidants, antimicrobial and anti-inflammatory effects, honey is a natural product widely consumed by humans for its therapeutic effects. The curative properties of honey have been known since ancient times in apitherapy [1]. A detailed analysis of honey is necessary to characterise this food due to its high complexity including carbohydrates, proteins, enzymes, amino acids and organic acids. In addition, using microscopy, the pollen of honey is also analysed to confirm its botanical and geographical origin. The analyses of honey ingredients (except pollen analysis) are mainly performed by spectroscopic methods (UV, FLD etc.), high-pressure liquid chromatography (HPLC), ion chromatography (IC), ^{1}H-NMR (nuclear magnetic resonance) spectroscopy profiling as well as LC-MS/MS (liquid chromatography tandem mass spectrometry). There are international standard parameters honey must satisfy such as diastase/amylase activity [2]. If this enzyme activity is low e.g. due to food fraud caused by adding sugar syrup or by handling the honey at high temperature during harvesting, a compensation could be performed by addition of foreign amylase. The enzyme activity tests for α-amylase or invertase are typically performed by spectroscopic tests. One international criterion for marketing honey is the diastase/amylase activity test with a diastase number (DN) not less than 8 DN in general (not less than 3 DN for low natural enzyme honey e.g. citrus honey) [3], however, these tests are not highly specific for enzymes from the honey bee *Apis mellifera*. Therefore, foreign enzymes from another organism, such as yeast (e.g. *Saccharomyces cerevisiae*) could be added to satisfy international standards (amylase/diastase activity criteria). With the enzyme activity tests currently used, these foreign enzymes are difficult to detect, because amylase or invertase from the bee can't be distinguished from other organisms. Therefore, it is important to eliminate the uncertainty whether diastase/amylase activity in honey is only of natural origin or enzyme activity was increased by an artificial enzyme.

This creates the necessity of having a specific method to detect foreign enzymes such as amylase or glycosidase to discover this kind of honey adulteration [4,5]. Our approach is based on targeted bottom-up proteomics. Using this method low amounts of enzymes or other proteins can be observed very specifically. This proteomics approach uses a targeted LC-MS/MS / LC-HRMS workflow of peptides after protein purification and digestion.

Proteomics of honey

Proteomics can reveal detailed characterization for the authenticity of food such as honey [6]. Using this method the same type of proteins such as the enzyme amylase from different organisms can be distinguished because the amino acid sequence of this protein differs even with the same biological enzyme activity. This can be used to distinguish the proteins from each other. In the proteomic workflow the proteins are purified (mainly separated from the sugar and small molecules). Then the proteins are cleaved selectively and digested by a protease. After digestion, the sample is measured via LC-MS/MS / LC-HRMS for peptide separation and detection. When the peptides are fragmented by collision induced dissociation (CID), b- and y-fragments are the mostly most abundant fragments. Therefore, these specific fragments can be used to identify the sequence of the peptides and to detect the protein in honey. This bottom-up proteomics approach can be used for an untargeted and a targeted workflow (figure 1).

Figure 1: Bottom-up proteomics workflow



Untargeted workflow

Using an untargeted workflow, all detectable peptides in the protein digest are measured by LC-MS/MS (all peptides are detected and fragmented via CID). The MS/MS spectra are searched via a database to identify the peptide sequence and to correlate this sequence with amino acid sequences of proteins in the database. Proteins found with this method are then listed with a probability score based on the sequence coverage of each protein (sequence coverage of detected peptides and their fragments). A prerequisite for a low false positive rate is the mass error of the detected peptide mass. This value must be low for a correct identification of the peptide sequence. Additionally, a high mass resolution of the mass spectrometer is required in complex samples to separate coeluting peptides and to resolve multiple and high charge states of peptides in the mass spectrum. That's why this method is typically used with Orbitrap (or q-TOF) instruments with mass errors below 5 ppm and a mass resolution of 30,000 or higher. This method is used to detect all proteins of a proteome in an organism or in complex samples such as honey. The untargeted workflow is used to find the peptides of artificial added enzymes to honey. The very accurate mass and fragments are needed to generate a precursor/qualifier list for the targeted workflow.

