Uric acid as a marker of milk microbiological spoilage

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Abstract. Uric acid is an important component of milk, which has antioxidant properties and protects it from rapid microbiological spoilage. The article studies the change in uric acid content during unwanted spoilage of pasteurized and unpasteurized milk using a developed voltammetric sensor based on a carbon veil. The sensor allows to estimate the content of uric acid in a wide range of concentrations without the influence of the complex matrix of the test sample on the determination result. It has been established that the uric acid content in pasteurized milk is several times less than in unpasteurized milk. With microbiological spoilage of milk, the uric acid content decreases by 40–100 %, which makes it possible to use this biologically active compound as a marker of milk spoilage. The developed sensor provides high reproducibility ($S_r \leq 4.6$ %) and accuracy of results, as evidenced by the recovery close to 100 %. The sensor can be used to control the quality and safety of milk used in technological processes for the production of dairy products.

1 Introduction

Milk is the most popular and widely consumed food product. Milk is the basis for the biotechnology of various dairy products, such as sour cream, kefir, yogurt, cottage cheese, curdled milk, cheese, etc. The properties of the final product of the biotechnological process largely depend on the properties of the raw milk used. The qualitative and quantitative composition of milk is not constant and varies depending on the type, breed, age, nutrition of the dairy animal, external environmental conditions, as well as the conditions of storage and transportation of the milk itself. In addition to macro- and micro-components, milk contains antioxidants. Antioxidants in milk are represented by vitamins (vitamins E, C), enzymes (superoxide dismutase, catalase, glutathione peroxidase and xanthine oxidase), ascorbic, uric acids, as well as whey proteins and caseins. Xanthine oxidase is a key enzyme in purine metabolism, contained in the milk fat globule membrane [1] and responsible for the catalytic oxidation of hypoxanthine and xanthine to uric acid. Uric acid in milk is considered a powerful antioxidant similar to α-tocopherol and provides oxidative stability to milk and dairy products [2]. Any imbalance between the anti- and pro-oxidant activities of these antioxidants can reduce the oxidative stability and nutritional quality of milk and ultimately cause off-odor and taste [1]. It has been established that uric

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acid can inhibit by 28% the formation of light-induced hydroperoxides in milk lipids and protect milk from the degradation of riboflavin under the influence of ultraviolet light [3].

In the nutrient medium of milk, along with beneficial microflora, harmful bacteria and microorganisms entering from the external environment can develop, causing microbiological spoilage of the product, changes in the content of useful substances and a decrease in the nutritional value of milk. In this regard, it was of interest to study the change in the content of uric acid, as one of the most important antioxidants in milk, during the development of its microbiological spoilage.

To determine uric acid in milk, various analytical methods have been developed, including titrimetric [4], spectrophotometric [5], chromatographic [6], and enzymatic methods [7]. Most of these methods are labor-intensive, requiring the use of complex instruments, toxic, unstable solvents and reagents, and a lengthy, multi-step preparation and analysis process. Many of the above laboratory methods require trained personnel. Given that uric acid is easily electrochemically oxidized in aqueous solutions, its detection by electrochemical methods (cyclic, linear, differential pulse voltammetry and amperometry) is reliable, fast and simple, as well as sensitive and selective. Electrochemical analytical methods use a variety of electrochemical sensors to determine uric acid. To increase sensitivity, various modern materials are used in such sensors. The sensing layer may include graphene or graphene oxide with its large active surface area and high electrical conductivity [8, 9]; metal and oxide nanoparticles [10], which are catalytic and adsorption centers; polymers that selectively react to uric acid [11], etc. There is a narrow linear range of detectable uric acid concentrations and insufficient sensitivity or selectivity in some existing sensors. Thanks to its excellent analytical characteristics, our previously developed sensor based on a carbon veil activated at +2.0 V for the determination of uric acid [12] can be promising and successfully used for its determination in milk. As can be seen from Table 1, the linearity range of the developed sensor is wider, and the detection limit is lower than that of most of the compared devices. Other advantages of this sensor include ease of manufacture and low cost.

