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Analytical methods

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Rapid and accurate determination of malate, citrate, pyruvate and oxaloacetate by enzymatic reactions coupled to formation of a fluorochromophore: application in colorful juices and fermentable food (yogurt, wine) analysis

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Abstract

A fluorometric-coupled reaction for the accurate and rapid determination malate, citrate, pyruvate and oxaloacetate is presented. The method was found useful for an accurate and rapid determination of these metabolites in low volumes of milk, yogurt, apple and lemon juice and wines without considerable pretreatment. In particular, this method was found valuable in characterizing the outcome of maloactic acid fermentation (MLF) in wine and outlined for the first time fundamental differences in MLF between red and white wines. Thus, this method has merit in analyzing these substances in heterogeneous, opaque and colorful foods.

Keywords: malate, citrate, pyruvate, oxaloacetate, analysis, fluorometric, food, yogurt, wine, colorful juices
1. Introduction

Citric acid (citrate), malic acid (malate) pyruvic acid (pyruvate) and oxaloacetic acid (oxaloacetate) are important components of the intermediate metabolism and occurs in the cells and tissues of virtually all living things. These organic acids are also normal components in food products, such as in milk and various fruits. Citrate is relatively significant constituent of milk that characterized lactogenesis (Peaker & Linzell, 1975).

In bovine milk, citrate at typical concentration of ~ 10 mM forms one of the main buffer systems that regulate the equilibrium between Ca\(^{2+}\) and H\(^+\) ions and thus affects milk coagulation (Faulkner & Peaker, 1982). Citrate affects the aroma of dairy products because its fermentation products yield distinct flavors characteristic of fermented milk products (Urbach, 1995). The biological role of citrate in milk is thought to be maintenance of fluidity through its effects on structure of the casein micelles (Faulkner & Peaker, 1982). In comparison to citrate, malate and pyruvate are minor constituents of milk that are presented in milk at the µM levels, and analysis on their content in milk and yogurt were scarcely conducted. Because malate and pyruvate are consumed by bacteria, they were mainly studied as indicators for bacterial quality of milk (Cousins, Rodrigues, & Fuluford, 1981; Matias, Jaspe, & SanJose, 1994; Senyk, Shipe, Ledford, & Weinberg, 1984; Spohr, & Schutz, 1990). We could not found in the literature information about oxaloacetate concentration in milk. Very little information is available regarding the concentration of the tested acids in yogurts. The situation is more complicated than in milk, because these acids may be consumed or generated during different stages of fermentation and storage (Fernandez-Garcia, & McGregor, 1994).
Together with yeasts, lactic acid bacteria (LAB) are the most important microorganisms in various fermentative processing, leading to products such as yogurts and wine. Yeasts are responsible for alcoholic fermentation, whereas LAB carry out the main process in yogurt-making and malolactic (MLF) stage of fermentation in wine-making (Liu, 2002, 2003; Urbach, 1995).

In wine-making, MLF takes place after alcoholic fermentation and contributes to the microbial stability of the final product and its organoleptic quality (Maicas, 2001; Versari, Parpinello, & Cattaneo, 1999). Phenolic compounds, which are much richer in red wine in comparison to white wine affects LAB fermentation (García-Ruiz, 2008, 2009; Liu, 2002). However, there is scant information how this properties affect the composition of red and white wines.

So far, the main analytical approaches for the determination of organic acids in milk included HPLC (Garnsworthy, Masson, Lock, & Mottram, 2006), capillary electrophoresis (Izco, Tormo, & Jiménez-Flores, 2002) and spectrophotometric (Mezzour, Neffati, & Najjar, 2005; Millan, 1987; Moellering, & Gruber, 1966) methods (see Mato, Suarez-Luque, & Huidobro, 2005 recent review in wines). Though accurate records can be obtained with HPLC and capillary electrophoresis, the use of fluorimetric and spectroscopic methods is more simple and convenient, particularly for routine or high throughput analysis. However, applying monochromatic absorbance photometry for analysis of milk and dairy products is frequently difficult and problematic because they are heterogeneous and complex substances that contain fat droplets of varying size that scatters light in an unpredictable way and contains opaque and colloidal solution of proteins (see discussion in Silanikove, & Shapiro, 2010). Overcoming these problems necessitates the use of various procedures in order to minimize the above described problems.
However, in many cases such pretreatments only partially resolve the problem. In addition, in many cases these procedures are cumbersome and time consuming.

