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Application of cyclic voltammetry to analyse uric acid and reducing agents in commercial milks

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Abstract

Cyclic voltammetry (CV) and high performance liquid chromatography (HPLC), were compared to quantify uric acid and antioxidant reducing agents in 36 milk samples. The enzymes uricase and ascorbate oxidase were used to remove uric and ascorbic acids and showed that the peaks obtained by CV and HPLC did not contain contributions from other unknown compounds. The levels of uric acid obtained by CV compared well to those determined using HPLC, with only a few exceptions, and the average difference was around 6%. CV measurements were made using the main anodic peak seen at approximately 330 mV (Ag/AgCl), while a later oxidation peak at approximately 650 mV can be associated with further reducing agents present in milk. The electrochemical method was quicker to apply than HPLC that included a pre-treatment step, and provides an inexpensive and simple method for the reliable analysis of uric acid in milk.

Keywords: Milk, cyclic voltammetry, HPLC, uric acid, PEDOT, glassy carbon electrode, uricase, ascorbate oxidase.

1. Introduction

Uric acid (UA) as one of the main antioxidant biomarkers found in milk, is the final product of the metabolic breakdown of unwanted purine nucleotides such as guanine and adenine (Hediger, Johnson, Miyazaki, & Endou, 2005). UA is present in milk and contributes to the dietary intake of antioxidants along with ascorbic acid (AA), vitamin E, tyrosine and cysteine (Lindmark-Månsson & Åkesson, 2000; Østdal, Andersen, & Nielsen, 2000), and to the intake of organic acids, alongside citric acid, orotic acid, formic acid, lactic acid, hippuric acid and pyruvic acid (Marsili, Ostapenko, Simmons, & Green, 1981). An imbalance in the milk UA levels may affect milk flavour and its detection can also provide information about the health and metabolism of cows and other mammals (Nielsen, Østdal, & Andersen, 2002).

Conventionally, UA is analysed by enzymatic methods (Y. Zhao, Yang, Lu, Liao, & Liao, 2009), spectrophotometry (Yamaguchi et al., 2007), gas chromatography (GC) (Chen, Calder, Prasitkusol, Kyle, & Jayasuriya, 1998; March, Simonet, Gráses, Muñoz, & Valiente, 2003; Matsumoto & Kuhara, 1996), high-performance liquid chromatography (HPLC) (Li & Franke, 2009), high performance capillary electrophoresis (HPCE) (Yao, Wang, & Chen, 2007), and electrochemical sensors (Chen, Gorton, & Åkesson, 2002; Jawad, Dorie, & Murr, 1991; Motshakeri, Travas-Sejdic, Phillips, & Kilmartin, 2018a). Among the electrochemical techniques, cyclic voltammetry is one of the most straightforward and informative methods, which has also been used to analyse different micro-constituents in foods, such as glucose (Haldorai, Choe, Huh, & Han, 2018), H_2O_2 (Cai et al., 2019), and glutathione (Pang et al., 2012).

Most of these techniques are complex, time and solvent consuming, and suffer from interferences from other compounds such as dopamine and ascorbic acid that are also found in biological fluids. With the exception of electrochemical sensors, these methods usually all

require expensive equipment and unstable reagents. Thus, recent efforts have been made to improve the electrochemical quantification of uric acid in standard solutions in biological fluids (Aslanoglu, Kutluay, Abbasoglu, & Karabulut, 2008; Brajter-Toth, El-Nour, Cavalheiro, & Bravo, 2000; Cai, Kalcher, & Neuhold, 1994; Gao & Huang, 1998; Kasai et al., 2002; Li, Zeng, Liu, & Tang, 2006; Rocheleau & Purdy, 1991; Tukimin, Abdullah, & Sulaiman, 2017; Wang, Du, & Zou, 2002; Zen & Chen, 1997).

Excellent separation of peaks for UA and AA were obtained at a poly(3,4-ethylenedioxythiophene) (PEDOT) electrode, with extension to a microchip sensor (Kumar, Mathiyarasu, Phani, Jain, & Yegnaraman, 2005). In previous research (Motshakeri, Travas-Sejdic, et al., 2018a), we have shown the potential of a modified PEDOT electrode for the sensitive and selective determination of UA in milk. The conducting polymer PEDOT was successfully deposited on a glassy carbon electrode, and the conditions of use to quantify UA optimized. In this study, we have applied the new electrode and voltammetric technique to the direct analysis of UA in 34 different pasteurised milk samples available in the local supermarket and 2 unpasteurised samples of milk taken directly from the farm. The milks were analysed by voltammetry without any sample pretreatment. The UA results were then compared with standard analytical analysis using reversed-phase HPLC.

2. Materials and Methods

2.1 Reagents and materials

All chemicals and solvents used were of analytical grade. Di-sodium hydrogen phosphate dihydrate (Na_2HPO_4) and boric acid (H_3BO_3) (Scharlau Chemie SA, Barcelona, Spain), di-sodium tetraborate ($Na_2B_4O_7.10\ H_2O$) (BDH Chemicals Ltd., Poole England), ortho-phosphoric acid (85%, Merck, Darmstadt, Germany), chloroform (95%, Loba Chemie India),

and sodium perchlorate, lithium perchlorate, 3,4-ethylenedioxythiophene (EDOT), propylene carbonate, sodium dihydrogen phosphate (NaH_2PO_4), uric acid, ascorbic acid, uricase from porcine liver (Type V, soluble powder ≥ 10 unit/g protein), and ascorbate oxidase from Cucurbita sp. (Lyophilized powder, 1000-3000 units/mg protein) were all purchased from Sigma-Aldrich. Milli-Q water (18.2 MΩ/cm at 25°C, Barnstead Nanopure Diamond Water Purification System, Thermo Scientific, USA) was used for preparation of aqueous solutions.