Table 1. Comparison of analytical characteristics of sensors for determining UA in milk.

Sensor	LD, µM	LR, µM	Method	Reference
PEDOT/GCE PEDOT/Au	7	6-200	CV	[11]
rGO/AuNPs /ITO-PET	7.1	10-1000	LV	[13]
HPC/GCE	0.06 0.07	0.13-806 0.2-100	Amp DPV	[14]
Au/Cys/Bnt/GCE	0.93	1-200	DPV	[15]
rAu-PtNPs/GQDs	0.0003	0.001-0.1	SWV	[16]
nafion/AuNPs /AzA/MWCNT /GCE	0.028	300-10000	CV	[10]
nafion /AuNPs / CSPE	0.25	0.5-600	LV	[17]
CVE_{act}	0.05	0.09-700	LV	[12]

LD – detection limit; LR – linear range; PEDOT/GCE – poly(3,4-ethylenedioxythiophene) on a glassy carbon electrode; PEDOT/Au – electropolymerized poly(3,4-ethylenedioxythiophene) on a gold microelectrode; rGO – reduced graphene oxide; AuNPs – gold nanoparticles; ITO-PET – indium tin oxide deposited on a flexible polyethylene terephthalate substrate; HPC – hierarchical porous carbon; Cys – cysteine; Bnt – bentonite; PtNPs – platinum nanoparticles; GQDs – graphene quantum dots; AzA – AzureA; MWCNT – multi-walled carbon nanotubes; CSPE – carbon screen-printed

electrode; CV – cyclic voltammetry; LV – linear voltammetry; Amp – amperometry; DPV – differential pulse voltammetry; SWV – square wave voltammetry

The purpose of this work was to study changes in uric acid content during microbiological spoilage of milk using a voltammetric sensor based on an activated carbon veil (CVE_{act}).

2 Materials and methods

2.1 Reagents and materials

$Na_2HPO_4 \times 12H_2O$ (JSC Vecton, St. Petersburg, Russia), KH_2PO_4 (LLC NevaReaktiv, St. Petersburg, Russia), H_2SO_4 (JSC Khimreaktivsnab, Ufa, Russia), uric acid (Acros Organics, Geel, Belgium), acetone (JSC ECOS-1, Moscow, Russia). All reagents were chemically pure and used without further purification. Working solutions were prepared using deionized water. Carbon veil (M-Carbo, Minsk, Belarus), polyethylene terephthalate sheets (Fellows Inc, Itasca, IL, USA) were used to produce working electrodes.

2.2 Equipment

Electrochemical measurements were carried out using a semi-automatic voltammetric analyzer IVA-5 with a three-electrode cell (IVA, Yekaterinburg, Russia), which included a working electrode (sensor) based on an activated carbon veil (CVE_{act}), a silver chloride reference electrode EVL-1M3.1 (Ag/AgCl/KCl, 3.5 M) (JSC GZIP, Gomel, Belarus) and a carbon rod as an auxiliary electrode. An LM-260iD laminator (Rayson Electrical MFG, China) was used to produce working electrodes in accordance with the technology described in [18].

2.3 Objects of research

The objects of the study were pasteurized (samples 1 and 2) and unpasteurized (sample 3) cow's milk. Samples 1 and 2 were purchased from supermarkets, and sample 3 was provided by a private individual immediately after milking the cow. The fat content of samples 1 and 2 was 3.2 %. The protein content per 100 g of product for these samples was in the range of 2.8 – 3 g, carbohydrates – 4.7 g, respectively. For sample 3 these parameters are not set. Milk samples were used without additional processing. Between analyses, samples were stored in a refrigerator at +4 °C. The days of the study were counted from the moment the milk package was opened (samples 1 and 2) and the day after the evening milking of the cow (sample 3). After the appearance of visible defects in milk consistency (formation of a gelatinous clot and separation), the “supernatant” whey was taken for analysis.