What's common to citrate, malate, pyruvate and oxaloacetate is the involvement of NAD\(^+\)/NADH as cofactors in the enzymatic reactions that regulate their metabolism. Oxidation of metabolites in NAD-coupled reactions can be estimated from the increased absorption of NADH. A common alternative consists of measuring NADH disappearance by coupling its oxidation to NAD\(^+\) with enzyme such as diaphorase (Shapiro, Shamay, & Silanikove, 2002). In the new procedure, we added resazurin to the coupling reaction, which in turn is converted stoichiometrically to the highly fluorescent substance resorufin (Shapiro and Silanikove, 2010). This method was found useful for an accurate determination of D and L-lactate, lactose and galactose in milk, yogurts and colorful drinks, such as red wine and beer, without pretreatments (Shapiro and Silanikove, 2010).

In the present report, we extend this approach for the accurate and rapid determination of citrate, malate, pyruvate and oxaloacetate in milk, yogurt, apple, orange and lemon juice and in red and white wines.

2. Materials and method

2.1 Assay principle

Malate was converted to oxaloacetate with malic dehydrogenase (MDH) in the presence of NAD\(^+\); thereby simultaneously reducing NAD\(^+\) to NADH + H\(^+\). The primary reaction was coupled with diaphorase for the conversion of NADH + H\(^+\) to NAD\(^+\) and the non-fluorescent resazurin to the highly fluorescent substance resorufin (Dejong & Woodlief, 1977):

1. \[ \text{Malate + NAD}^+ \xrightleftharpoons{\text{MDH}} \text{oxaloacetate + NADH + H}^+ \]
2. \[ \text{NADH} + H^+ + \text{resazurin} \rightarrow \text{diaphorase} \rightarrow \text{NAD}^+ + \text{resorufin} \]

Pyruvate in the presence of fixed concentration of excessive NADH was converted to L-lactate and NAD\(^+\) with L-lactic dehydrogenase (L-LDH, reaction 3). The residual non-reacting NADH can be converted to NAD\(^+\) with diaphorase according to reaction 2.

3. \[ \text{Pyruvate} + \beta\text{-NADH} \rightarrow \text{L-lactic dehydrogenase} \rightarrow \text{L-lactate} + \beta\text{-NAD}^+ \]

Citrate was converted to oxaloacetate and acetate with citrate lyase (reaction 4). Oxaloacetate in the presence of fixed concentration of excessive NADH was converted to L-malate and NAD\(^+\) with MDH (reaction 5). The residual non-reacting NADH can be converted to NAD\(^+\) with diaphorase according to reaction 2.

4. \[ \text{Citrate} + H_2O \rightarrow \text{citrate lyase} \rightarrow \text{oxaloacetate} + \text{acetate} \]

5. \[ \text{Oxaloacetate} + \beta\text{-NADH} \rightarrow \text{MDH} \rightarrow \text{L-malate} + \beta\text{-NAD}^+ \]

For the direct determination of oxaloacetate, the procedure started from reaction 5. Citrate concentration is determined by subtracting the concentration of oxaloacetate (reactions 5 and 2) from citrate +oxaloacetate concentration (reactions 4, 5 and 2).

