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CALORIMETRIC ANALYSIS OF THREE HYDROXY ACIDS AS MARKERS FOR QUALITY AND SAFETY IN FOOD

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A new analytical methodology to quantify three hydroxy acids (orotic, ascorbic, *L*-malic acids), by isothermal solution microcalorimetry, was outlined and applied to different foods. Three specific enzymatic reactions were used to ensure the correctness of the results. The considered acids can be considered as markers in food quality for their biochemical peculiarities.

The enzymatic microcalorimetric method is very reliable and linearity is satisfied in the concentration ranges useful for food analyses. The analytical results of the underlined method are very accurate, precise, sensitive and in good agreement with the values obtained with other common methods.

Keywords: *ascorbic acid, enzymatic microcalorimetry, food analysis, L-malic acid, orotic acid*

Introduction

Some hydroxy acids, natural constituents of different foodstuffs can be considered as markers of good quality of foods. In particular three acids have been taken into account: ascorbic, orotic and *L*-malic acid, very important because of their anti-oxidant and nutritional properties.

The correct and reliable determination of their concentrations in natural and commercial foods represents a good index of quality and safety in foods. The two first mentioned acids are micro-nutrients, especially in milk (a very important feed for growth): it is well known the fundamental action of the ascorbic acid (Vitamin C) in human health [1, 2] and also the orotic acid is important because it is a precursor in the nucleic acids metabolism [3, 4]. The concentration of the *L*-malic acid is a very critical parameter in foods [5, 6] because it is responsible of acidity and also of good taste and flavour in fruits and wine.

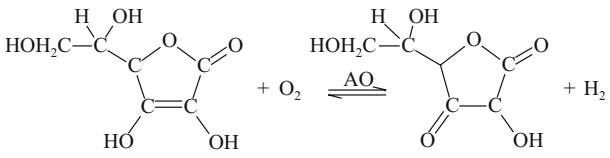
The use of solution microcalorimetry combined with specific enzymatic reactions ensures analytical results accurate and sensitive, because it allows a thermodynamic parameter (the heat quantity associated with each reaction under examination) to be measured directly [7].

The analytical methods described in this study have been validated by comparison with other common analytical methodologies, such as spectrophotometry and/or HPLC.

Ascorbic acid (Vitamin C) determination

A man can assume Vitamin C through vegetables, fruits and other foods as milk and meat, which contain it. Or he can take special drugs containing vitamins as integrators. It is very important to know the exact concentration of vitamin C in the different substances, so as to take the right quantity, which can be daily assimilated.

The method here proposed is based on the microcalorimetric determination of ascorbic acid by means of its enzymatic oxidation in the presence of the ascorbate oxidase (AO) enzyme and oxygen.



ascorbic acid

The microcalorimetric method was validated by comparison with a known HPLC method [8], by analysing some replicates of drug sample.

Orotic acid determination

Being an intermediate in the uracile base synthesis [9], the orotic acid plays an important role in the mammalian growth. In fact it is present essentially in milk and it can be considered a useful marker of the quality of this food.

The determination of the orotic acid has been performed by microcalorimetry and using the enzy-

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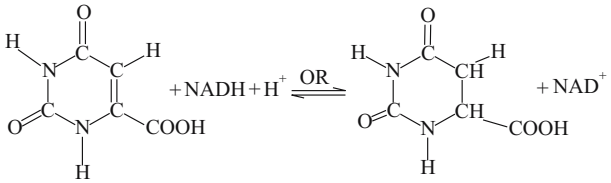
matic reduction reaction in the presence of the orotate reductase (OR) enzyme and NADH.



orotic acid

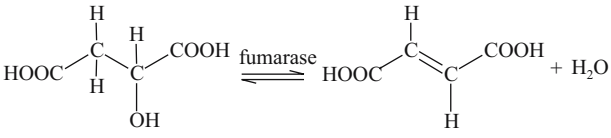
The results obtained with the method here proposed have been compared with those obtained by a known spectrophotometric method [10], by analysing some replicates of milk sample.

