Detection of Foreign Enzyme Addition into the Adulterated Honey

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Abstract: Natural honey contains several enzymes, which are produced by bees (salivary secretion) and some are found in the nectar or pollen. The most important enzymes are amylases, invertases, glusidases, catalases, fosfatases and other. The activity of diastase (α-, β-, γ-amylase) is the important quality parameter of honey, according to the Directive 2001/110/CE the diastase activity (diastase number) must not be less than or equal to 8, for some kinds of honey also higher or equal to 3 (in these cases the HMF must not be higher than 15 mg/kg). Diastase is used as a marker to evaluate the freshness or the heat damage of honey. When honey is adulterated by addition of inverted sucrose or hydrolysed starch namely high fructose corn syrup (HFCS), then such dilution of honey leads to the reduction of diastase number. Such adulteration can be masked by addition of foreign amylases, e.g. bakery mould amylases. Recently several suspect samples of honey with inconsistent diastase number were found in the market. The possibilities of detection of foreign amylase addition based on the comparison of diastase determination using the Schade and Phadebas procedures are evaluated. The both tests are based on the determination of hydrolytic activity (the Schade number is expressed as g of starch hydrolysed 1 h at 40°C per 100 g honey), but the results depend on the substrate used for the trial (according to the standard procedure an insoluble blue dyed cross-linked type of starch should be used). The results of Schade test are therefore often affected by the choice of substrate. The model samples of honeys with addition of foreign amylase (*Aspergillus oryzae*) were analysed, the methods of adulteration detection based on the substrate specificity of enzymes is proposed.

Keywords: falsification; honey; diastase number; Schade; amylase addition

INTRODUCTION

Natural honey contains several enzymes, which are produced by bees (salivary secretion) and some are found in the nectar or pollen. The most important enzymes are amylases, invertases, glusidases, catalases, fosfatases and other. The activity of diastase (α-, β-, γ-amylase) is the important quality parameter of honey, according to the Directive 2001/110/CE the diastase activity (diastase number) must not be less than or equal to 8, for some kinds of honey also higher or equal to 3 (in these cases the HMF must not be higher than 15 mg/kg).

Diastase is used as a marker to evaluate the freshness or the heat damage of honey. When honey is adulterated by addition of inverted sucrose or hydrolysed starch namely high fructose corn syrup (HFCS), than such dilution of honey leads to the reduction of diastase number. Such adulteration can be masked by addition of foreign amylases, e.g. bakery mould amylases.

The traditional method for the measurement of diastase activity in honey is the Schade procedure (Schade *et al*. 1958). One unit of diastase activity (or more specifically, a-amylase), Diastase Number (DN), is defined as that amount of enzyme which will convert 0.01 gram of starch to the prescribed end-point in one hour at 40°C under the conditions of the test. In this assay, a standard solution of starch, which react with iodine to produce a colour in a defined range of intensity, is used as substrate for honey enzymes under the standard conditions. The similar methodology is described in CSN 57 0190 Testing methods for bee honey (according

to Schade modified by Duisberg). More recently, an alternative procedure employing Phadebas® test tablets was introduced (Persano & Pulcini 1999). This method is based on the use of an insoluble, dyed starch substrate. As this substrate is hydrolysed by α-amylase, soluble dyed starch fragments are released into solution. The reaction is terminated and insoluble substrate removed by centrifugation. The absorbance of the supernatant solution (at 620 nm) is directly proportional to the diastase activity of the sample. This procedure has been widely adopted within the honey industry due to the convenience of a commercially available substrate and the simpler assay format.

The objective of this short communication is to evaluate the factors which are affecting the results of diastase number determination.

MATERIALS AND METHODS

Material. 15 samples of honeys of diverse botanical and region origins, bakery mold α-amylase (*Aspergillus oryzae*, VERON® AX, AB Enzymes GmbH, Germany), 3 soluble potato starches of different origin.

Methods. Diastatic activity was determined spectrophotometrically by Schade and Phadebas method according to Harmonisation methods of International Honey Commission (Bogdanov 1999).

RESULT AND DISCUSSION

Influence of Starch in Schade method

Different honeys samples contain various enzymes, the enzymes produced by bees could be to some extent similar but some higher variability could result from the different sources of nectars. Diastase number is an objective criterion quantifying the general enzyme activity in honey, which is expressed as amylase activity on standardised substrate. Honey amylases have to differ in substrate specificity, so that the substrate used for the determination is the principal factor affecting the results. The Schade procedure (Schade *et al.* 1958) specify the substrate as starch reacting with iodine yielding the absorbance at 660 or 662 nm. The mechanism of iodine reaction with starch is not fully clear yet, but it is supposed, that polyiodide chains I_3^- or I_5^- or bigger are formed with reaction of potassium iodide with amylose or straight chain portion of starch, which form helices where iodide molecules assemble (Rendleman 2003). The amylopectin or branched portion of starch form much shorter helices and iodine molecules are unable to assemble, leading the color to be of different hue (it does not absorb at 660 nm)(Thoma & French 1960). Such specification of starch (according to the range of absorbance at 660 nm mentioned above) need not to be enough for the various substrate specificity of honey enzymes, and this is probably one of the reasons of the variability of diastase determination results (Kervliet & Putten 1973). This variability is obvious also from the results given in Table 1, where the diastase number was determined using three different starch samples for five honey samples.

