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Short communication

Determination of proline in honey: Comparison between official methods, optimization and validation of the analytical methodology

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ABSTRACT

The study compares official spectrophotometric methods for the determination of proline content in honey – those of the International Honey Commission (IHC) and the Association of Official Analytical Chemists (AOAC) – with the original Ough method. Results show that the extra time-consuming treatment stages added by the IHC method with respect to the Ough method are pointless. We demonstrate that the AOACs method proves to be the best in terms of accuracy and time saving. The optimized waiting time for the absorbance recording is set at 35 min from the removal of reaction tubes from the boiling bath used in the sample treatment. The optimized method was validated in the matrix: linearity up to 1800 mg L^{-1}, limit of detection 20 mg L^{-1}, limit of quantification 61 mg L^{-1}. The method was applied to 43 unifloral honey samples from the Marche region, Italy.

1. Introduction

Proline is the predominant free amino acid of honey and it is a measure of the level of total amino acids (Iglesias, de Lorenzo, Polo, Martin-Àlvarez, & Pueyo, 2004). The proline content of honey is measured as a criterion for estimating the quality (Bogdanov, 2002; Von der Ohe, Dustmann, & von der Ohe, 1991) and the antioxidant activity of the honey (Meda, Lamien, Romito, Millogo, & Nacoulma, 2005; Saxena, Gautam, & Sharma, 2010) and it may be used also for characterization on the basis of botanical origin (Bogdanov, Ruoff, & Persano Oddo, 2004; Persano Oddo, Piazza, Sabatini, & Accorti, 1995; Soria, González, De Lorenzo, Martinez-Castro, & Sanz, 2004).

Over time many spectrophotometric methods have been used to determine proline content (Ough, 1969; Troll & Lindsley, 1955; White & Rudyj, 1978; Wren & Wiggal, 1965). To date, the analytical methods reported in the literature for the determination of proline in honey refer to official methods of the International Honey Commission, IHC (Bogdanov, 2002), and of the Association of Official Analytical Chemists (AOAC, 2005). These methods are derived from the original Ough method (1969), in which the content of proline was measured by spectrophotometry from the colour developed with ninhydrin at a wavelength of 510 nm. The IHC method introduces some significant changes that lengthen the time of analysis, the most important of which involves the use of a water bath at 70 °C for 10 min following the boiling bath included in the original method. The AOAC method follows the original procedure, but includes the subtraction of the interference due to the colour of honey on the absorbance recording of the reacted test solution, as provided for in the work of White and Rudyj (1978). From the analytical point of view, it is important at this point to know if different methods give the same (or different) results and to understand if it is possible to compare results of different studies which use different methods of analysis. Another critical point is the lack of a complete validation of the proposed methods: the official methods are validated for accuracy (studied with a recovery test), and precision (in terms of repeatability and reproducibility), but they provide no information about linearity range, limits of detection or quantification in matrix for the 2 official methods proposed nor for the original Ough method.

The purpose of the present study was to compare the 2 official methods for proline determination in honey, the IHC method and the AOAC method, with the original Ough method (1969) and to choose the best method in terms of accuracy and time saving. In order to verify the performance of the best method chosen and optimized for the waiting time for absorbance recording, quality parameters such as accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), and linearity range were also evaluated.

The method was applied to 43 unifloral honey samples from the Marche region, Central Italy.

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2. Materials and methods

2.1. Sample collection

Forty-three different unifloral honey samples produced during Spring–Summer 2009 in the Marche Region, Italy, were collected by the Centro Agrochimico Regionale A.S.S.A.M. (Agenzia Servizi Settore Agroalimentare Marche), Ancona. The unifloral honey samples were: 21 from acacia (*Robinia pseudoacacia*), 6 from chestnut (*Castanea sativa*), 3 from coriander (*Coriandrum sativum*), 2 from lime (*Tilia* spp.) and 9 from sunflower (*Helianthus annuus*). Two honeys were *Metcalfa* honeydew samples. A palinological study of the honey samples was performed in order to guarantee their labelled botanical origin (Louveaux, Maurizio, & Vorwohl, 1978). The samples were maintained at a temperature of 4 °C until the time of analysis.

