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A rapid method for the determination of honey diastase activity

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ABSTRACT

A new rapid method for the determination of honey diastase activity using direct potentiometric principles has been proposed. A platinum redox sensor has been used to quantify the amount of free triiodide released from a starch triiodide complex after starch hydrolysis by honey diastase. The method was tested on honey samples with varying diastase activities. The first 5 min of data for each sample were used for linear regression analysis in order to calculate diastase activity. The new method was compared with classical Schade and commercial Phadebas procedures. The results showed good correlations with both methods and offered a simple method for unit conversion to DN units for diastase activity, making the method suitable for routine analysis.

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1. Introduction

Honey is a sweet food made by bees using nectar from flowers. The physical and chemical properties of honey vary depending on water content, the type of flora used to produce it, temperature, and the proportion of the specific sugars it contains. Honey contains small amounts of different enzymes, notably, diastase (α- and β-amylase), invertase (α-glucosidase), glucose-oxidase, catalase and acid phosphatase, which come from nectar sources, salivary fluids and the pharyngeal gland secretions of the honeybee [1].

A diastase is any one of a group of enzymes that catalyze the breakdown of starch into maltose [2]. Alpha amylase degrades starch to a mixture of the disaccharide maltose, the trisaccharide maltotriose (which contains three α (1-4)-linked glucose residues) and oligosaccharides known as dextrins (which contain the α (1-6)-linked glucose branches).

Diastase activity can be measured using the classical Schade procedure [3,4] or by the commercial Phadebas [5] and Megazymes [6] tablets. The Schade procedure uses a standard starch solution that, when developed with triiodide, produces a color in a defined range of intensity. The starch solution is treated with the sample being investigated under standard conditions, and the resulting diastase activity shows a decrease in blue coloration that is measured spectrophotometrically at 660 nm at defined intervals. Importantly, the Schade procedure is limited by several procedural elements. First, when selecting the absorbance range of the starch–triiodide solution, the absorbance range should be limited to a small absorbance area, and several solutions of different concentrations should be prepared to achieve the required absorbance. Second, almost every step is accompanied by dilutions and the addition of volume to the analytical mixture, thereby increasing the associated error. Third, large variations in diastase activity among honeys cause limitations in the measurement. For example, if the activity is low, analysts spend up to 45 min or more on the analysis of one sample. When analyzing the data, only one range of absorbance areas should be taken as calibration points for calculating the diastase activity. If the analyst misses the time for taking the aliquots to achieve the given absorbance range, an additional measurement is needed, and therefore, more time is required to complete the analysis. The commercial methods are based on the use of an insoluble, dyed starch substrate [7]. As this substrate is hydrolyzed by diastase, soluble dyed starch fragments are released into solution. The reaction is terminated, the insoluble substrate is removed by centrifugation, and the absorbance of the supernatant, which is proportional to diastase activity, is measured at a characteristic wavelength. These commercial methods are quite simple to use, but they are more expensive per sample than the Schade method. Also, if the diastase activity is too high, they require additional dilution, and the time for analysis is 30 min.

Diastase activity is expressed as the diastase number (DN) in Schade units and is defined as follows: one diastase unit corresponds to the enzyme activity of 1 g of honey, which can hydrolyse 0.01 g of starch in 1 h at 40 °C.

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The interaction of starch and triiodide ions results in the formation of starch–triiodide complex [8] with characteristic colors. The proposed methodology was based on the direct potentiometric determination of levels of triiodide ion released from a starch–triiodide complex using a platinum redox electrode as a detector after biocatalytic starch degradation by diastase.

In previous work [9], we have reported a direct potentiometric method for alpha-amylase determination in pure systems and detergent cogranulates. This analytical methodology is based on the direct potentiometric determination of levels of triiodide released from a starch–triiodide complex during the hydrolysis reaction. A platinum redox electrode was used as a sensor to detect changes in triiodide concentration.

The aim of this paper was to determine honey diastase activity using sensing principles similar to those we have previously reported by direct potentiometry measurements and starch–triiodide complex formation. The new method should be simpler, faster and cheaper compared to offered standard methods.