L-malic acid determination

L-malic acid is a component of fruits and wine and is present in industrial foods as soft drinks and fruit juices. Its principal characteristic is to give to foods the acidic flavour and taste. In particular it is a fundamental component of wine (together with the tartaric acid represents about the 90% of wine total acidity) and is the substrate of a bio-transformation process named malo-lactic fermentation [11], due to some bacteria naturally present in wine, which is a spontaneous phenomenon really unpredictable. If this fermentation goes on too much, the wine becomes too much de-acidified and could presents a disagreeable taste and flavour.

So the correct control of the *L*-malic acid concentration is an important goal in the food industry and also in the wine-making process.

The calorimetric methodology here presented makes use of the transformation of *L*-malic acid into fumaric acid catalysed by the *Fumarase* enzyme [12].



L-malic acid

The proposed method has been compared with a recommended spectrophotometric one, which uses a cascade of enzymatic reactions [13], by analyzing some replicates of wine sample.

Experimental

Materials

All chemicals were commercial products of analytical grade (purity ≥99%). Ascorbic acid standard was from Carlo Erba, *Ascorbate oxidase* enzyme lyophilised powder from *Cucurbita species* (E.C. 1.10.3.3) was from Sigma; orotic acid standard and *Orotate reductase* enzyme lyophilised powder from *Zymobacterium oroticum* (E.C. 1.3.1.14), *L*-malic acid standard and *Fumarase* enzyme buffered suspension from porcine heart (E.C. 4.2.1.2) were from Sigma–Aldrich.

The enzymatic kit (*L*-malic UV test) used for the spectrophotometric assay of the *L*-malic acid was from Boehringer–Mannheim.

Real food or pharmaceutical samples were common commercial products. Buffer solutions were prepared with analytical grade chemicals.

All solutions were prepared daily with deionised water redistilled on $KMnO_4$ and maintained at 4°C until used.

Apparatus

Batch solution calorimetry at 25.00+0.01°C was done with an LKB mod. 2107 instrument, as reported elsewhere [14]. Performance was checked by measuring the dilution heat of aqueous sucrose [15].

The spectrophotometric apparatus used was a UV-visible Philips Pye-Unicam 8800, equipped with quartz vessels of 1 cm o.p.

A Gibertini pH-meter equipped with a combined glass electrode Ingold was also employed.

In order to check the purity of the standards a thermogravimetric apparatus Perkin-Elmer TGS-2 thermo-balance was used.

A homogeniser Ika Ultra turrax T 18 Basic and an ultrasound sonicator apparatus AGE Elettronica mod. Acivar were employed.

Methods

Calorimetry

Calorimetric measurements allow the acid concentration to be determined directly, because the heat quantity associated with each enzymatic reaction ($Q_{reaction}$) is linearly related with the substrate concentration (each acid under examination represents the substrate of the specific enzymatic reaction used): $Q_{reaction} \propto C_{substrate}$. This relationship is linear if the appropriate kinetic conditions are realised: that is in excess of enzyme so that the K_M of the appropriate enzyme>>$C_{substrate}$. 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 submitted by the curve (voltage function of time) which is the instrumental output in each run.

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 substrate solution from the total heat reaction. The dilution heat of the enzyme has been checked by preliminary experiments, and it was neglected if it was found quite null, otherwise it has been subtracted in each calculation. The reported concentrations refer to the solutions inside the calorimeter before mixing.

For each enzymatic system examined, to obtain the calibration curve and also to measure the real samples, preliminary experiments were run to find out the best operative conditions. The optimum enzyme activity was each time determined by checking the calorimetric responses fixing the substrate concentration and changing the enzyme activity.

Spectrophotometry

The spectrophotometric analyses performed for the orotic and *L*-malic acids are based on the absorbance measurements and by means of the known Lambert–Beer law the concentration values are obtained through a calibration curve: absorbance in function of concentration.

For the orotic acid analysis [10] the absorbance of NADH is measured at λ=282 nm in function of time, the decrease of absorbance *vs.* time is proportional to the orotic acid concentration.

For the *L*-malic determination [13] the increasing of absorbance of NADH at λ=340 nm is monitored in function of time and related with the *L*-malic concentration.