2.2 Foods

Milk was sampled from the commingled milk of six cows with bacterial-free udders. Bacterial-free samples were defined so following absence of bacterial
identification in during 3 sampling taken once every 3 to 4 weeks. The sampling procedure and bacterial identification were carried out according to internationally recognized standard (Leitner, Krifucks, Merin, Lavi, & Silanikove, 2006). The milk sampled for chemical analysis was stored on ice, skimmed (Silanikove, & Shapiro, 2007) and analyzed in 3 replications within the same day. This procedure was repeated over 3 days. Two types of white plain yogurts were bought from local supermarket, stored in a refrigerator and analyzed in triplicates over 3 days. Recovery of the respective analyzed acids was carried out as described before (Shapiro and Silanikove, 2010). The information regarding the producers and product composition is identical to those coded A and B in Silanikove and Shapiro (2010).

Six varieties of red and white wines were bought from local stores. Five of the white wines were locally produced and one of them was imported from Chile (Sauvignon blan). All the white wins were made in 2009; their variety included Muscat (2), Semilllon (2) and Sauvignon blan (2). Five of the red wines were locally produced and one of them was imported from Chile (Pinot Noir). All the red wines were made in 2009; their variety included Sauvignon red (2), Merlot (2) Cabernet (1) and Pinot Noir (1). The chemical composition and the recovery of the tested acids were analyses in the wines in triplicates. In addition L and D-lactate in the wine samples was analyzed according to Shapiro and Silanikiove (2010).

2.3 Chemicals

The following chemicals were obtained from Sigma (Rehovot, Israel): DL-malic acid, MDH from porcine heart, citric acid, citrate lyase from Enterobacter Aerogenes, oxaloacetic acid, pyruvic acid, diaphorase (100 units/ml) from
Pseudomonas fluorescens 100 u/ml, β–NAD⁺, potassium chloride, resazurin, Trisma base and Triton X-100.

2.4. Reagents

All types of reagent solutions were freshly prepared once a week.

2.4.1 Stock solutions that contained the starting reaction mixture (solution A)

For malate determination, the starting reaction mixture (solution A) was composed of NAD⁺ 20mM and 0.1 µl of malic dehydrogenase (700 units/mg protein) dissolved in 50 mM potassium phosphate buffer, pH – 7.6.

For citrate, the starting reaction mixture (solution A) was composed of NADH 200 µM, 0.2 µl malic dehydrogenase (700 u/mg protein) and 0.2 µl citrate lyase (0.2 u/mg solid) dissolved in buffer solution composed of Tris/HCl buffer 20 mM, pH – 8.2 that contained 1 mM MgCl₂.

The same mixture, less the content of citrate lyase was used as the starting reaction mixture for oxaloacetate.

For pyruvate, the starting reaction mixture (solution A) was composed of NADH 200 µM, 0.2 µl of L-lactic dehydrogenase (800-1200 units/mg protein) dissolved in buffer solution composed of Tris/HCl buffer 20 mM, pH – 8.2 that contained 1 mM MgCl₂.

2.4.2 Stock solutions that contained the coupling reaction mixture (solution B)

The coupling reaction mixture (solution B) was composed of β–NAD⁺ 1 mM, resazurin 48 µM, diaphorase 1u/ml that were dissolved in Tris/HCl buffer 75 mM, pH 8.9, which included: KCl 100 mM, Tritton X-100 0.0004%.
2.4.3 Stock solutions of standards (solution C)

Malic acid was dissolved in 50 mM potassium phosphate buffer, pH – 7.6, whereas citrate, oxaloacetate and pyruvate were dissolved in Tris/HCl buffer 20 mM, pH – 8.2 that contained 1 mM MgCl₂.

2.5 Standard curve

Standards were prepared by serial dilutions of stock solution of the test substances in distilled water to yield concentrations of 1, 5, 10, 25, 50, 100, 250, 500 and 1000 µM.

2.6 Sample preparation

For malate determination in skim milk, samples were diluted with the buffer of solution C. For citrate the samples were diluted 50 folds with buffer of solution C, whereas for oxaloacetate and pyruvate the sample was diluted 5 folds with the buffer of solution C.

Yogurt samples, 1 g, were homogenized for 10 min in 10 ml of potassium phosphate buffer 50 mM, pH – 7.6. For malate determination, the homogenate was diluted 10 folds with the same buffer. The homogenates were diluted 50 fold for citrate, 10 folds for oxaloacetate and 10 fold or without dilution in the case of pyruvate in the buffer of solution C.