2. Experimental

2.1. Reagents and preparation of solutions

Determination of starch dry weight was performed by drying soluble starch at 130 °C until the constant mass was achieved. The equivalent of 0.500 g of anhydrous starch was dissolved with previously prepared acetate buffer (pH 6) in a 100 mL volumetric flask. The suspension was swirled and boiled gently for 3 min. When the starch suspension was completely dissolved, the solution was rapidly cooled under running water to make it ready for further usage. The solution must be prepared on the day of use to avoid microbial degradation and retrogradation.

Honey samples were purchased from local stores in Croatia. Ten honey samples were used; thorn, mandarin, forest 1, forest 2, linden, acacia, floral 1, floral 2, lavender and mountain lea honey. Honey solutions (HS) were prepared by adding 15 g of honey sample to a 100 mL volumetric flask that was then filled with a previously prepared acetate buffer (pH 6) solution containing 6 mM $CaCl_2$ and 20 mM NaCl. It is essential that the honey should be buffered before coming into contact with sodium chloride because diastase activity rapidly decreases in the presence of sodium chloride at pH values below 4.0. The prepared solution was stirred without heating, at room temperature, until the honey was completely dissolved. When prepared, the honey solutions should be used within a few hours to avoid self-decomposition and a decrease in diastase activity.

The potassium triiodide solution was prepared by dissolving solid iodine (100 μM) in a 0.05 M potassium iodide in small water volume. When solid iodine was completely dissolved, acetic acid was added to a final concentration of 1 M. The acetic acid triiodide solution (ATIS) was used as a diastase inhibitor and the reagent for producing colored starch–triiodide complexes.

2.2. Apparatus

Direct potentiometric measurements were performed using a 780 Metrohm pH Meter (Metrohm, Switzerland) combined with a IJ64 platinum redox electrode (Ionode, Australia) as a detector and a silver/silver (I) chloride reference electrode. Spectrophotometric measurements were performed using an AvaSpec 2048-2 UV–Vis spectrophotometer (Avantes, Netherland). A thermostat (PolyScience, USA) was used for the diastase incubation.

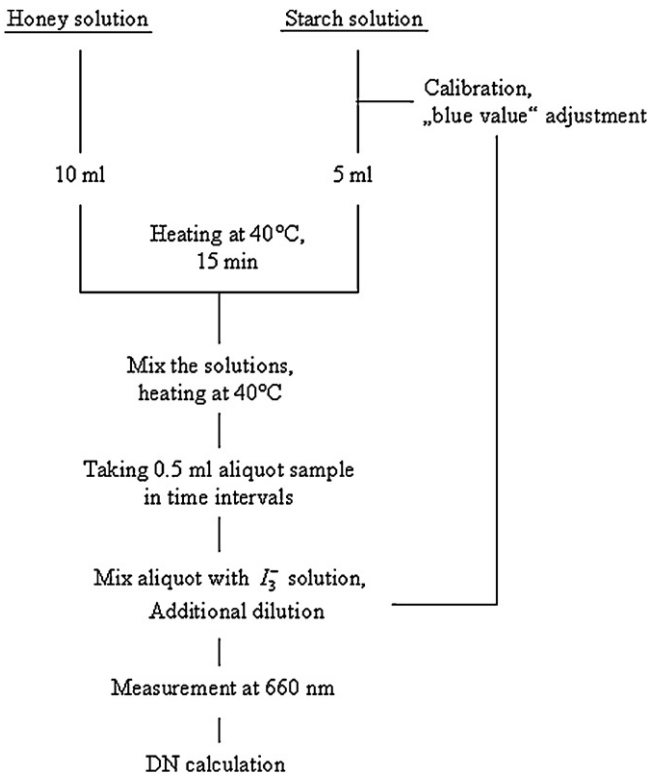

Fig. 1. Procedure of the official Schade method.

2.3. Procedures

2.3.1. Standard Schade method

The unit of diastase activity, the Gothe unit, is defined as the amount of enzyme that will convert 0.01 g of starch to the prescribed end-point in 1 h at 40 °C under defined conditions. The results are expressed in Gothe units (or Schade units) per gram of honey.