Table 1. Characterisation of different starches (absorbance of standardised soluble starch, required dilution) and the results of analyses of DN with them

Honey sample/Starch	A	B	C
Absorbance at 660 nm*	0.75	0.75	0.63
Absorbance at 662 nm**	0.62	0.51	0.54
1	10.56	12.80	9.96
2	14.58	17.04	14.85
3	15.58	18.78	14.47
4	2.42	3.23	2.43
5	17.22	19.11	16.97

*Schade method, permitted range 0.745–0.77

** CSN 57 0190, permitted range 0.5–0.55

Comparison of the results of Schade and Phadebas method

Similar disproportions in results are also evident if the results of diastase number obtained by Schade and Phadebas are compared (Figure 1). The results generally correlate, but the differences between the results for the same honey sample obtained by the both methods can reduce the reliability of the evaluation and subsequent conclusions. According to our results there is a systematic deviation between Schade and Phadebas method. The problem is the same as within the results of the Schade test, i.e. the variability of substrate.

Foreign amylase addition

Diastase number is very useful criterion to detect the adulteration of honey or to confirm not

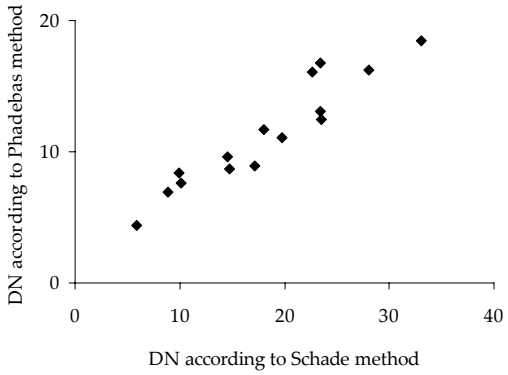

Figure 1. Diastase number (DN) obtained for the same samples by Schade and Phadebas methods

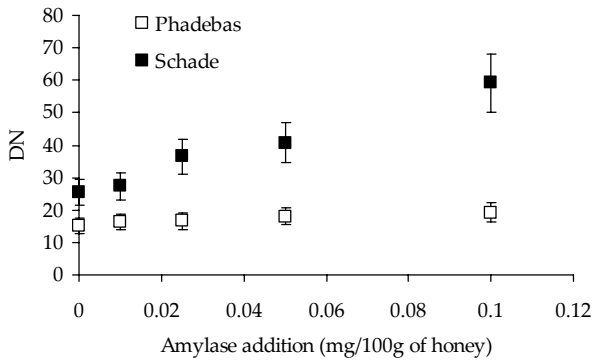

Figure 2. The comparison of diastase activity determined (DN) by Schade and Phadebas procedure for the one honey sample spiked with defined amount of bakery mold amylase

permitted heat treatment or incorrect storage conditions, etc. If adulterated honey is falsified by dilution with sugar or high fructose corn syrups or by similar "diluents", the reduced diastase number could be increased by addition of foreign amylase. To detect the addition of foreign amylase the comparison of diastase number for different substrates can be used. In Figure 2 there is the comparison of diastase activity determined by Schade and Phadebas assays for the one honey sample spiked with defined amounts of bakery mold amylase (*Aspergillus oryzae*, VERON® AX, AB Enzymes GmbH, Germany). With higher portions of added enzyme, the results of Schade test were more different from those obtained by Phadebas procedure. In our experiment the starch used for the Schade test probably matched more with substrate specificity of the added mould amylase comparing with substrate in Phadebas tablets. We used the described results to eliminate suspect honey samples and the falsification of those samples was confirmed by the analyses of the other markers, such as sucrose content, 5-furan-2-carbaldehyde (hydroxymethylfurfural) and minor oligosaccharides profiles. The differences between Schade and Phadebas test was in our case based on the individual properties of the starch substrate and the batch of Phadebas tablets used for the assays, for another combination the differences need not to be so important and usable for the detection of suspect samples. But these results indicate the possibility to develop a new procedure to proof the foreign amylase addition, which will be based on the Schade test done with two different starches being more described not only with iodine test. One starch sample being as close as possible to the requirements of the Schade procedure, another substrate should be of enough different properties and more corresponding with the substrate specificity of easily available and cheap amylases which could be used for the falsification.

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