2.2 Milk samples and stock solutions

34 commercial milk samples were purchased from local supermarkets in Auckland, New Zealand. Two samples of fresh raw milk was also obtained from a local farm in Auckland, and sodium azide (0.02%), as a preservative, was added to one of the samples. Each milk sample was divided into two equal portions (15 mL each). One portion was subject to electrochemical analysis without any pre-treatment (untreated sample). The other matched milk sample first underwent removal of fats and proteins by addition of acid and chloroform (treated sample) and was then subject to standard reversed-phase HPLC analysis. The treated samples for HPLC were obtained by mixing the milk sample with 1% ortho-phosphoric acid solution (1:1) and standing for 30 minutes to remove proteins (Kondyli, Katsiari, & Voutsinas, 2007). The mixture was then centrifuged at 2000 g at 4°C for 15 minutes, and the supernatant was decanted and mixed with chloroform (95%) at a ratio of 1:1 to dissolve the fat. The mixture was vortexed for one minute and then centrifuged again at 2000 g at 4°C for 20 minutes. Finally, the supernatant was passed through a syringe filter (0.22 μm PTFE) and then analysed in triplicate on the HPLC. The type of milk sample used for CV optimization was a commercial whole milk, and was compared to UA standard solutions. All freshly-prepared solutions were degassed for 5 min with nitrogen before analysis. All milk samples, either treated or untreated, were analysed at room temperature (22 ± 2°C). A borate buffer with pH adjusted at 8.5 was also prepared for use in enzyme studies.

2.3 Chromatographic conditions

Detection of UA was carried out by reversed-phase HPLC (HP 1200 series, Agilent Technologies, USA) at a C18 column (250 x 4.6 mm I.D.; 5 µm particle size, Phenomenex, USA), following methods reported previously with minor modifications (Havemose, Weisbjerg, Bredie, Poulsen, & Nielsen, 2006; Østdal et al., 2000). Sodium dihydrogen phosphate (NaH_2PO_4, 10 mM, pH = 4.8) was used as the mobile phase, and was first filtered through a Nylon filter paper (0.22 µm pore size, 47 mm diameter) and kept at a flow rate of 1 mL/min. UA concentration was monitored at a wavelength of 290 nm using a UV detector and was quantified from its peak area. Satisfactory linearity was gained in the detector response using six concentration levels of UA standards (3.125, 6.25, 12.5, 25, 50, 100 µM) in 0.1 M phosphate buffered saline (PBS) at pH 6.6, which was then lowered to pH 2.3 using 1% ortho-phosphoric acid solution to match the pH of the treated milk samples. Blank injections were made between each sample run to check for possible sample carry-over, and for this purpose the blank consisted of a mixture of PBS:Acid (1:1) without UA.

2.4 Electrochemical analysis

Electrochemical experiments were performed on a BAS100A system (Bioanalytical Systems) employing the cyclic voltammetry (CV) technique. A three-electrode cell was used with a PEDOT modified glassy carbon working electrode (GCE, 3 mm diameter), a reference electrode (Ag/AgCl in 3 M NaCl), and an auxiliary electrode (platinum wire). The modified electrode was prepared by electropolymerization of 0.1 M 3,4-ethylenedioxythiophene monomer (EDOT) in 0.1 M lithium perchlorate in propylene carbonate, followed by acclimatization in aqueous sodium perchlorate solution (Motshakeri, Travas-Sejdic, et al., 2018a; Motshakeri, Travas-Sejdic, Kilmartin et al., 2018b). Afterwards, the modified electrode was inserted into the untreated milk sample for determination of UA. A 30 second hold time

was employed prior to the voltammetric run without any applied potential and without stirring the sample, following the procedure already described (Motshakeri, Travas-sejdic, et al., 2018a; Motshakeri, Travas-Sejdic, et al., 2018b). The electrode surface was cleaned and polished on an alumina slurry (0.05 mm), sonicated in ethanol and in Milli-Q water before and after each run. The voltammograms were obtained between -100 to +800 mV at a 100 mV/s scan rate and with 15 s quiet time at -100 mV prior to the scan. The final voltammograms are presented after subtraction of a blank run of the PEDOT electrode in the PBS buffer to remove the background capacitive-type currents due to PEDOT internal redox processes (Motshakeri, Travas-sejdic, et al., 2018a; Motshakeri, Travas-Sejdic, et al., 2018b). The calibration curve for UA was plotted using peak currents of different UA concentrations, which was used to determine the UA concentration in the untreated milk samples. All measurements were carried out in triplicate. All milk samples were used prior to their "best before date", and stored in 4°C in their original containers while waiting to run the analyses (cyclic voltammetry/HPLC). However, the temperatures of milk samples came to room temperature by keeping them out of the refrigerator prior to analysis.

