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A microcalorimetric sensor for food and cosmetic analyses: L-Malic acid determination

Marta Letizia Antonelli *, Claudio Spadaro, Rosalia Fortunata Tornelli

Dipartimento di Chimica, Università degli Studi di Roma "La Sapienza", Piazzale Aldo Moro, 5, 00185 Rome, Italy

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Abstract

Enzymatic microcalorimetry has been successfully employed in the reliable determination of the L-malic acid concentration in some foods and cosmetic products. The L-malic acid concentration during the wine-making process is particularly useful in order to control the progress of the malo-lactic fermentation. Total acidity, taste and flavour characteristics of wine depend on the L-malic acid quantity still present.

To point out the analytical methodology the dehydration process of L-malic acid, in the presence of *Fumarase* enzyme, has been used. The new method has been compared with a common spectrophotometric one.

By the proposed calorimetric method the L-malic acid concentration in different types of food (white and red wines, fruits and soft beverages) has been determined. In some cosmetic products too the L-malic acid was quantified.

The method outlined resulted simple, direct and reliable (good accuracy and precision), in particular it does not require any pre-treatment or clean up of the samples, save the dilution in buffer.

Keywords: L-Malic acid; *Fumarase* enzyme; Food; Cosmetics; Calorimetric sensor

1. Introduction

The L-isomer of malic acid is the natural form of this acid, therefore, it is present in many foods, for example, fruits and wine; only if the synthetic racemate is used as an additive in food industry, the D-malic acid could be found [1].

In the food industry, in particular in soft drinks, fruit juices and wine production, the L-malic acid determination is widely employed [2]. The L-malic acid being a component of those products gives the particularly acidic flavour and taste if present in the proper quantity, otherwise the good characteristics of those foods are altered.

Together with the tartaric acid the L-malic acid is a fundamental component of wine, they represent about the 90% of the wine total acidity [3]; L-malic acid is the substrate of a bio-transformation process named the malo-lactic fermentation (MLF) [4]. This is an important process in wine production, not only due to the partial de-acidification determined by the transformation of L-malic in L-lactic acid, but also because it contributes in the *bouquet* of fragrances and in the microbiological stability of wine [5]. The MLF fermentation, due to some bacteria naturally present in wine, is a spontaneous phenomenon really unpredictable, especially when the initial L-malic concentration in must is high: in these cases the final product could present a disagreeable taste and flavour [6].

In the literature many different methods for L-malic acid determination are reported: the chromatographic ones [7,8] present high sensitivity and precision, but need long and tedious clean up and generally are not so recommended if only one analyte must be quantified. Many other methods based on enzymatic reactions (i.e., photometric and electrochemical ones) make use of the enzymatic transformation of L-malic acid to oxaloacetic acid in the presence of NAD^+/NADH. Being this reaction unfavourable from a thermodynamic point of view, it is always necessary to force it by means of secondary reactions and particular pH conditions [9]. The electrochemical methods too necessitate of an enzymatic reactions chain in order to obtain a final product easily measurable by ISE electrodes [10,11].

The new method here outlined is direct and easy, is based on the microcalorimetric technique and uses the enzymatic dehy-

* Corresponding author. Tel.: +39 0649913618; fax: +39 06490631.
E-mail address: marta.antonelli@uniroma1.it (M.L. Antonelli).

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dration of L-malic acid catalysed by the *Fumarase* enzyme [12,13].

By this methodology [14] the L-malic acid concentration is directly correlated with the heat quantity associated with the enzymatic reaction, a main parameter of the reaction employed (ΔH_{react}), without chain reactions. Moreover, by this technique samples as emulsions and suspensions can be immediately analysed [15], therefore, the proposed method can be usefully adopted to control food products and also the wine-making process.

Owing to the fact that in the last years the acids named AHA (α-hydroxy acids) such as the L-malic acid, have been widely used in the cosmetic field, especially for their anti-aging effect on skin, but at the same time they showed some sensitisation effects of the skin to the UV radiations, the accurate determination of their concentration in the cosmetic products has become of interest [16]. The method has been, therefore, applied to the analysis of some cosmetic creams containing AHA in hydrophobic matrices.