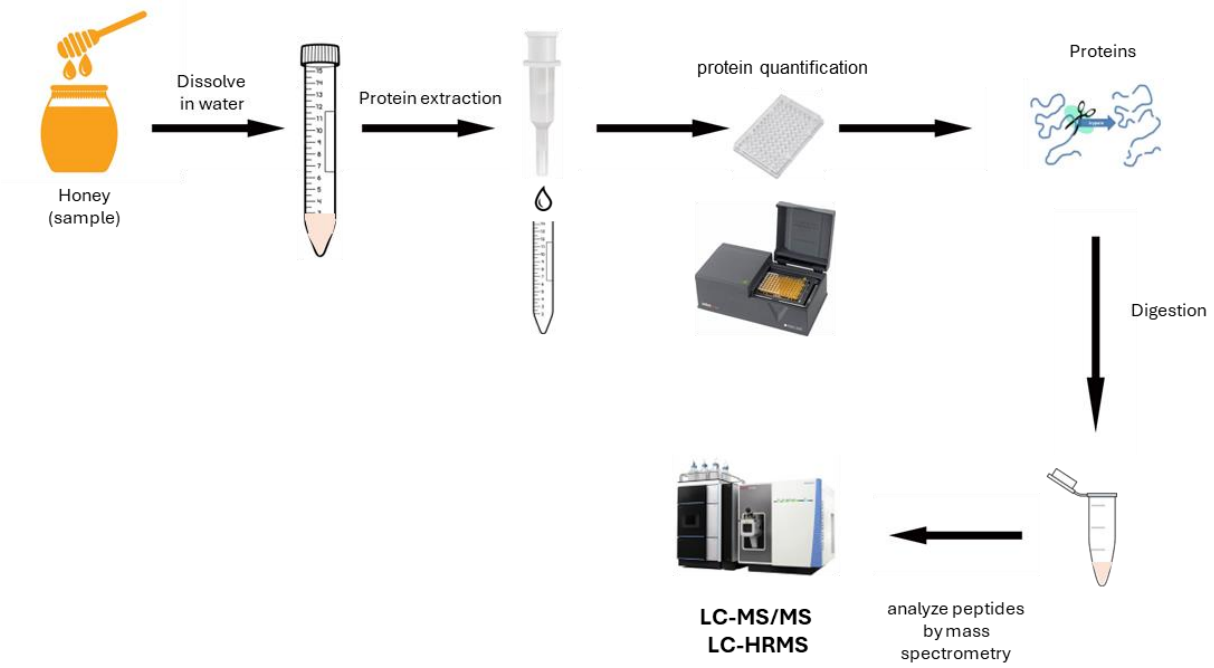
Targeted workflow

The targeted workflow is an approach to detect specific peptides and their qualifier (b- and y- fragments) of specific proteins/enzymes using LC-MS/MS / LC-HRMS. This workflow has the advantage that mass spectrometers with unit mass resolution such as a triple quad (qQq) instrument can be used. This MRM (multiple reaction monitoring) mode is known for its high sensitivity and selectivity. That´s why this method is commonly used for pharmaceutical analyses. A qQq instrument uses single or multiple qualifier fragments of precursors masses and the retention time for correct identification of the precursor which could be a small molecule or a peptide. It should be noted that the targeted workflow can also be performed with an LC-HRMS method especially when combined with the SIM (selective ion monitoring) mode. The combination of SIM for precursor selection and targeted MS/MS with an Orbitrap instrument increases this sensitivity to detect low amount peptides. This extremely sensitive method results also in a low false positive rate due to accurate precursor mass detection. In addition, the targeted proteomics workflow does not need the database search which can be time consuming depending on the size of the database.

Proteomics workflow for the detection of foreign enzymes at Eurofins

The proteomics workflow for the detection of foreign enzymes can be separated into the following steps (see figure 2):

Figure 2: Proteomics workflow for detection of foreign enzymes in honey



Sample Preparation

The honey sample is diluted with the extraction buffer and mixed for a certain time to dissolve all compounds of the honey in the solvent. The density of the solution is measured to calculate the protein concentration in the honey.

Sample purification

The honey solution is filtered through a size-exclusion chromatography (SEC) column. SEC columns are often used for buffer exchange or desalting of protein solutions, but they can also be used to separate high molecular species such as proteins with MW > 10 kDa from the small molecular species in honey such as sugars, polyphenols, amino acids, organic acids etc. with MW < 1 kDa. SEC uses a porous resin as the stationary phase and molecules that are larger in size are prevented from entering the pores, while smaller molecules can penetrate the pores and take a longer time to elute. First the honey solution is pipetted onto the SEC column and then the proteins are eluted from the column by adding the eluent water.

Quantification of protein in honey

The amount of protein in honey is determined by a protein assay. The assay is a dye-binding assay for fast and simple protein quantification. The assay is performed at room temperature. The assay reagent is pipetted onto a microtiter plate. Standard (bovine serum albumin at different concentrations) and sample solutions are pipetted into the microtiter plate. Then the absorption is measured by a spectrometer. The quantification of protein in honey is needed for the protein digestion protocol.

Digestion of proteins

The proteins are dissolved in a buffer solution and digested with a protease enzyme. It cleaves the proteins with high specificity, resulting in a positive charge at the peptide C-terminus, which is advantageous for MS analysis.

Detection of foreign enzymes by LC-HRMS or LC-MS/MS

The protein digest is measured with a standard targeted workflow for bottom-up proteomics using a reverse phase (RP) column and LC-MS/MS / LC-HRMS.