2.5 Enzyme reactions

Uricase enzyme (0.5 unit/mL) and UA (80 μM) solutions were prepared by dissolving the proper amount in a freshly-prepared 25 mM borate buffer at pH 8.5. These solutions, and whole milk sample (Blue cap, Fonterra) with the pH adjusted to 8.5 using small additions of concentrated sodium hydroxide, were then incubated in a water bath at 25°C. Then, 5 mL of the milk sample or UA solution was analysed by cyclic voltammetry (CV) using the PEDOT-modified sensor. In the next step, enzyme reaction mixtures containing uricase and UA, or uricase and milk sample were prepared separately. In this case, 2.8 mL of UA solution or milk sample were mixed with 0.2 mL of uricase solution, vortexed for one minute, and then incubated for 90 minutes at 25°C in a water bath, prior to CV analysis. To investigate the effect

of the ascorbate oxidase enzyme (AOX, 11.1 unit/mL) on current due to ascorbic acid oxidation, a standard 0.5 mM AA solution was prepared in PBS (4 mM, pH 5.6). Next 1 mL of the AA standard solution was oxidised with 0.5 mL of enzyme at 37°C for 90 minutes incubation in a water bath. Milk pH was also adjusted to 5.6 using some drops of 1 M HCl, before enzyme addition under the same conditions. The milk sample (1 mL) was then treated with 0.5 mL of the AOX enzyme, resulting in a 33% dilution effect. The samples were cooled down to room temperature before CV analysis. For HPLC analysis of enzyme-treated milk samples, uricase was added to the whole milk sample (pH 8.5, 25°C) to oxidise UA before going through acid/chloroform treatment (the final pH before HPLC injection was 2.3). However, AOX was added to the whole milk sample after acid/chloroform treatment and the pH changed to 6 by using a few drops of sodium hydroxide. This was a known optimum pH for AOX activation (the final pH before HPLC injection was 6).

2.6 Surface characterization techniques

2.6.1 Scanning Electron Microscopy (SEM)

SEM analysis was performed to check the surface morphology of the bare glassy carbon and the PEDOT-modified glassy carbon electrodes (prepared as in section 2.4), using a FEI ESEM Quanta 200 FEG (USA), with a SiLi (Lithium drifted) Super Ultra Thin Window EDS detector. The electrode head was placed horizontally on a metal block with a certain angle above the stage, and then it was secured by a carbon tape attached to the stage.

2.6.2 Raman spectroscopy

The Raman spectra of the PEDOT-modified glassy carbon electrode were taken on a Renishaw Raman Imaging Microscope, consisting of a single grating spectrograph (System 1000), a Leica microscope, and a CCD (charge couple device) array detector (400 × 578 Si pixels). The Renishaw diode laser emitting a line in the near-infrared region at 785 nm (350 mW max) was

used as the excitation source. The laser power was 10%, objective of 50x, accumulation 10, and detector time of 10 seconds. WiRE instrument control software, and GRAMS32 data analysis software were used to collect and analyse the spectra.

2.6.3 Fourier Transform Infrared Spectroscopy (FTIR)

Analyses of the PEDOT-GC electrode was also performed using a FTIR spectrometer (PerkinElmer, Spectrum Two) with a resolution of 4 cm^{-1}, over the wavenumber region from 400 to 1600 cm^{-1}. The OMNIC spectroscopic software was used to analyse the spectra.

3. Results and discussion

3.1 Surface characterisation of the PEDOT sensor

The SEM images of the bare electrode and PEDOT electrode are presented in Fig. S7 at different magnifications. The bare GC electrode was very smooth and flat with some scattered scratches on the surface due to polishing. However, after PEDOT electropolymerization, a porous layer was formed on the surface which consisted of an aggregation of mountainous or flake-like particles, growing regularly towards the electrode edges (Fig. S7a to S7b).

Raman spectra at 785 nm provided information on the vibration modes of the thin PEDOT structure, as shown in Fig. S8a. The bands at 437, 700, 991, and 1252 cm^{-1} correspond to the C-O-C deformation, symmetric C-S-C bending, deformation of oxyethylene ring, and C_α-C_α inter-ring stretching, respectively (Zhao, Jamal, Zhang, Wang, & Abdiryim, 2014). The major band at 1420 cm^{-1} is due to symmetric stretching of C_α=C_β (Cα and Cβ are the carbon atoms connecting with sulfur atoms), and indicated a high degree of conjugation in the PEDOT structure (Zhao, Jamal, Zhang, Wang, & Abdiryim, 2014). The peaks at 1372 and 1100 cm^{-1} are assigned to the C_β-C_β inter-ring stretching, and C-O-C stretching, respectively. The 991 cm^{-}

204 [1] peak is related to C-C anti-symmetrical stretching. The Raman spectrum was in agreement
205 with PEDOT spectra from previous reports and confirms the presence of conjugated PEDOT
206 on the electrode surface (Sakmeche et al., 1999; Tamburri, Orlanducci, Toschi, Terranova, &
207 Passeri, 2009; Zhou et al., 2011).

208 An FTIR spectrum was recorded (Fig. S8b), which showed transmittance peaks typical of
209 PEDOT. Peaks at 1520 cm^{-1} (C=C), 1329 cm^{-1} (C-C), 930 cm^{-1} (S-O), and 674 cm^{-1} (C-S)
210 correspond to thiophene ring of PEDOT, while the vibrations at 1200, 1148, 1089, and 1064
211 cm^{-1} are assigned to (C-O-C) bond stretching in the ethylenedioxy group (Zhao et al., 2014).
212 The vibration seen at 1064 cm^{-1} is related to C-O bond stretching. These results were similar
213 to the FTIR spectrum of a PEDOT:PSS nanocomposite (Rattan, Singhal, & Verma, 2013), and
214 to PEDOT nanowires (Zhu et al., 2015), and are indicative of PEDOT polymerisation on the
215 GC surface (Zhao et al., 2014).