HPLC (high performance liquid chromatography)

The chromatographic method is based on a reported one [8], it uses a HPLC instrument and the measurements were done by isocratic analysis at a flow rate of 1.0 mL min^{-1} with a solvent mixture formed by tetra-butyl-ammonium hydroxide (adjusted to pH 5.0 with formic acid) and acetonitrile (80:20 *v*/*v*). Detection was made by measuring the absorbance at λ=254 nm.

Results and discussion

Ascorbic acid (Vitamin C)

The calibration curve to determine the ascorbic acid concentrations, obtained with solutions of pure ascorbic acid standards, is linear in the range 3–270 mg L^{-1}. The plot is described by the equation: $Q_{reaction}=0.4525C_{ascorbic}$ and its r^2 value is 0.9979. The best operative conditions were: T=25°C, acetate buffer 0.1 mol L^{-1} at pH 5.6 and the AO enzyme in excess was 11 IU mL^{-1}.

In these operative conditions different food samples have been tested, the results are listed in Table 1, note that very concentrated samples have been diluted in buffer before analysis.

Table 1 Vitamin C content in various food and drug samples (values are means of three analyses of each sample), determined by microcalorimetry

Drink samples	[Ascorbic acid]/mg L^{-1}±S.D.
Orange juice	355±5
Fresh orange juice	530±5
Sport drink	317±6
ACE juice	162±2
Food/drug samples	**[Ascorbic acid]/g per 100 g±S.D.**
Candies	2.4±0.4
Vitamin C tablets	4.8±0.1
Integrator	0.52±0.07
Aspirin C	9.5±0.3
Milk samples	**[Ascorbic acid]/mg L^{-1}±S.D.**
Whole fresh	11.05±0.04
UHT	3.1±0.2

In order to compare the calorimetric method with the HPLC one, samples of Aspirin C tablets have been tested and the values for the vitamin C content resulted in a very good agreement (the equation of this correlation is $Y_{cal}=1.2197X_{spect}-2.0204$, with $r^2=0.9985$). It must be noticed that by means of the calorimetric method no particular treatments or clean-up of the samples, even if they are non homogeneous or solids, are needed contrarily to the chromatographic method.

The effect of time and light on the shelflife products, containing vitamin C, has been studied by analysing the vitamin C levels during conservation in transparent and non-transparent containers, in standards and real samples (milk solutions and orange juice solutions). The results showed that samples with a lower starting concentration of vitamin C reduced their content more rapidly that the others. Moreover it was observed that the standard solutions lose their content of vitamin C faster than the real samples. All samples maintained in transparent containers lost vitamin C very faster than those conserved in non-transparent containers, as expected.

Table 2 Comparison between the results (orotic acid concentrations, mg L^{-1}) obtained by calorimetry and spectrophotometry for the same samples of two different marks of pasteurised milk, values are averages of three measurements ±S.D of each sample

Method	Milk sample	High quality	Whole	Semi-skimmed	Skimmed
Calor.	1	25.97±0.01	26.61±0.02	26.75±0.02	29.11±0.01
Spectro.	1	25.98±0.04	23.55±0.05	26.84±0.06	28.97±0.05
Calor.	2	25.70±0.02	21.89±0.02	25.90±0.01	33.98±0.02
Spectro.	2	25.77±0.06	21.93±0.04	26.00±0.05	34.08±0.06

Table 3 Orotic acid concentrations (mg L^{-1}) determined by microcalorimetry in cow milk samples (values are means of three measurements ±S.D. of each sample)

Milk sample	Pasteurised	UHT	Sterilized	Powder	High digestibility
High quality	25.97±0.01				
Whole	23.61±0.02	23.10±0.01		37.70±0.02	
Semi-skimmed	26.75±0.02	28.06±0.01	39.31±0.01		22.70±0.02
Skimmed	29.11±0.01	33.06±0.02			

The observed behaviour for the vitamin C content must be ascribed to the oxidation phenomenon, due to light and oxygen; moreover the different trend observed for the standards during time, with respect to drinks/or milk solutions, can be ascribed to the competition in oxidation among vitamin C and the other components of the complex matrices of real samples.