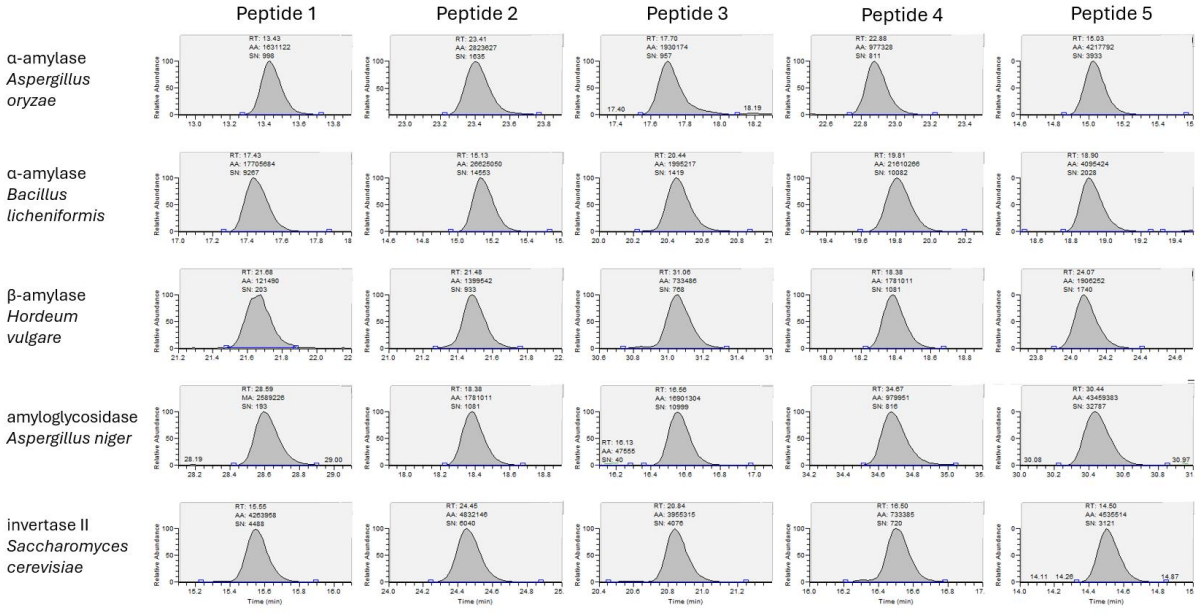
The following enzymes (see table 1) are currently measured with our novel workflow, but this list can be easily extended for new enzymes expected as artificial enzymes in honey. Beside the foreign enzymes, the highly abundant protein MRJP1 (major royal jelly protein) in honey as well as α-glucosidase *Apis mellifera* are also measured to see irregularities of MRJP1 and the naturally occurring enzyme α-Glucosidase caused by addition of high amount of sugar syrup or rigorous processing of the honey.

Table 1: Proteins searched for using the targeted bottom-up proteomics workflow

Name of protein
α-amylase *Aspergillus oryzae*
α-amylase *Bacillus licheniformis*
β -amylase *Hordeum vulgare*
amyloglucosidase *Aspergillus niger*
invertase *Saccharomyces cerevisiae*
MRJP1 (major royal jelly protein)
α-glucosidase *Apis mellifera*

To prove the existence of the foreign enzyme in the honey, all peptides and qualifiers must be detected (see figure 3) to report this enzyme positively found in honey.

Figure 3: Traces of added qualifiers for all peptides of foreign enzymes of a reference honey spiked with a mixture of these enzymes.



Summary

Artificial enzymes can be added to honey to increase the diastase/amylase enzyme activity to satisfy international standards (amylase/diastase activity criteria) needed for honey marketing. Proteomics can be used to detect proteins in honey very specifically. The targeted bottom-up proteomics workflow at Eurofins can detect foreign enzymes which can't be detected with common enzyme activity tests (Phadebas/Schade). The proteomics method is so specific and sensitive that even low amounts of an artificial enzyme can be discovered. Therefore, we recommend using this test for the detection of honey adulteration.

▶ What is the limit of detection of foreign enzymes in honey?

The result of this test is qualitative. However, the limit of detection of enzymes in honey is in the low µg/g range depending on the equipment and the amount of honey used for protein extraction.

▶ Can you quantify each foreign enzyme added to the honey?

The quantification of the foreign enzyme in honey is possible, but we do not have a protocol to determine the exact amount of each protein in honey. Therefore, our results are qualitative due to the fact that the addition of foreign enzymes to honey is prohibited.

▶ Do you need a protein database for this proteomics workflow?

There is no database needed for our targeted proteomics workflow at Eurofins. We use the amino acid sequence of the protein (enzyme) to determine the peptides by *in-silico* digestion. We constantly update our protein database with enzymes (amylases and diastases). Enzymes are tested on a HRMS (Orbitrap) system to determine the retention time of the most abundant peptides of this protein. Then, this information is used for our targeted workflow.

Literature

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