Wine samples were diluted 10 folds for L- and D-lactate and for malate concentration in red wine, whereas 5 fold dilution was applied for the rest of the test substances.

2.7 Reaction procedure

All the procedures were carried out in 96 wells plates suitable for fluorimetric reading.

2.7.1 Reaction procedures for malate assay
First, 10 µl of solution A and 10 µl of standard, or sample were incubated for 60 min at 37°C, followed by addition of 200 µl of solution B. After 30 min incubation at room temperature, the plate was red in a fluorimeter (BioTek Instruments, USA) under Ex/Em = 540/590.

2.7.2 Reaction procedures for pyruvate, citrate and oxaloacetate assays

First, 20 µl of solution A and 5 µl of standard, or sample were incubated for 30 min at 30°C, followed by addition of 200 µl of solution B. After 30 min incubation at room temperature, the plate was red in a fluorimeter (BioTek Instruments, USA) under Ex/Em = 540/590.

2.8. Calculation, validation parameters and statistical treatment

Determination of tested acid concentration, determination of linearity by regression analysis, derivation of limit of detection for each metabolite and assay between samples and day-to-day repeatability and recovery test were carried out essentially as described in Shapiro and Silanikove (2010). Differences in the organic acids composition in the red and white wines were evaluated by the pair-comparison student t-test. The relationships between malic and L-lactic acids were analyzed separately in the white and red wines by standard linear regression analysis. Analysis of differences between the slopes was done by t-test analysis.

2.9. Analysis of organic acids in food samples by the present developed assays, commercial enzymatic kit assays and a HPLC method

Milk sampled from six cows as described above, the red and white wine samples described above and fresh juice from six apples (red Hermon variety), six oranges (Jaffa variety) and six lemons were analyzed for the tested organic acids by
the current developed method. The samples were analyzed by the Biovision Inc. (Mountain View, California) commercial kits for malate (K637), citrate (K655), pyruvate (K609) and oxaloacetate (K659) according to the manufacturer instructions. The samples were analyzed for the organic acids by HPLC, based on the method of Garnsworthy et al. (2006) at the Hebrew University, Faculty of Agriculture (Rehovot), Analytical Service Center. Between samples R.S.D. were calculated as described above following their analysis in 3 replicates. Differences between 3 methods were evaluated by the student t-test analysis.

3 Results and Discussion

3.1 Performance of the standard curves

In accordance to the reactions order, malate concentration was positively related to resorufin emission (Table 1). On the other hand, as the detections of citrate, oxaloacetate and pyruvate are based on non-reacting NADH, their concentration was inversely related to resorufin emission (Table 1).

Linearity of the calibration curves ($R^2 = 0.972$ to 0.998), and estimation of the accuracy (R.S.D. of the estimate of 3 to 4%), repeatability (day-to-day R.S.D of 3 to 5%) and sensitivity (minimal detection limit of 10 to 50 µM; 1-5 nM/well) of the test substances are reported in Table 1. The within day R.S.D and day-to-day R.S. D. were similar, indicating that the results were consistent over time. The limit of detection, except that of malate, was inferior to that achieved with D and L lactate, galactose (5 µM), where the same reactions for formation of the fluorochromophore were applied. The improved performance of lactate, galactose and malate over that of citrate, oxaloacetate and pyruvate suggests that linear chain enzymatic reactions allows more accurate detection than those based on residual non-reacting substrate. Nevertheless,
the current methodology still offers considerable improvement in detection limit and reduced volume of samples in comparison with equivalent colorimetric methods (Milan, 1987, reporting detection limit of 1 mM for citrate; Mezzour et al; 2005 and Moellering, & Gruber, 1966, reporting limit of detection at the range of ~200 µM), or commercial kits (e.g. the Boeringer uv-kits for citrate and malate, reporting typical limit of detection at the range of ~200 µM). The present methods offer significant improvement in accuracy, simplicity and throughput in comparison to the above mentioned colorimetric methods and the fluorimetric method of O'nell, & Sakamoto (1969) for detecting citrate in urine samples.