216 **3.2 Cyclic voltammetry**

217 **Dilution effect.** Dilution of a biological sample is important because it assists in determining
218 any matrix interferences. Cyclic voltammograms of a whole milk sample (Blue cap, Fonterra),
219 and after dilution in pH 6.6 PBS buffer, are shown in Fig. 1a. The sharp peak at around 330
220 mV is ascribed to the oxidation of UA (Motshakeri, Travas-sejdic, et al., 2018a; Motshakeri,
221 Travas-Sejdic, et al., 2018b), while further current beyond 600 mV is due to oxidation of
222 additional reducing agents. Interestingly, what appeared as a single peak in the undiluted milk,
223 separated out into two oxidation peaks as the milk sample was diluted, centred at approximately
224 600 and 680 mV. These peaks are most likely due to oxidizable amino acids such as tyrosine,
225 tryptophan and cysteine (Motshakeri, Travas-Sejdic, et al., 2018a), and to further unknown
226 compounds. Given that these compounds do not interfere with the UA peak, they can be
227 examined independently of UA after dilution of the milk. Dilution to 50% milk fraction resulted

in a higher peak at 600 mV compared to the feature at 680 mV. However, once the milk fraction was lowered to 25%, the two peaks were almost equal in intensity. Conversely, dilution to lower milk proportions led to a higher anodic current with the 680 mV peak. While the origin of this change in ratio is unclear, different extents of pre-adsorption of the oxidisable compounds at different milk dilutions could be a factor. As can be seen in Fig. 1b, the anodic peak current ($I_{p.a}$) for UA changed linearly with the milk dilution factor. The error bars represent the standard deviations for three repeat runs which were very low at some points.

Temperature effect. The effect of sample temperature on the performance of the sensor was also investigated. This is important to consider, as the response of milk from the refrigerator maybe different to that at physiological temperatures. As can be seen in Fig 2a and 2b, the UA standard solution (100 μM, pH 6.6) and whole milk sample (Blue cap, Fonterra) were incubated at different temperatures (5, 15, 25, 37°C) in a water bath prior to running the voltammetry. When the UA standard solution was analysed, a partially reversible redox process was observed, with the lowest (ca. 17 μA) and the highest (ca. 20 μA) anodic peak currents at 5 and 15 °C, respectively (Fig. 2a). Moreover, increasing the temperature from 5 to 37 °C led to a progressive decrease of 47 mV in the UA peak potentials. Fig. 2b displays the effect of temperature on the whole milk sample, again showing two anodic peaks for UA and additional reducing agents. One noticeable difference in the voltammograms was the loss of the return UA cathodic peak in the milk samples, (Motshakeri, Travas-Sejdic, et al., 2018a), even when the voltammetric scan was reversed at 500 mV, suggesting some interaction of oxidised uric acid with other milk components. The anodic peak due to UA in the milk decreased gradually in intensity with increasing temperature, by around 5 μA between the highest and the lowest current responses (Fig. 2c). There was again a 45 mV negative shift of the anodic peak potential with temperature increase from 5 to 37 °C. There was enough of a shift in response to indicate

252 that all samples should be run after the milk has reached a common room temperature, and that
253 controlled temperature conditions should be considered for more accurate analytical work.

254 **Effect of uricase enzyme.** Treatment with uricase (EC 1.7.3.3) enzyme was used to confirm
255 the oxidation current contributions of UA in a whole milk sample (Blue cap, Fonterra), to
256 ensure that what is measured is not merged with other compounds. A milk sample was chosen
257 without a significant earlier peak due to a measureable level of ascorbic acid, which is the
258 subject of the following section. One unit of activated uricase oxidizes one micromole of UA
259 per minute, at 25°C and pH 8.5, to produce allantoin, H_2O_2 and CO_2. Fig. 3a shows the expected
260 sharp oxidation peak of about 26 μA for 80 μM UA on top of the background PEDOT redox
261 processes before enzyme addition, both run in pH 8.5 borate buffer at 25°C. The peak position
262 of 225 mV is consistent with the expected shift in moving from a pH 6.6 to a pH 8.5 solution
263 (Motshakeri, Travas-Sejdic, et al., 2018a). As can be seen in Fig. 3b, after treatment with
264 uricase for 90 min, the UA peak was completely eliminated from the UA solution. The response
265 of a whole milk sample was similarly investigated before and after adding the uricase enzyme.
266 Fig. 3c illustrates the voltammogram of milk adjusted to pH 8.5 on top of the background for
267 the PEDOT electrode in the pH 8.5 borate buffer. Even though the pH had been increased, the
268 two distinct anodic peaks found in milk samples were still seen (Motshakeri, Travas-sejdic, et
269 al., 2018a; Motshakeri, Travas-Sejdic, et al., 2018b). It can be noted that the second peak at
270 around 620 mV had also shifted in position by about 30 mV compared to values found in milk
271 without pH adjustment. The position of this peak had been found to be changed very little in
272 lower pH solutions, reaching only 670 mV at pH 3.0. After enzyme addition the UA peak was
273 again completely removed, and only the later peak at around 620 mV was retained, which was
274 also a little smaller in size due to dilution effects of around 7% from the addition of the uricase
275 solution (Fig. 3d). It can also be noted that the compounds that contribute to this peak begin to

oxidise as the potential exceeded 400 mV, with no sign of additional reducing agents that might interfere with UA determination at the 330 mV peak.

