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A microcalorimetric enzymatic method for trehalose determination in food

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Abstract As trehalose is a glucose font and also an additive in food, a new reliable method for trehalose determination is proposed. The analytical method uses an isothermal microcalorimeter, directly relates the analyte concentration with the heat variation of the enzymatic decomposition of trehalose into two glucose molecules. The enzymatic reaction is performed inside the calorimeter in the presence of *trehalase* enzyme immobilized on amino activated glass beads. Through the calibration curve, the trehalose quantity in some food samples (mushrooms and honey) has been determined. The calorimetric procedure was compared to a previously identified methodology based on an amperometric biosensor.

Keywords Enzymatic calorimetry · Food analysis · Immobilized *trehalase* · Trehalose

Introduction

The α,α-trehalose (1-*O*-(α-D-glucopyranosyl)-α-D-glucopyranoside) is a disaccharide in which two α-D-glucose molecules are linked by a 1,1 bond ($C_{12}H_{22}O_{11}$; M.W. 342). It is present in many microorganisms and also in many foods: mainly honey, apples, and mushrooms. In humans, this disaccharide is metabolized by the *trehalase* enzyme [1].

Trehalose plays a protective role on the cells against stress. In fact this sugar, in the absence of water, preserves membrane on protein structures [1]. This compound is strongly present in edible mushrooms. It can cause intolerance to people who are lacking or have only low levels of the *trehalase* enzyme. Because of its peculiar physicochemical properties, this saccharide is also used as a food additive; it maintains the integrity of the products during the freezing or drying procedures by the retention of water surrounding the product [2, 3].

The particular flexibility of the glicosidic bond between the two anomeric carbon ions of trehalose allows the sugar and macromolecules interactions to be easy realized. This means that even in low temperature conditions, proteins and also nucleic acids are entrapped in a trehalose surface and so protected from degradation due to the crystallization of the water molecules. Trehalose shows a capacity to maintain in part its amorphous glass state, instead of getting the crystalline form directly at low temperatures and also in dry conditions; it can act as a preservative of food macromolecules in those stressing conditions, probably because it can control the water activity in complex matrices. It does seem that trehalose could substitute water molecules in the surroundings of macromolecules by means of hydrogen bonds with the polar groups of phospholipids or of aminoacids [4].

The accurate determination of this sugar in food plays an important role in analytical chemistry.

Some methods are reported in the literature (chromatographic, FIA, and spectrophotometric ones [5–8]), but in all cases they need some pre-treatment of the samples and in some cases the detection limits are quite high.

The microcalorimetric method here proposed makes use of an enzymatic reaction, very specific for the trehalose substrate; it can be applied directly on the real samples without pre-treatments or extractions. The microcalorimetric technique is also very useful for analytical

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applications [9]. It is very sensitive and it allows direct measurements on the sample under examination, also in a non-homogeneous state. The analyte concentration by means of a bioreceptor (such as a specific enzyme) put inside the calorimetric reactor is simply detected, via an enzymatic reaction occurring in the calorimetric core with a heat effect which is revealed directly and reliably.

The proposed method, together with another method, based on an amperometric biosensor [10], has been compared.

Experimental

The calorimetric methodology here described has already been reported in previous papers [11–13].

The microcalorimeter is an isothermal batch instrument [14] of the heat conduction type (LKB Model 2107), equipped with two gold vessels of about 7 mL total volume (Fig. 1), a multi-temperature cooling circulator (LKB Model 2209), a control unit (LKB Model 2107-350), and a potentiometric recorder (LKB Model 2210). Each vessel consists of a chamber partially divided into two compartments (2.5 and 4.5 mL) by an interior wall. The reactants are inserted into the two compartments of each vessel separately. When the experiment begins, the calorimetric drum rotates, thereby the reactants are mixed and the reaction takes place.

All the equipments are housed in a thermostatic room at 25 ± 1 °C, and all measurements were made at 25.00 ± 0.01 °C. The calorimetric accuracy was checked by measuring the sucrose dilution heat. The results were always consistent with the literature values within 0.5% [15].

The instrument output is a curve $V = f(t)$, where V is voltage in mV and t is time in seconds. The curve area A_{react} (in cm^{-2}) is then converted in Q_{react} (heat quantity involved in the reaction) expressed in mcal, by means of an instrumental constant $\varepsilon = Q_{cal}/A_{cal}$ expressed in mcal cm^{-2}, determined by a series of electrical calibrations (performed with a calibrated resistance lain inside the measuring vessel).

Fig. 1 Inside view of the batch microcalorimeter

The *trehalase* enzyme was immobilized on amino activated glass microbeads (Sigma-Aldrich Co.).

Preliminary measurements have been performed to find the best operative conditions with respect to the enzymatic hydrolysis of trehalose and also for the calorimetric responses. In particular, a lot of effort has been made in finding out the best immobilizing procedure [16] for the enzyme and also in selecting the optimum activity in excess of the immobilized enzyme. Finally, the *trehalase* enzyme has been immobilized on amino-activated glass microbeads 0.2 IU mL^{-1} equivalent to 0.01 IU mg^{-1} of beads (1 International Unit of *trehalase* converts 1 µmol of trehalose to 2 glucose µmol per min); pH was equal to 6.5 in phosphate buffer 0.1 mol L^{-1} and working temperature was $T = 25$ °C.

The solution volumes inside the calorimetric vessels (reference and measuring) were always 1 mL on each side of each vessel. The measuring vessel was filled with 1 mL of *trehalase* suspension (0.2 IU mL^{-1}) and 1 mL of trehalose solution (X mol L^{-1}) separately, while the reference vessel was filled with 1 mL of buffer solution and 1 mL of the same trehalose solution (X mol L^{-1}).