2.2. Reagents and standards

High-purity water was Milli-Q from Millipore (Bedford, MA, USA). Deionized water was Elix 3 (Millipore). All the reagents for the spectrophotometric methods were from Carlo Erba, Milan, Italy, except Formic acid 98%, from Baker (Austin, Texas). The L(-)proline reference solution was prepared by daily diluting the stock solution (containing 40 mg L^{-1} proline, 97% purity, in 50 ml) with Milli-Q water to give a solution containing 0.8 mg/25 ml. Ninhydrin (1,2,3 triketohydrindene) was made up as a 3% solution (wt/vol) in 2-methoxyethanol. Isopropanol was reagent grade diluted 1:1 by volume with water.

2.3. Proline determination

The sample analyzed should be representative of the honey lot, so all honey samples were prepared according to the IHC method (Bogdanov, 2002). About 5 g of homogenized honey was weighed and dissolved in water, then quantitatively transferred to a 100 ml volumetric flask and diluted with water.

Van Slyke, Dillon, MacFadyen, and Hamilton (1941), affirm that the molar extinction coefficient of proline is not constant, as it depends on the pH of the solution. As such, Bogdanov (IHC, 2002) underlies the need to measure, for each series of measurements, the molar extinction coefficient of the proline standard solution. In our experiments, we followed the IHC method in order to investigate whether the molar extinction coefficient of proline is constant or not. The absorbance was determined using a Lambda 25 double-beam spectrophotometer UV/Vis, Perkin Elmer, Waltham, Massachusetts, USA. A spectrum of proline from 440 to 560 was performed to evaluate the wavelength at a maximum absorbance, which was 513 nm.

2.4. Official analytical methods

The sample treatment procedure for proline determination using the original Ough method is shown schematically in Fig. 1. The IHC method features substantial differences (Fig. 1, left pane), the most significant of which is the temperature of the bath following the boiling bath: the original method provides for a cooling bath at 70 °F (~22 °C), whereas the IHC method transfers samples to a water bath at 70 °C. Another change is the waiting time from the beginning of the cooling time and absorbance recording, which

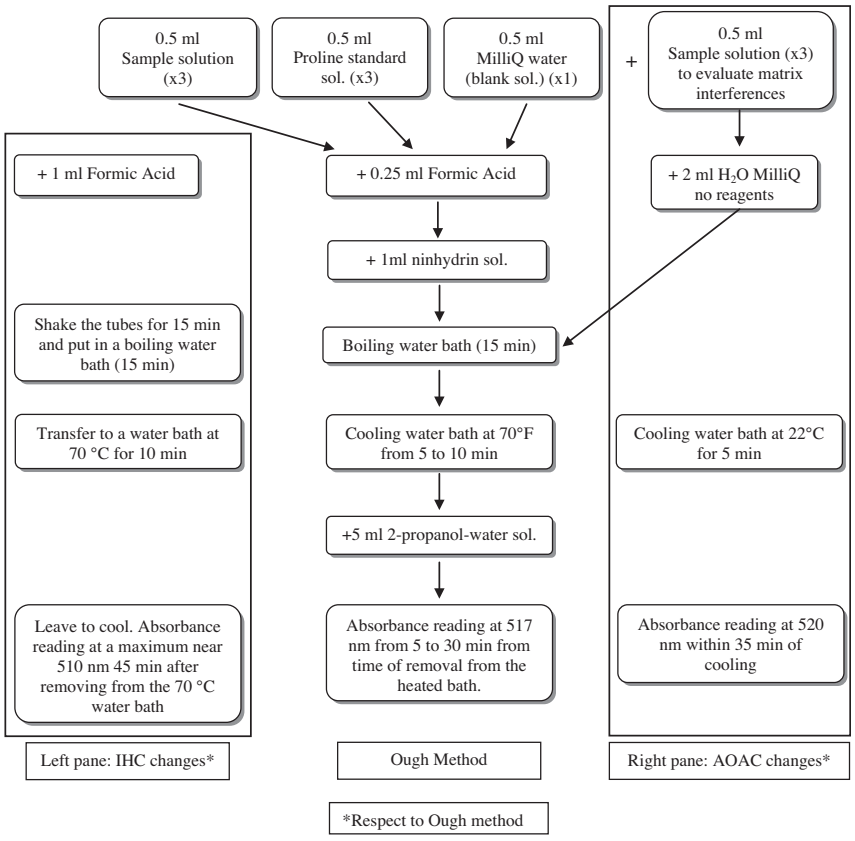

Fig. 1. Timetable of events (diagram) of sample treatment for proline determination with the Ough method. In the right pane the changes introduced by the AOAC method, in the left pane the changes introduced by the IHC method.