Effect of ascorbate oxidase enzyme. Ascorbate oxidase (AOX) (EC 1.10.3.3) catalyses oxidation of L-ascorbic acid in the presence of oxygen to L-dehydroascorbic acid and H_2O. Fig. 4a clearly indicates a well-defined and broad oxidation peak at about 200 mV for AA, while this peak was absent (Fig. 4b) after treatment with the enzyme (blue line). When this experiment was repeated with whole milk sample (Blue cap, Fonterra) that was found to contain a small amount of AA (Fig. 4c), there was no AA detected in the milk after adding enzyme and incubating at 37 °C for 90 min (Fig. 4d). We note that Fig. 4d shows an expected 33% reduction in the observed peak currents, as a result of dilution after adding ascorbate oxidase to the milk sample. Thus, it was found that AA does not interfere with UA peak in the CV analysis of untreated whole milk.

3.3 RP-HPLC-UV analysis

Effect of acid concentration, pH, and centrifuge. To compare the results obtained by CV with an HPLC method for uric acid, an HPLC protocol was employed. The optimum ortho-phosphoric acid concentration used for pre-treating the whole milk sample was found to be 1%, as it showed the highest UA HPLC peak value, which was unchanged after increasing the acid concentration to 4% (data not shown). Moreover, centrifuging the milk sample at a relative centrifugal force of 2000 g showed a higher UA peak compared to a 12000 g run (data not shown).

The obtained supernatant (pH about 2.3) from the pre-treatment step was analysed by HPLC and CV immediately, and once its pH had been changed to 6.6. Fig. S1 displays how changing the pH affected the UA analysis for a whole milk sample, and for a 100 μM UA standard solution prepared at these two different pH values (6.6 and 2.3). It is clear that the UA peak in

HPLC shifted to an earlier retention time for the higher pH injection. Interestingly at pH 6.6, the UA peak exhibited significant tailing and showed signs of some shoulder features (Fig. S1a). When the pH is higher than the pK_a of UA (pK_a =5.4), the speciation will lead to more urate ion present than undissociated uric acid. Upon injection, a sample at pH 6.6 will initially contain more urate relative to uric acid, but this will gradually change as the injected sample becomes mixed with the HPLC mobile phase buffered to pH 4.8, where the uric acid form will be the major species present. The urate ion is expected to be less strongly retained on the reversed phase column compared to uric acid, and to elute earlier, but with some tailing expected as some of the compound in the uric acid form is retained on the stationary phase. With a pH 2.3 injection, nearly all of the UA will be present as uric acid to begin with, producing a sharper and later HPLC peak.

With regards to the milk samples, injection of the supernatant directly after acid/chloroform treatment (pH 2.3) gives a clear peak for use in quantitative analysis (Fig. S1b). However, raising the pH of the treated milk sample back to 6.6 led to a peak with tailing and greater overlap with potential interfering species. Although the pH of treated milk samples, mobile phase and acid were the same in all of the HPLC experiments, there was still some variation in UA retention times for different milk samples. It can also be noted that the HPLC UA peaks for the milk samples were located over a minute later than peaks seen for UA standards on their own.

HPLC analysis of UA standard solutions. Different concentrations of UA were prepared in a PBS buffer (0.1 M, pH 6.6) and then diluted by 1% ortho-phosphoric acid in a 1:1 proportion to assess matrix effect based on the slope of the obtained calibration curve. The final pH of the UA standard solutions was 2.1. Then solutions were analysed by HPLC, and a calibration curve was obtained with a linear regression equation of y=11.7x-18.4 (Fig. S2), and excellent linearity was apparent from the data obtained. The measurements were repeated 3 times and a

very low standard deviation was obtained. The limit of detection (LOD = 3*s*/*b*) and limit of quantification (LOQ = 10*s*/*b*), where *s* is the standard error of the intercept and *b* is the slope of the calibration curve, were calculated to be 0.6 μM, and 1.7 μM, respectively, while noting that this analysis does not involve a consideration of variations from the milk pre-treatment step.

Enzymatic treatment of milks prior to HPLC analysis. Incubation of a milk sample (Blue cap, Fonterra) with uricase enzyme at pH 8.5 for 90 minutes, followed by acid/chloroform treatment led to removal of the HPLC peak for UA (Fig. S3a). In this chromatogram the UA peak appeared at around 9.3 minutes in non-enzyme treated milk samples, while no extra peaks were left after removal of UA by uricase in enzyme-treated samples. As shown in Fig. S3b, addition of ascorbate oxidase (AOX) to a treated milk, known to contain AA, with pH adjusted to 5.6 (optimum pH for activation of AOX) resulted in removal of the AA peak that appeared at 3.5 minutes. Thus, in the HPLC analysis of UA there is no interference coming from AA present in the milk.