2. Experimental

2.1. Materials

All common chemicals were commercial products of analytical grade (purity ≥ 99%). L-Malic acid was a Sigma–Aldrich Co. product; Fumaric acid 99% of purity was from Sigma. *Fumarase* enzyme (EC 4.2.1.2) (1 IU-International Unit- will convert a 1.0 μmol of L-malate to fumarate per minute at pH 7.4 at 25 °C) suspension from porcine heart was from Sigma–Aldrich Co.

L-Tartaric acid and L-ascorbic acid were from Carlo Erba. The enzymatic kit (L-malic UV test) used for the spectrophotometric assay was from Boehringer-Mannheim.

Industrial wine samples were kindly provided from "Cantina Sivestri" and those of artisan production from "Teatini" and "Ambrosini" families. All other food and cosmetic samples were common shelf products. Buffer solution at pH 7.4 was obtained with KH_2PO_4 and Na_2HPO_4 (5×10^{-3} mol L^{-1}) salts. All solutions were prepared in doubly distilled water and maintained at 4 °C until used, if necessary.

2.2. Apparatus

The microcalorimeter used is an isothermal batch instrument [17] of the heat conduction type (LKB Model 2107), equipped with two gold vessels of about 7 mL total volume, 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 in two compartments (2.5 and 4.5 mL) by an interior wall. The reactants are inserted into the two compartments separately. When the experiment begins the calorimetric drum rotates, thereby the reactants are mixed and the reaction takes place.

All the equipment was 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 consistent with the literature values within 0.5% [18].

In order to check the purity of the L-malic acid standard a thermo-gravimetric measurement, by using a Perkin-Elmer TGS-2 thermo-balance, has been performed. Water content resulted less than 1% at low temperatures and after that the total decomposition of L-malic molecule was observed.

Spectrophotometric measurements were performed by means of the UV/vis Philips Pye-Unicam PU 8800 spectrophotometer.

The pH of the solutions has been measured with a pH-meter Gibertini with an Ingold glass-combined electrode.

A homogeniser Ika Ultra turrax T 18 Basic was used.

To sonicate some cosmetic and food samples the ultrasound apparatus AGE Elettronica mod. Acivar was employed.

2.3. Methods

2.3.1. Calorimetric method

The enzymatic reaction used in this analytical method is the dehydration of the L-malic acid to fumaric acid in the presence of the *Fumarase* enzyme. This reaction in fact is of the equilibrium type, but it is stereo-specific for the L-malic substrate, while it is not in the case of the fumaric acid [19]. The concentration and pH conditions strongly influence the reaction, and moreover, the low solubility of fumaric acid [13] allows the L-malic acid to be transformed in fumaric easier than the inverse.

The reaction is rapid and it is overseen by microcalorimetry [20,14], by using the linear relationship between the involved heat ($Q_{reaction}$) and the L-malic acid concentration:

$$Q_{reaction} \propto C_{substrate}.$$

This relationship is linear if the appropriate kinetic conditions are realised: that is in excess of enzyme, first order kinetics with respect to the substrate. The heat quantity involved in each run is obtained by the equation:

$$Q_{reaction} = \varepsilon A_{reaction}$$

where ε is an instrumental constant (expressed in mcal cm^{-2}), determined by a series of electrical calibrations, which depends on the physical features of the calorimeter in different experimental conditions of temperature, sensitivity and record scale. $A_{reaction}$ is the area (expressed in cm^2) subtended by the curve (voltage function of time), which is the instrumental output in each run and $Q_{reaction}{:}A_{reaction} = Q_{calib}{:}A_{calib}$. By means of a calibration curve ($Q_{reaction}$ in function of L-malic acid concentration) obtained with standards, it is possible to calculate the analyte content in samples.

The instrument was filled as follows: the two compartments of the sample vessel were filled with 2 mL of the enzyme solution and 2 mL of the substrate solution, respectively, while the reference vessel was filled with 2 mL of the buffer solution and 2 mL of the substrate solution, respectively, in order to subtract the dilution heat of the L-malic solution from the total heat of reaction. The dilution heat of the enzyme has been checked by preliminary experiments, and it was subtracted from the total heat measured in each measure.