2.4 Methods of research

Before measurements, the working surface of the electrode was washed with a water-acetone mixture (1:1) with constant stirring for 15 minutes and activated in a solution of 0.05 M sulfuric acid at a potential of 2.0 V for 5 minutes.

The procedure for conducting electrochemical measurements was as follows: electrodes were lowered into an electrochemical cell containing 9.5–9.0 ml of a phosphate buffer solution with pH 6, and a background linear voltammogram was recorded from 0.25 V to

0.65 V at a scanning potential scanning speed of 0.05 V/s. Then an aliquot of milk or whey (0.5–1.0 ml) was added, mixed thoroughly, and the voltammogram of the sample was recorded three times. Then, additives of a standard solution of uric acid were added to the cell and the corresponding voltammograms were recorded. The uric acid content in the sample was determined using the addition method. The effect of the matrix on the determination of uric acid was assessed using the "added-found" method.

All measurements were carried out in five parallels. The results were calculated with a confidence level of P = 0.95. The results were presented as X±ΔX, where X is the mean value, ΔX is the standard deviation. The recovery (R) was calculated according to IUPAC [19].

3 Results and discussion

The amplitude of the integral voltammogram at a potential of 0.41 V was used as an analytical signal of the electrochemical oxidation of uric acid (Fig. 1). Figure 1 shows linear sweep voltammograms (with baseline correction) recorded using CVE_{act} in a phosphate buffer solution of pH 6 (background electrolyte), in a milk sample and with the addition of a standard uric acid solution.

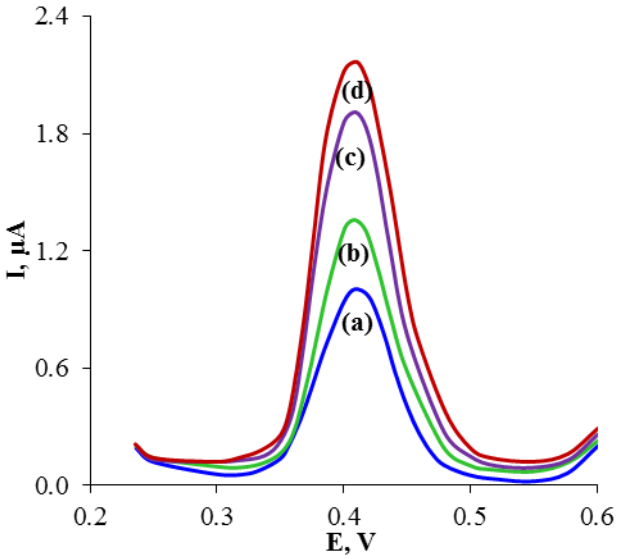

Fig. 1. Linear voltammograms with baseline correction obtained using CVE_{act} in a phosphate buffer solution pH 6 containing 0.5 ml of milk (**a**) and additives 2 (**b**), 4 (**c**), 6 (**d**) µM uric acid.

Figure 1 demonstrates that when 0.5 ml of a milk sample is added into the background electrolyte (curve a), a well-defined signal is recorded at a potential E = 0.41 V, growing with increasing concentration of uric acid. This signal was accepted as analytical and was subsequently used to determine the uric acid content in milk samples during the process of microbiological spoilage.

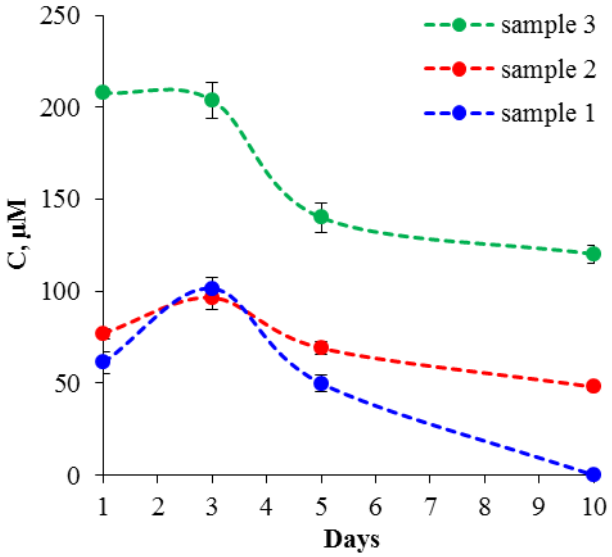

Fig. 2. Changes in the content of uric acid in milk samples during the process of microbiological spoilage.