The principle of this method is as follows: a standard solution of starch that is capable of producing color in a defined range of intensity when treated with iodine is acted upon by the enzyme in the sample being investigated under standard conditions. The resulting reduction in blue coloration is then measured at defined intervals. A plot of absorbance relative to time or a regression equation is used to determine the time (t_x) required to reach the specified absorbance of 0.235. The diastase number is calculated as 300 divided by t_x. This method is based on the original work of Schade [3], which has been subsequently modified [4,10], as presented by the Codex Alimentarius method. The standard Schade methodology is briefly described in Fig. 1.

The diastase activity is calculated as diastase number (DN) as follows:

$$\mathrm{DN} = \frac{60\ \mathrm{min} \times 0.1}{t_x} \times 0.02 \quad (1)$$

A calibration of the starch solution (also known as adjustment of blue value) is carried out to determine the amount of water that has to be added to the reaction mixture so that the absorbance range of the iodine starch solution is 0.745–0.770. The procedure requires several steps of dilution and subsequent absorbance analyses until the set absorbance range is reached. The amount of water added for each dilution determined in this way is then used as the standard dilution for every determination carried out with the starch solution.

2.3.2. Phadebas honey diastase test

The diastase activity of samples was also measured using the Phadebas method according to the Harmonized Methods of the European Commission of Honey [5]. A tablet of an insoluble blue-dyed, cross-linked starch was used as the substrate for the degradation reaction.

After dissolving 1.00 g of honey in the acetate buffer (0.1 M, pH 5.2) in a 100 mL volumetric flask, 5.0 mL of the honey solution was transferred to a test tube and was incubated in a water bath at 40 °C for a few minutes. A blank was prepared by adding 5.0 mL of the acetate buffer solution and was treated in the same manner as a sample solution. After placing the Phadebas tablets (Pharmacia Diagnostics AB, Uppsala, Sweden) into both test tubes, a timer was started. The tubes were quickly removed from the water bath, stirred and then returned to the water bath. After 30 min, the reaction was terminated by adding 1.0 mL of 1 M sodium hydroxide solution, and the mixture was stirred again and filtered using filter paper. The absorbance of the sample was measured at 620 nm using deionized water as a reference, and the absorbance of the blank was subtracted from that of the sample solution (ΔA_{620}). If the absorbance was higher than 1.0, then the sample was diluted, and the dilution factor was taken into account when calculating the final activity. The measured absorbance of the solution is directly proportional to the diastatic activity of the sample.

The diastase activity, expressed as DN or diastase number, was calculated from the absorbance measurements using Eqs. (2) and (3) for high (8–40 diastase units) and low (up to 8 diastase units) activity values, respectively:

$$\mathrm{DN} = 28.2 \times \Delta A_{620} - 2.64 \tag{2}$$

$$\mathrm{DN} = 35.2 \times \Delta A_{620} - 0.46 \tag{3}$$

Eqs. (2) and (3) were proposed by the above mentioned methodology of Bogdanov. Diastase activity was referred to as DN in the Schade scale, which corresponds to the Gothe scale number, or as g of starch hydrolyzed per hour at 40 °C per 100 g of honey.

There is a strong correlation between the diastase activity expressed in Schade units (the traditional Schade method) and the absorbance measured in the Phadebas test, so a linear relationship can be used to calculate the diastase activity in Schade units from the UV–vis absorbance.

2.3.3. Potentiometric measurement of diastase activity

The honey solution (30 mL) was pipetted into a 100 mL flask and placed in a 40 °C water bath with a second flask containing 30 mL of starch solution. After 15 min, 20 mL of the honey solution was added into the starch solution and mixed. After 1 min and at regular time intervals (30 s) thereafter, 3 mL aliquots were added to 6 mL of the ATIS solution. The solution was mixed thoroughly, and the potential was immediately measured using the platinum redox sensor. Blank or zero point potential was measured separately using the same procedure as described above, except that the honey solution was replaced with the same volume of deionized water. For each honey type solution the same measurement was repeated five times, including standard and commercial method