3.2 Concentration of the tested substances in milk and yogurt

The concentrations of the test substances in milk and yogurt are reported in Table 2. The recovery of the tested substances when added to milk and yogurts ranged 94 to 98%. The predominance of citrate over the other tested organic substances and the actual concentration levels of citrate, malate and pyruvate in milk are consistent with previous reports (Faulkner, & Peaker, 1982). We could not find in the literature reports on oxaloacetate concentration. Our results suggest that its concentration is 2 to 3 fold higher than those of malate and pyruvate. Thus, this organic acid may be found suitable to be used as a maker of bacterial quality during milk storage.

We could not detect pyruvate in yogurts. Yogurt typically contains very high level of L-lactate, including in the studied products (~ 100 mM, Shapiro and Silanikove, 2010). As pyruvate is detected through it conversion to L-lactate with L-lactic dehydrogenase, the high level of lactate most likely prevented the conversion of pyruvate to L-lactate. In agreement, we found using pure substances that the presence of 40 mM of L-lactate in the reaction solution prevented the conversion of pyruvate to
L-lactate. Thus, this study shows once more that the use of reversal enzymatic reactions cannot be applied when one of the reaction products is presented in the sample in excessive concentration.

The larger concentration of malate in yogurt and the lower concentration of oxaloacetate and citrate in comparison to milk are consistent with finding that organic acids exhibited varying degrees of increases and decreases during fermentation and storage of yogurts (Fernandez-Garcia & McGregor, 1994).

3.3 Concentration of the tested substances in red and white wines

The recovery of the tested substances when added to red and white wines ranged 96 to 102%. The concentration of L-lactate in the red wines (~ 5 mM) was significantly higher than in the white wines (~ 2 mM) (Table 3). However, based on the present result we cannot determine if these differences are real, or reflect the choice of wines included in the study. On the other hand, the concentration of malate in red wines (~ 0.8 mM) was dramatically (~93%) lower than in the white varieties (~12 mM). Thus, our results strongly suggest that the MLF is fundamentally different in red and white wines. A plot of the concentration of malic against L-lactate concentration yielded a significant inverse linear correlation for both types of wines (Fig. 1). However, the slope of this inverse relationship was significantly higher in the white wines in comparison the red wines (P < 0.004), indicating that the MLF was more extensive the white wines. To the best our knowledge, this is the first time where such dramatic outcome of difference in MLF between white and red wines is demonstrated. However, our results are consistent with the much higher contents of phenolics in red wines than in white wines and with the fact that wine phenolics were shown to affect LAB fermentation, hence MLF products (see Liu, 2002 for review).
Though D-lactate was a bit higher in red wines than in the white wine, the differences were not significant (Table 3). In addition, D-lactate concentration was significantly related to malate concentration (data not shown), hence to MLF. Thus, our results are consistent with the conclusion that the levels of D-lactate in wines are particularly valuable for detection of wine-spoilage due to excessive content of LAB (Shapiro & Silanikove, 2010).

Citrate concentration in red wines (~1.5 mM) was significantly lower than in the white wines (~2.7 mM) (table 1). Citrate, a major component in grapes, is metabolized during wine processing by LAB (Liu, 2002; Sarantinopoulos, Kalantzopoulos & Tsakalidou, 2001). Thus, the lower content of citrate in red wines in comparison to white wines is consistent with the conclusion that LAB fermentation is lower in red wines in comparison to white wines due to the higher content of phenolics in red wines.

Besides the MLF route, most LAB can catabolise citrate via the pathway: citrate $\rightarrow$ acetate + oxaloacetate $\rightarrow$ pyruvate + CO$_2$ $\rightarrow$ lactate (Sarantinopoulos et al., 2001). Thus, the lack of detection of oxaloacetate in white wines while it was detected in red wines (Table 1), is another indication that LAB fermentation is essentially different in the two types of wines. The results suggest that this avenue is activated when the direct MLF route is suppressed by high content of phenolics.