Figure 2 illustrates the change in uric acid content in milk samples during microbiological spoilage. There are significant differences in the uric acid content in the initial milk samples with different treatments. Thus, the uric acid content in samples 1, 2 on the first day of the study is 60-80 μM, which is almost 3 times less than sample 3, in which the uric acid content is at the level of 208 μM. The observed differences can be explained by different milk processing methods. Milk samples 1 and 2 were pasteurized, while sample 3 (raw milk) was not subjected to any heat treatment. Short thermal exposure during pasteurization promotes the inactivation of enzymes and the oxidation of antioxidants, which leads to a decrease in the uric acid content in pasteurized milk compared to unpasteurized milk. As can be seen from Figure 2, the uric acid content in samples 1 and 2 increases slightly in the first days of standing pasteurized milk in air and reaches its highest value on the third day. The increase in uric acid content during the first three days can be explained by the irreversible oxidation of xanthine and hypoxanthine under the action of the enzyme xanthine oxidase, which is released from the membranes of milk fat globules into the aqueous phase [1]. After three days of milk souring, a gradual thickening and separation into two phases was observed: a white curdled clot, which is a coagulated casein protein, and whey. Protein coagulation is accompanied by a decrease in the uric acid content in the liquid phase of curdled milk. It is known that milk proteins are able to reduce the concentration of uric acid, causing the so-called uricosuric effect [20]. From the fifth to the tenth day of milk souring, the content of uric acid decreased more noticeably in sample 1 and slightly in samples 2 and 3. The decrease in the concentration of uric acid during this period of milk souring can be explained by the occurrence of non-enzymatic spontaneous reactions of milk fat oxidation with the initial formation of peroxide compounds of the free radical type , and then aldehydes, ketones and other compounds that have an unpleasant odor and taste. Uric acid, as an antioxidant, inhibits the oxidation process and interrupts free radical reactions, being consumed in the process [21].

Table 2 presents the results of the determination of uric acid in milk samples during its microbiological spoilage using CVE_{act} and the “added-found” method.

Table 2. Results of voltammetric determination of uric acid on different days of microbiological spoilage of milk using CVE_{act} (n=5, P=0.95) and the “added-found” method.

Day of milk spoilage	Found, µM	S_r, %	Added, µM	Found in milk with additive, µM	Found additive µM	S_r, %	R, %
Sample 1							
1	61±6	3.8	50	111±7	50±4	3.2	99
3	102±6	4.5	100	200±6	98±9	7.6	98
5	50±5	3.7	50	101±11	51±4	4.7	101
10	-	-	50	-	49±4	2.7	98
Sample 2							
1	77±3	2.8	60	139±8	61±4	2.6	101
3	96±6	1.3	100	195±13	99±6	2.8	99
5	69±4	1.1	60	128±9	59±4	2.6	99
10	48±2	3.9	40	81±6	41±4	4.3	103
Sample 3							
1	208±1	4.6	200	412±19	212±15	2.9	106
3	204±10	1.9	200	399±34	197±19	3.8	97
5	140±8	2.3	120	258±19	121±5	5.2	101
10	120±5	3.3	100	221±5	101±7	5.5	101

S_r – relative standard deviation; R – recovery

As can be seen from Table 2, the relative standard deviation (S_r) of the results of determining uric acid in milk does not exceed 4.6 %, and the S_r of the additive found is not more than 7.6 %. The obtained S_r values indicate good reproducibility of the results of determining uric acid using the CVE_{act} sensor. The recovery values range from 96–106 % and are close to 100 %, which indicates the absence of systematic errors in the determination of uric acid in milk using CVE_{act}.