3. Results and discussion

3.1. Direct potentiometric determination principle

Direct potentiometric determination was based on the measurement of free triiodide released from the starch triiodide complex. Potassium triiodide was prepared by dissolving iodine in a large excess of potassium iodide solution. The equilibria between triiodide and iodide ions is defined by the following redox reaction:

$$I_3^- + 2e^- \rightleftharpoons 3I^- \tag{4}$$

The corresponding redox potential can be described using the Nernst equation:

$$E = E^0 + \frac{RT}{2F}\ln\frac{[I_3^-]}{[I^-]^3} = E^0 + S\log\frac{[I_3^-]}{[I^-]^3} \tag{5}$$

where R is the universal gas constant (8.314472 J K^{-1} mol^{-1}), T is the absolute temperature (°C), E^0 is the standard cell potential (mV), F is the Faraday constant (96 485 C/mol), $[I_3^-]$ is the triiodide concentration (mol L^{-1}), and $[I^-]$ is the iodide concentration (mol L^{-1}).

Starch (S) forms a complex with triiodide ion according to the following equation:

$$[KI_3]_0 + S \rightleftharpoons [KI_3 \cdot S] + [KI_3]_f \tag{6}$$

where $[KI_3]_0$ and $[KI_3]_f$ are the initial and free potassium triiodide concentrations, respectively.

Iodide should be present in large excess and is assumed to be constant.

After the addition of a known amount of the starch solution to the solution of iodide–triiodide, the following equilibria will be established:

$$[I_3^-]_f = [I_3^-]_0 - [I_3^-]_b \tag{7}$$

where $[I_3^-]_f$ is the free triiodide concentration, $[I_3^-]_0$ is the initial triiodide concentration and $[I_3^-]_b$ is the concentration of triiodide bound to starch.

By insertion of Eq. (7) into Eq. (5), the following expression is obtained:

$$E = E^0 + S\ \log\frac{[I_3{}^-]_0 - [I_3{}^-]_b}{[I^-]^3} \tag{8}$$

Diastase catalyses the hydrolysis of starch, causing the release of triiodide and a decrease in $[I_3^-]_b$.

The increase in the concentration of triiodide causes an increase of the triiodide/iodide redox couple ratio, which in turn results in an increase in the electrode potential of a redox sensor according to Eq. (8). These changes can be directly correlated to diastase concentration.

3.2. Direct potentiometric determination of honey diastase

The diastase activity of samples of 10 varieties of honey was determined. A plot of the response of the platinum redox sensor as a function of time for each variety of honey is shown in Fig. 2 (some curves presented are displaced vertically for clarity). The shape and the slope of the curve depend directly on the observed diastase activity. A period of 20 min appeared to be sufficient to observe variation in diastase activity between the different honey samples. Following the standard Michaelis–Menten enzyme kinetic model, the enzyme activity is highest during first few minutes of analysis. After this period, there is a slight decrease in activity, eventually resulting in a plateau in activity at certain point when there is no more starch to hydrolyze.

The sensor responds linearly in the first 5 min, which proved to be sufficient to distinguish the differences in diastase activity and calculate the diastase activity value using linear regression. The list of all results is shown in Table 1. The displayed regression line parameters including corresponding correlation coefficients demonstrated satisfactory linear correlation of the potentiometric sensor responses as a function of hydrolysis time.

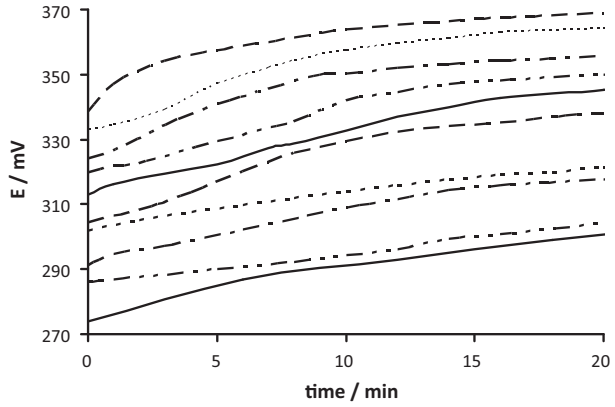

Fig. 2. Data plots of the platinum redox sensor response as a function of time during analysis of solutions of 10 honey samples containing starch after inhibition of diastatic hydrolysis with ATIS (honey samples from the lowest curve up-mandarin, floral 1, accacia, floral 2, linden, forest 2, forest 1, lavender, mountain lea and thorn).