Cyclic voltammetry of UA and different milks. A calibration curve was produced using standard solutions of UA at different concentrations (6.25, 12.5, 25, 50, 100 μM), as previously reported (Motshakeri, Travas-sejdic, et al., 2018a). The linear fit obtained for the 330 mV peak ($y=0.174x+0.4$; $R^2 = 0.998$), was used for calculation of UA concentration in the following analyses of commercial milk samples (Fig. S4). The CVs were run on fresh milk samples without any pre-treatment, and the results are presented in Table 1. All CV data were reported after subtracting the current due to the background solution (PBS pH 6.6). The peak for UA oxidation ($I_{p.a2}$) was observed between 325 and 350 mV in the different milk samples. A small earlier peak current at around 30 mV of ca. 1-2 μA ($I_{p.a1}$) (~ 30-55 μM) that can be associated with AA was observed in some samples, such as the raw milk. In these cases, the background was adjusted to take account of the continued AA current when analysing the peak for UA.

The intensity of the later 650 mV peak was also recorded ($I_{p.a3}$). It is interesting that the intensity of this peak was the highest in raw milk preserved with sodium azide compared to all other analysed milk samples (Fig. S5). Sodium azide is used to prevent microbial growth during storage (Suzuki et al., 2007), but its presence led to the creation of a broad current response across the region of the 3rd anodic peak. To check on the electrochemical oxidation of sodium azide, a 0.02% (W/W) solution in PBS (pH 6.6) was prepared, and current was seen from 250 mV and continued to form a broad peak at about 800 mV (Fig. S6). The impact of sodium azide was also checked in a commercial pasteurised milk, with voltammetry run immediately after the addition of sodium azide, and again a large broad additional oxidation current was observed. There was also a shift of the UA peak to an earlier potential at around 280 mV, which was not due to a change in milk pH, which did not change significantly after the addition of azide, but to some other interaction between azide and the uric acid. A small cathodic peak of unknown origin was seen on the reverse scan, also at around 280 mV, due to the reduction of some product of the interaction of oxidised sodium azide and milk (Fig. S6).

Comparison of two analytical methods (CV vs. HPLC). The reproducible CV peaks confirm the good performance of the PEDOT sensor for the analysis of milk, as previously reported (Motshakeri, Travas-sejdic, et al., 2018a; Motshakeri, Travas-Sejdic, et al., 2018b). The precision of both assays were calculated for three replicates of each milk sample as a relative standard deviation (%RSD). The CV method provide an average 4.7% for the %RSD. While the repeat HPLC injections were very reproducible, with an average 1% for the %RSD (Table S1), greater variation was observed from repeating the acid/chloroform treatment procedure. Table S2 displays an example of the high variation associated from three replicates in the processing method on the UA level measured in two different milk samples. Although HPLC values for each milk sample seemed to be more precise with lower %RSD than the voltammetric method, there was a significant extra variation of about 3.8-4.4% in the RSD

obtained from triplicates of the milk treatment step (Table S2). This is not a source of error for the CV method, given that no sample pre-treatment is needed.

Table S1 and Fig. 5 compare the UA concentration measured by CV in this study with the conventional method, HPLC, for 36 different milk samples. As seen in Fig. 5, an acceptable linear regression was obtained ($y=1.10x-1.97$; $R^2 = 0.80$), and the average difference in UA concentrations between the CV and HPLC methods was 6 %. In addition, analysing the data with TOST statistical analysis (Two One Sided Test) showed that the confidence interval describing the difference in the two mean values falls completely within the upper and lower range limit defined by theta (i.e. the acceptable statistical range (% $\theta \sim 40$)), indicating that on this test, the mean values of the two data sets were equivalent.

The highest UA concentration in this experiment was found in the A2 milk which lacks the presence of type A1 of β-casein proteins and it has been reported that people who drink milk with A1 casein are more susceptible to develop diabetes or allergy than those consuming A2 milk (Lacroix & Li-Chan, 2014). Although UA level in some milk samples such as UHT (Meadow), Trim (Fonterra) or Calci$^+$ Trim (Anchor), and Gray-non-homogenized (Lewis Road) were higher than average, the UA concentration values were not extreme and fell within the range of 50-110 μM. The lowest UA levels were observed in Homebrand, Calci-Trim Bottle (Meadow), Gray-whole-non-homogenized (Puhoi Valley), and raw milk with sodium azide. The remaining variations in the levels of UA between the different milk samples might be due to factors such as the breed of cows, and to various regional and seasonal factors.

In the present study, each PEDOT electrode was prepared separately, which involved electrode cleaning (7 min), electropolymerisation steps (10 min), and sample run (3 min) which together can be completed within 20 minutes. This can be compared to the time involved in HPLC column conditioning (45 min), the milk pre-treatment step (c. 1 hour and 15 min), and HPLC

399 runs (20 min). However, more significant time-savings can be expected through the use of
400 screen-printed and disposable PEDOT electrodes.

401 ## 4. Conclusions

402 This study describes a simple method to determine the UA concentration in milk using a
403 voltammetric method that is sensitive, selective, and rapid. The voltammetric scan also shows
404 the presence of ascorbic acid, and the contribution of additional reducing agents, seen at lower
405 and higher potential values, respectively. The mean values of UA in milks analysed by HPLC
406 were slightly higher on average than those obtained by the voltammetric method. The method
407 is advantageous compared to HPLC procedures, not only because there is no need to pre-treat
408 milk samples to remove fat/proteins before analysis, but also due to the single and sharp peak
409 that CV provides for UA analysis. In conclusion, the PEDOT sensor was highly selective
410 against interference from coexisting reducing compounds such as ascorbic acid, amino acids
411 etc., and the methodology can be used for routine UA analysis in milks.