3.4 Comparative aspects

The concentrations of the tested organic acids in milk, wines, and 3 types of fresh juices did differ from those measured by commercial enzymatic kits and by HPLC (table 4). The R.S.D. of the enzymatic methods was lower than in the HPLC
method, indicating that the reproducibility of these methods was better. The present method is much cheaper in comparison to the enzymatic kits and much less laborious in comparison to HPLC. The values obtained with the orange, lemon and apple juices are consistent with previous publications (e.g., Cunha, Fernandes & Ferreira, 2002).

4. Conclusions

The fluorimetric methodology presented here overcomes major deficiencies in using monochromatic colorimetric methods for detection of citrate, malate, pyruvate and oxaloacetate in milk and fermented food products, such as yogurts and wine while eliminating elaborated preparation procedures, relatively high sample volume (ml range), low detection and low precision associated with previously published techniques. The present results were found particularly valuable in characterizing the MLF in wines: We have demonstrated for the first time (to the best of our knowledge) that the MLF process in red wine is basically different in red wine than in white wine in accord with previous finding that wine phenolics affect LAB fermentation and with the fact that red wine are much richer in phenolics. In addition, this method ought to have valuable applications in food science, such as in monitoring the bacterial quality of milk and studying the dynamic of organic acids evolvement during fermentation and storage of fermented products. The versatility of present fluorimetric methodology as demonstrated by Shapiro & Silanikove (2010) and in the present results most likely will allow applying the method for analyzing the test substances in other types of biological fluids and foods, as demonstrated for the case of colorful juices.
References


hilgardii and Pediococcus pentosaceus) by wine phenolic compounds.

*Journal of Applied Microbiology*, 107, 1042-1053.


Figure legends

Figure 1. Interrelationships between L-lactate and malate concentration in white and red wines
Table 1: Regression equations of the calibration curves and analysis of linearity, accuracy, repeatability and sensitivity of malate, pyruvate, citrate and oxaloacetate

<table>
<thead>
<tr>
<th>Substance</th>
<th>Range (μM)</th>
<th>Regression equation</th>
<th>$R^2$</th>
<th>R.S.D* of the estimate (%)</th>
<th>Day-to-day R.S.D (%)</th>
<th>Limit of detection (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>malate</td>
<td>1 - 1000</td>
<td>Y=58.683x+16809</td>
<td>0.9998</td>
<td>3</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>pyruvate</td>
<td>1 - 1000</td>
<td>Y= -53.933x+49096</td>
<td>0.9916</td>
<td>4</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>citrate</td>
<td>1 - 1000</td>
<td>Y= -34.9408x+75186</td>
<td>0.9723</td>
<td>4</td>
<td>4</td>
<td>50</td>
</tr>
<tr>
<td>oxaloacetate</td>
<td>1 - 1000</td>
<td>Y= -18.135x+77082</td>
<td>0.9822</td>
<td>4</td>
<td>4</td>
<td>50</td>
</tr>
</tbody>
</table>

*R.S.D. - relative standard deviation
Table 2. Concentration and pooled intra-day and day-to-day repeatability of the test substances in milk yogurt

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration</th>
<th>R.S.D. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Milk</strong>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrate (mM)</td>
<td>9.1</td>
<td>15</td>
</tr>
<tr>
<td>Oxaloacetate (µM)</td>
<td>872</td>
<td>7</td>
</tr>
<tr>
<td>Pyruvate (µM)</td>
<td>303</td>
<td>6</td>
</tr>
<tr>
<td>Malate (µM)</td>
<td>398</td>
<td></td>
</tr>
<tr>
<td><strong>Yogurt</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrate (mM)</td>
<td>8.0</td>
<td>5</td>
</tr>
<tr>
<td>Oxaloacetate (µM)</td>
<td>452</td>
<td>5</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>Malate (mM)</td>
<td>6.6</td>
<td>4</td>
</tr>
</tbody>
</table>

*Milk of 6 cows, tested 3 times over 3 days, see materials and methods

**From two commercial products, tested tested 3 times over 3 days, see materials and methods

n.d. not detected
Table 3. Comparison between the concentration of the test substance in white and red wines (mean ± S. E.)