4 Conclusion

Uric acid is one of the important components of the antioxidant defense system of milk against oxidative damage. The change in uric acid content during microbiological spoilage of pasteurized and unpasteurized milk was studied using a voltammetric sensor based on an activated carbon veil. It has been determined that heat-treated milk contains several times less uric acid than raw milk. During the observed ten-day microbiological spoilage of milk, the uric acid content decreased by 40–100 % relative to the original milk. An explanation for the observed processes is given. A sensor based on a carbon veil makes it possible to determine uric acid in milk without additional separation and concentration operations and can be used for the purpose of qualitative control of biotechnological processes.

References

1. P. F. Fox, A. L. Kelly, Int. Diary J., **16**, 500–516 (2006)
2. J. H. Nielsen, H. Østdal, H. J. Andersen, ACS Symp. Ser., **807**, 126–137 (2002)
3. H. Østdal, M. J. Bjerrum, J. A. Pedersen, H. J. J. Andersen, Agric. Food Chem., **48**, 3939–3944 (2000)
4. E. Bitziou, M. E. Snowden, M. B. Joseph, S. J. Leigh, J. A. Covington, J. V. Mac Pherson, P. R. Unwin, J. Electroanal. Chem., **692**, 72–79 (2013)
5. M. R. Moghadam, S. Dadfarnia, A. M. H. Shabani, P. Shahbazikhah, Anal. Biochem., **410**, 289–295 (2011)

6. H. E. Indyk, D. C. Woollard, J. AOAC Int., **87**, 116–122 (2004)
7. Y. Zhao, X. Yang, W. Lu, H. Liao, F. Liao, Microchim. Acta., **164**, 1–6 (2009)
8. F. Mazzara, B. Patella, G. Aiello, A. O'Riordan, C. Torino, A. Vilasi, R. Inguanta, Electrochim. Acta., **388**, 138652 (2021)
9. J.-H. Lee, S.-J. Park, J.-W. Choi, Nanomater, **9**, 297 (2019)
10. H. Filik, A. A. Avan, S. Aydar, Arabian J. Chem., **9**, 471–480 (2016)
11. M. Motshakeri, A. R. J. Phillips, J. Travas-Sejdic, P. A. Kilmartin, Electroanalysis, **32**, 2101–2111 (2020)
12. M. A. Bukharinova, N. Yu. Stozhko, E. A. Novakovskaya, E. I. Khamzina, A. V. Tarasov, S. V. Sokolkov, Biosensors, **11**, 287 (2021)
13. F. Mazzara, B. Patella, F. Ganci, A. O'Riordan, G. Aiello, C. Torino, A. Vilasi, C. Sunseri, R. Inguanta, Chem. Eng. Trans., **87**, 421–426 (2021)
14. L. Dong, L. Fan, D. Yigang, J. Electrochem. Soc., **166**, B1585–B1593 (2019)
15. D. K. Yadav, R. Gupta, V. Ganesan, P. K. Sonkar, Microchim. Acta., **184**, 1951–1957 (2017)
16. M. L. Yola, N. J. Atar, Electrochem. Soc., **163**, B718–B725 (2016)
17. N. Stozhko, M. Bukharinova, L. Galperin, Kh. Brainina, Biosensors, **8**, 21 (2018)
18. N. Yu. Stozhko, M. A. Bukharinova, E. I. Khamzina, A. V. Tarasov, S. V. Sokolkov, Chemosensors, **8**, 78 (2020)
19. D. T. Burns, K. Danzer, A. Townshend, IUPAC Reccomendations 2002. Pure and Applied Chemistry, **74**, 2201–2205 (2003)
20. D. R. Garrel, M. Verdy, C. PetitClerc, C. Martin, D. Brule, P. Hamet, Am. J. Clin. Nutr., **53**, 665-669 (1991)
21. M. Caropres, M. G. Ciliberti, M. Albenzio, R. Marino, A. Santillo, A. Sevi, Small Ruminant Res., **180**, 79–85 (2019)