Table 1
Regression line parameters calculated from the linear segment (first 5 min) of the platinum redox sensor responses of the honey samples investigated.

Honey sample	Slope (S)	R^2
Thorn	4.3391	0.9974
Mandarin	1.0743	0.9861
Forest 1	1.9857	0.9795
Forest 2	2.3373	0.9778
Linden	1.8451	0.9845
Acacia	1.7405	0.9726
Floral 1	0.8373	0.9257
floral 2	1.3151	0.9841
Lavender	2.4305	0.9991
Mountain lea	2.7746	0.9698

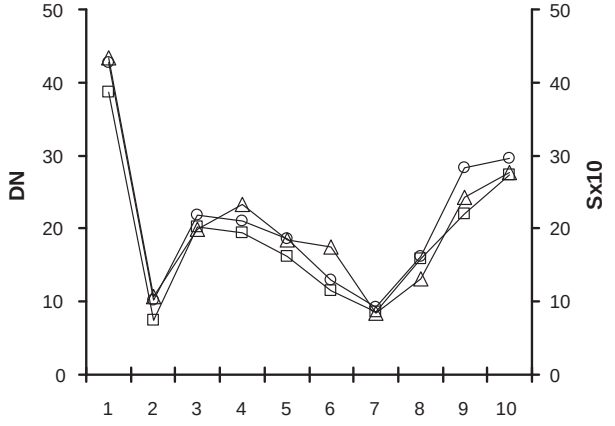

Fig. 3. Comparison of the results obtained for the diastase activity of honey samples by direct potentiometric measurement ($S \times 10$, △), standard Schade methodology (DN, □) and the commercial Phadebas method (DN, ○). Numbers represent honey samples (1-thorn, 2-mandarin, 3-forest 1, 4-forest 2, 5-linden, 6-accacia, 7-floral 1, 8-floral 2, 9-lavender and 10-mountain lea).

3.3. Direct potentiometric diastase activity determination vs. standard methods

The determined values of diastase activity of different varieties of honey were compared with calculated DN values obtained using the Phadebas and Schade methodologies. The activity results from all three methods are shown in Fig. 3, where slope values (S) of the potentiometric method are multiplied by a factor of 10 ($S \times 10$) to achieve the same value range as the DN values of other two methods. The results obtained using the proposed redox sensor were compared with those obtained using the standard spectrophotometric Schade method [3] and method using Phadebas tablets. There are no significant differences between mean values at the 95% confidence level after evaluation using Student's t-test by comparing the proposed redox method and standard Schade method, except for the sample 6 (acacia) where this statement is valid at the 99% confidence level. No significant difference was also observed between mean values at the 95% confidence level by comparing the redox method and method using Phadebas tablets, except for the sample 6 (acacia) and 9 (lavender), where this assertion is valid at the 99% confidence level.

It is noticeable that unification of diastase activity unit (DN) can be easily achieved by a very simple conversion.

The unification of these measurements is additionally complicated by the variation of the wavelength of the absorbance maximum of the starch–triiodide complex among different starches. The proposed method offers an absorbance independent measurement, thus eliminating the necessity of using some recommended starch types for the analysis in order to obtain achieve the recommended absorbance values.

There is also no need to use filtration or centrifugation to separate insoluble fragments or to adjust the "blue value" before starting the measurement, both of which indicate that this methodology is much simpler and faster than those previously described. This method also yields measurements to be recorded without additional solution dilutions and volume adjustments, thus making the error associated with the procedure lower than that of the Schade method.

4. Conclusion

The use of direct potentiometric principles for determination of honey diastase activity was presented. The methodology is based on measurement of free triiodide released from a starch triiodide complex by using a platinum redox sensor during the biocatalytic degradation of starch.

The mechanism of the sensor response was proposed, and the method was tested on 10 honey samples of varying diastase activity. The new methodology exhibited a good correlation with both standard methods and offered a simple unit conversion to DN units for diastase activity.

The described method requires low cost instrumentation and less volume manipulations, additional calibrations and calculations, thus making the measurements more accurate and suitable for routine analysis and miniaturization in microfluidic systems.

The analysis time was reduced to 5 min per honey sample.

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