<table>
<thead>
<tr>
<th>Substance</th>
<th>Red wines</th>
<th>White wines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate (mM)</td>
<td>1.47 ± 0.08</td>
<td>2.67 ± 0.30</td>
</tr>
<tr>
<td>L-lactate (mM)</td>
<td>4.82 ± 0.85</td>
<td>2.81 ± 0.42</td>
</tr>
<tr>
<td>D-lactate (mM)</td>
<td>3.73 ± 0.53</td>
<td>3.10 ± 1.10</td>
</tr>
<tr>
<td>Malate (mM)</td>
<td>0.85 ± 0.27</td>
<td>11.67 ± 1.74</td>
</tr>
<tr>
<td>Oxaloacetate (mM)</td>
<td>1.59 ± 0.13</td>
<td>Not detected</td>
</tr>
<tr>
<td>Pyruvate (µM)</td>
<td>446 ± 72</td>
<td>139 ± 36</td>
</tr>
</tbody>
</table>

*Values in raw marked by different superscript values are significantly different, P < 0.01 or lower.
Table 4. Concentrations (mM) of tested organic acids in milk, red wines, white wines and fresh juices made of orange, lemon and apple (mean and R.S.D. as % of the mean)

<table>
<thead>
<tr>
<th>Method</th>
<th>Present-enzymatic</th>
<th>Commercial kit- enzymatic</th>
<th>HPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acid and RSD</td>
<td>C. RSD</td>
<td>M. RSD</td>
</tr>
<tr>
<td>milk</td>
<td>9.11 3.3 0.398 3.5 0.305 3.6 0.874 3.1</td>
<td>9.20 3.4 0.396 3.2 0.307 3.5 0.872 3.4</td>
<td>9.52 4.8 0.385 5.5 0.315 5.1 0.865 5.9</td>
</tr>
<tr>
<td>Red vine</td>
<td>1.51 3.4 0.86 3.5 0.448 0.03 1.58 3.4 1.48 3.3 0.84 3.5 0.446 0.04 1.59 3.6</td>
<td>1.57 6.2 0.81 5.7 0.425 0.02 1.65 6.0</td>
<td></td>
</tr>
<tr>
<td>White vine</td>
<td>2.69 3.5 11.70 3.3 0.142 3.5</td>
<td>N.D.</td>
<td>2.66 3.6 11.66 3.6 0.140 3.5</td>
</tr>
<tr>
<td>Orange juice</td>
<td>51.2 3.6 19.9 3.4</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Lemon Juice</td>
<td>297 3.1 32.11 3.5</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Apple juice</td>
<td>N.D.</td>
<td>82.17 3.6 0.757 3.7</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

C. – citrate; M. malate; P. pyruvate and OA – oxaloacetate
N.D. – not detected
Red wine
\[ y = -1.4867x + 6.0781 \]
\[ r^2 = 0.813, P < 0.01 \]

White wine
\[ y = -0.5402x + 9.1189 \]
\[ r^2 = 0.9391, P < 0.001 \]
1. A fluorimetric method for detection of organic acids in milk, juices and fermented food products is presented.
2. These include citrate, malate, pyruvate and oxaloacetate.
3. The method is precise, fast and requires low volume of samples.
L-lactic acid (mM)

malic acid (mM)

White wine
\[ y = -0.5402x + 9.1189 \]
\[ r^2 = 0.9391, P < 0.001 \]

red wine
\[ y = -1.4867x + 6.0781 \]
\[ r^2 = 0.813, P < 0.01 \]
1. A fluorimetric method for detection of organic acids in milk, juices and fermented food products is presented.
2. These include citrate, malate, pyruvate and oxaloacetate.
3. The method is precise, fast and requires low volume of samples.