Orotic acid

The calibration curve was obtained using standards of pure orotic acid and the values represent the average of three different calorimetric runs. The range investigated is 20–60 mg L^{-1}, the equation describing the plot is: $Q_{reaction}=0.0382C_{orotic}+43.9$, where $Q_{reaction}$ is expressed in mcal and C in mg L^{-1}; the r^2 value is 0.9993 and the trend is linear in the considered interval.

The operative conditions used are: T=25°C, phosphate buffer 0.07 mol L^{-1} at pH 6.2, the OA enzyme in excess was 1.197 IU mL^{-1}, NADH concentration was taken always 10 times the concentration of orotic acid and $MgCl_2$ concentration was 0.075 mol L^{-1}.

By the described method two different marks of pasteurised cow milk have been tested and the same samples were analysed by using the spectrophotometric method, in Table 2 the values (mean of three determinations) are listed.

As can be seen, there is a good agreement between the two methods, in particular the proposed calorimetric method appears accurate and it shows more precision than the spectrophotometric one.

Other kinds of cow milk were tested by the calorimetric method and the relative values in Table 3 are reported.

It is noticeable that the method here proposed allows the milk samples to be analysed for determining the orotic acid content, directly without any pre-treatment, while in the spectrophotometric analysis a preliminary deproteinization is required. The determination is not affected by the presence of other compounds present in milk matrix. The results show that the heat processing conditions of milk do not influence the orotic acid quantity; only the sterilisation process determines a significant effect on its concentration.

Because of its sensitivity, the proposed calorimetric method could be used to control new functional foods: for example milk with added orotic acid for nutritional purposes.

L-malic acid

The calibration curve to determine the *L*-malic acid concentrations, obtained with solutions of pure standards, is linear in the range 0.07–2.68 g L^{-1}. The plot is described by the equation: $Q_{reaction}=10.52C_{malic}$ and its r^2 value is 0.9991. The best operative conditions were: T=25°C, phosphate buffer $5\cdot10^{-3}$ mol L^{-1} at pH 7.4 and the *Fumarase* enzyme in excess was 18 IU mL^{-1}.

In these operative conditions wine, soft drinks and fruits samples have been analysed, the results are listed in Table 4, note that concentrated samples have been diluted in buffer before analysis.

As can be seen the *L*-malic acid content decreases during fruit ripening and therefore this analyte can be considered a marker quality control in fruits. In wine, owing to the fact that this acid is responsible of the acidic taste and flavour, the *L*-malic acid concen-

Table 4 *L*-malic acid concentrations found in different food samples by the calorimetric method (values are means of three analyses of each sample)

Liquid food products	[*L*-malic acid]/ g L^{-1}±S.D.
Red wine (North-West Italy)	2.84±0.02
White wine just buttled (Central Italy)	1.76±0.03
White wine 1 year old (Central Italy)	1.74±0.02
Soft drink	0.40±0.02
Apple juice (50% of fruit)	1.70±0.03
Solid food products	**[*L*-malic acid]/ (g per 100 g)±S.D.**
'Fuji' apple (ripe)	0.59±0.02
Mandarin (unripe)	0.24±0.03
Mandarin (ripe)	0.14±0.02

tration is a useful index to control the malo-lactic fermentation during the wine-making process.

A sample of wine, at different dilutions, has been used to compare the calorimetric results with those obtained by a spectrophotometric method which employs a commercial kit for the UV test of *L*-malic acid. The plot of the obtained results shows a linear trend with this equation: Y_{cal}=0.9978X_{spect}–0.0144 and a value of r^2=1, the reliability of the here proposed method is thus confirmed.

The proposed analytical method, based on the enzymatic microcalorimetry, appears very accurate, sensible and it allows the three hydroxy acids (orotic, ascorbic and *L*-malic acids) to be analysed in a very easy way. In fact, no pre-treatments of the samples are required, non homogeneous samples can be inserted inside the calorimeter, only the use of an appropriate buffer is required.

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