In order to obtain the calibration curve for the trehalose analysis, in the presence of *trehalase* enzyme, pseudofirst order kinetic condition with respect to the substrate has been set up by using of an excess enzyme (0.2 IU mL^{-1}). The concentration range of the saccharide studied ranged from 2.0×10^{-5} to 10.0×10^{-5} mol L^{-1}. Linearity has been respected up to 7.5×10^{-5} mol L^{-1}, and the linear part of the curve is described by the equation:

$$Y = -0.1251\,X + 0.8597\left(R^2 = 0.9914\right),$$

where Y is the heat quantity expressed in mJ and X the trehalose concentration in mol L^{-1} (standard deviations ranged between ±0.02 and ±0.05).

By means of the calibration curve, some food samples have been tested to determine the trehalose content.

The results of the microcalorimetric method have been compared with those obtained with a flow amperometric system of analysis (elsewhere described [10]).

The electrochemical system (Fig. 2) is composed of an amperometric detector (VA 641 Metrohm) equipped with a GOD (*Glucose Oxidase*) SPE (Screen Printed Electrode) electrode for Glucose and a flow reactor with *trehalase* enzyme immobilized on Immunodyne ABC membrane (Pall Corporation Co.).

All other reagents were analytical grade from Sigma-Aldrich Co.

The water used in all the prepared solutions was deionized water redistilled on $KMnO_4$.

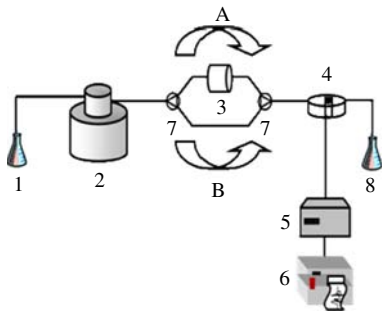

Fig. 2 Scheme of the flow electrochemical system for the trehalose determination (*1* sample solution; *2* peristaltic pump; *3* T*rehalase* reactor; *4* GOD biosensor; *5* amperometer; *6* recorder; *7* switch T valve; *8* waste). *A* direction of the total glucose assay; *B* direction of the intrinsic glucose in the sample assay

Results and discussion

The outlined method was applied to some mushroom samples (*Boletus edulis*) and to Italian honey samples. The mushroom samples were frozen, dried, powdered, and so prepared: a weighed sample aliquot minced and then homogenised with the buffer.

The honey samples, coming from different Italian Regions, were simply weighed and solved in buffer solution. One milliliter of these prepared solutions was directly inserted into the calorimetric vessels. The measurements of the real samples were performed in the same way as described for the calibration curve in the presence of the same enzymatic solution.

In Table 1 the trehalose concentrations found in the real samples are listed.

As can be seen from Table 1, the samples of honey from Lazio contain only traces of trehalose compared with other kinds of honey. With respect to the mushroom samples, the trehalose levels found were in good agreement with those reported in the literature [17].

The calorimetric method showed to be reliable because it allows very sensitive measurements (LOQ equal to 2×10^{-5} mol L^{-1} equivalent to 0.1 mg of trehalose g^{-1} of dried sample), with high accuracy and precision (SD $\leq$ 0.15 on real samples). The enzymatic reaction involved is very specific for substrate trehalose, and real samples can be easily analysed without pretreatments or cleaning. Owing to the fact that the disaccharide is highly soluble in water medium, the simple dilution in buffer aqueous solution is sufficient. Moreover, some preliminary experiments using the standard adding method were done to assess the complete availability of trehalose for the interaction with the specific *trehalase* enzyme. The results showed a recovery of about 87% in mushrooms and about 90/93% in honey samples. The reaction enthalpy associated with the enzymatic hydrolysis of trehalose is sufficiently high and therefore the calorimetric responses are large enough (in the range of tens and hundreds of millivolts) to obtain reaction curves which are easily measurable and so that concentration levels of 10^{-5} mol L^{-1} are detectable.

Table 1 Trehalose concentrations found in the real samples (the value are the average of three measurements of the same sample); SD $\leq$ 0.15

Sample	Trehalose/ concentration/mg g^{-1a}
Frozen mushroom (*Boletus edulis*)	1.2
Dried mushroom (*Boletus edulis*)	2.0
Powdered mushroom (*Boletus edulis*)	0.9
Honey from Calabria Region	0.9
Honey from Lazio Region	0.1

[a] Trehalose concentration/mg g^{-1} of dried sample

Table 2 Comparison between the results obtained for the trehalose concentrations in a mushroom sample by using the microcalorimetric and the amperometric methods

Mushroom sample	Trehalose (calorimetric) (concentration/ mg g^{-1a} ± SD)	Trehalose (amperometric) (concentration/ mg g^{-1a} ± SD)
Dried (*Boletus edulis*)	2.0 (±0.14)	1.9 (±0.19)
Frozen (*Boletus edulis*)	1.2 (±0.15)	1.4 (±0.13)

[a] Trehalose concentration/mg g^{-1} of dried sample

In order to validate the microcalorimetric method, some samples of the same mushroom have been analysed by means of an amperometric biosensor (elsewhere described [10]). The results are listed in Table 2.

This proposed method for trehalose assay can be usefully applied to quality control and also for verifying security in the food industry, whenever this disaccharide is used as an additive.

In conclusion, it can be asserted that the biosensing-based analytical methods forward a high reliability in food analyses, independently from the trasduction system used. In fact, they assure a good response especially when complex matrices are present.

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