412 ## Conflicts of interest

413 None.

414 ## Acknowledgments

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416 (MBIE) within the “High Performance Sensors” program.

417

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543

Table 1. Cyclic voltammetry parameters for different available milk products, measured at the developed PEDOT sensor measured at 100 mV/s after a 30 second holding time. All the data are the average of 3 replicates.

Milk type (Based on the label information on milk bottle)	$E_{p.a1}$ **(mV)**	$E_{p.a2}$ **(mV)**	$E_{p.a3}$ **(mV)**	$I_{p.a1}$ **(μA)**	$I_{p.a2}$ **(μA)**	$I_{p.a3}$ **(μA)**
UA standard solution (100 μM in PBS pH 6.6)	nd	328 ± 1	nd	nd	17.7 ± 2.1	nd
Blue (Anchor™)	29.3 ± 1.2	332 ± 1	649 ± 7	2.5 ± 0.2	14.5 ± 1.0	34.2 ± 1.1
Lite (Anchor™)	nd	332 ± 1	657 ± 3	nd	14.3 ± 1.0	32.2 ± 1.5
Green (Anchor™)	22.5 ± 0.7	329 ± 2	658 ± 2	2.1 ± 0.0	14.7 ± 0.9	34.3 ± 3.5
Blue (Meadow™)	23.5 ± 0.7	333 ± 5	658 ± 7	2.1 ± 0.1	15.3 ± 1.0	20.0 ± 0.9
Lite (Meadow™)	nd	331 ± 2	654 ± 7	nd	14.2 ± 0.2	28.1 ± 0.8
Trim (Meadow™)	nd	328 ± 4	642 ± 6	nd	12.6 ± 0.4	28.3 ± 1.9
Blue (Home brand™)	nd	329 ± 2	644 ± 6	nd	10.6 ± 0.6	18.0 ± 2.3
Lite (Home brand™)	nd	324 ± 2	643 ± 5	nd	10.3 ± 0.3	14.5 ± 0.8
Green (Home brand™)	nd	325 ± 2	647 ± 4	nd	9.5 ± 0.4	16.6 ± 3.6
Blue (Signature™)	30.0 ± 0.0	336 ±3	645 ± 6	1.3 ± 0.2	13.5 ± 0.4	20.8 ± 1.0
Lite (Signature™)	27.0 ± 0.0	334 ± 3	654 ± 5	1.5 ± 0.0	15.0 ± 0.4	21.7 ± 2.1
Lite (Puhoi organic™)	nd	351± 2	656 ± 2	nd	14.0 ± 0.5	34.6 ± 3.6
Fresh milk from local farm						
Raw	32.0 ± 0.0	332 ± 2	644 ± 5	1.6 ± 0.4	12.6 ± 0.3	22.7 ± 1.9
Raw + Sodium azide (NaN_3)	26.0 ± 2.6	288 ± 3	657 ± 7	2.5 ± 0.2	10.7 ± 0.3	49.6 ± 1.7
Full cream						
Half & half (Puhoi organic™)	26.3 ± 0.6	340 ± 2	660 ± 2	1.8 ± 0.2	14.4 ± 0.8	33.9 ± 2.0
Farmhouse (Meadow™)	nd	329 ± 2	647 ± 6	nd	12.7 ± 0.9	16.2 ± 1.4
Homogenized						
Blue (Lewis Road organic™)	21.3 ± 6.1	330 ± 4	651 ± 4	1.9 ± 0.0	14.9 ± 0.9	36.9 ± 6.9

Whole-permeate free (Lewis Road organic™)	nd	330 ± 2	657 ± 7	nd	12.8 ± 1.0	19.9 ± 1.2
A2 (Fresh Valley™)	23.7 ± 3.8	333 ± 2	661 ± 7	1.9 ± 0.2	16.1 ± 0.9	29.3 ± 1.9
Blue-pure (Puhoi organic™)	28.3 ± 2.3	331 ± 2	652 ± 4	1.1 ± 0.0	14.8 ± 0.8	23.2 ± 2.0
Blue (Kapiti organic™)	nd	331 ± 2	651 ± 3	nd	13.5 ± 0.8	32.0 ±2.5
Non-homogenized						
Whole (Lewis Road organic™)	nd	330 ± 7	643 ± 7	nd	12.1 ± 0.6	19.4 ± 2.9
Gray (Lewis Road organic™)	20.3 ± 6.0	330 ± 3	649 ± 5	1.5 ± 0.2	14.7 ± 1.2	34.5 ± 4.6
Whole-gray (Puhoi organic™)	nd	331 ± 1	666 ± 7	nd	11.2 ± 0.2	25.2 ± 4.7
Gray (Kapiti organic™)	nd	334 ± 3	656 ± 9	nd	14.4 ± 1.0	32.4 ± 1.4
UHT						
Blue (Meadow™)	nd	331	655 ± 5	nd	15.5 ± 0.6	20.0 ± 2.4
Lite (Meadow™)	29.0 ± 0.0	339	651 ± 3	1.5 ± 0.0	16.6 ± 0.4	23.0 ± 3.2
Calcium (Meadow™)	nd	336 ± 1	655 ± 4	nd	13.8 ± 0.1	15.2 ± 1.0
Skim (Home brand™)	nd	335 ± 1	651 ± 1	nd	14.1 ± 0.5	18.8 ± 2.6
Blue (Anchor™)	32.0 ± 1.4	336 ± 3	663 ± 5	1.5 ± 0.2	12.7 ± 0.2	28.6 ± 1.8
Lite (Anchor™)	29.0 ± 1.4	334 ± 2	653 ± 6	1.3 ± 0.4	14.8 ± 0.3	25.2 ± 0.8
Trim (Anchor™)	39.0 ± 4.2	338 ± 2	657 ± 6	1.5 ± 0.2	13.5 ± 0.8	26.5 ± 1.3
Full cream (Select™)	nd	338 ± 2	645 ± 7	nd	13.9± 0.9	15.6 ± 0.4
Calci^{+}/ Proto^{+}						
Calci Trim bottle (Meadow™)	nd	330 ± 3	648 ± 3	nd	10.5 ± 0.8	16.4 ± 1.2
Calci^{+} trim (Anchor™)	22.5 ± 4.9	337 ± 2	658 ± 1	1.9 ± 0.2	14.7 ± 0.8	34.0 ± 2.6
Proto^{+} lite (Anchor™)	30.3 ± 1.5	336 ± 4	657 ± 4	1.5 ± 0.1	13.6 ± 0.8	36.7 ± 1.1

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548

549 **Figure captions:**

550

551 **Fig.1.** (a) Cyclic voltammograms (background subtracted) of whole milk sample, and after
552 dilution in pH 6.6 PBS buffer, measured at 100 mV/s using PEDOT-modified glassy carbon
553 electrode; and (b) the anodic peak current for UA at 330 mV versus the percentage of milk
554 (Error bars = ± STD, n = 3).

555 **Fig. 2.** Cyclic voltammogram of (a) 100 μM UA in PBS pH 6.6, and (b) whole milk at different
556 temperatures (5, 15, 25, 37 °C), (c) $I_{p.a}$ of UA peak vs. Temperature (Error bars = ± SD, n = 3).

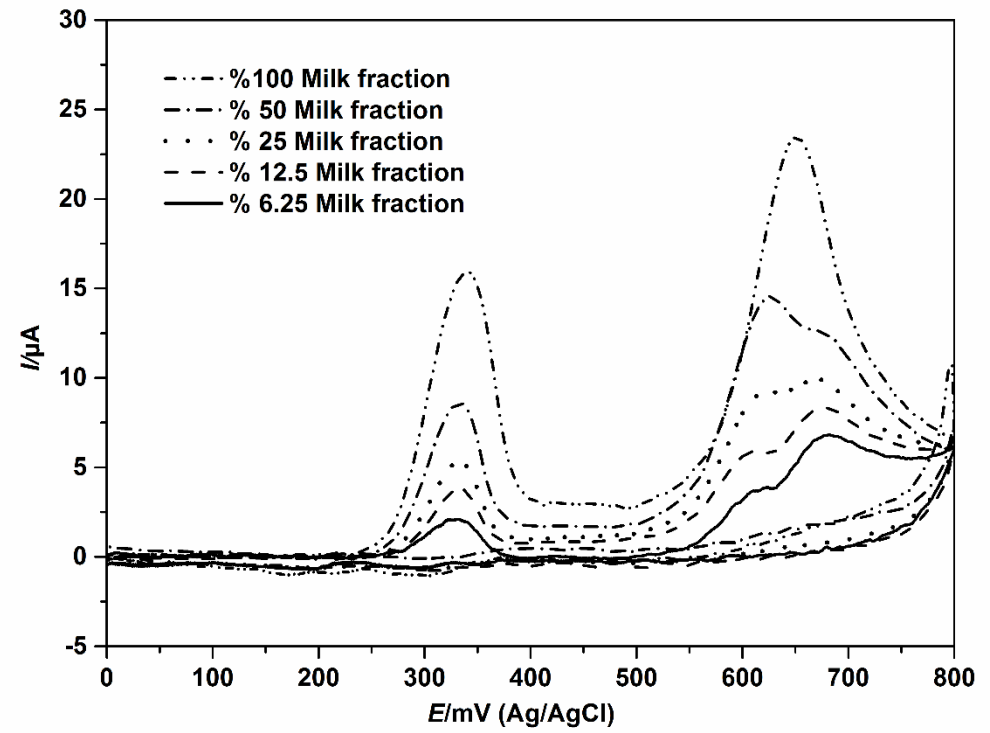
557 **Fig. 3.** Cyclic voltammograms of (a) 80 μM of UA (a) before and (b) after the addition of
558 uricase; a milk sample (c) before (d) after addition of uricase, using PEDOT-modified
559 electrode. All voltammograms were measured at 100 mV/s scan rate and are compared to the
560 response obtained with the background buffer.

561 **Fig. 4.** Cyclic voltammograms of (a) AA (1 mM) before enzyme addition and (b) after addition
562 of AOX to 0.5 mM AA; and milk (c) before and (d) after addition of AOX at pH 5.6 and 37°C,
563 using PEDOT-modified electrode. All voltammograms were measured at 100 mV/s scan rate
564 and are compared to the response obtained with the background buffer (PBS pH 5.6, 4mM).

565 **Fig. 5.** Comparison of the results obtained by the HPLC and the CV methods for the analysis
566 of UA in 36 different samples of milks.

567

568 Figure 1



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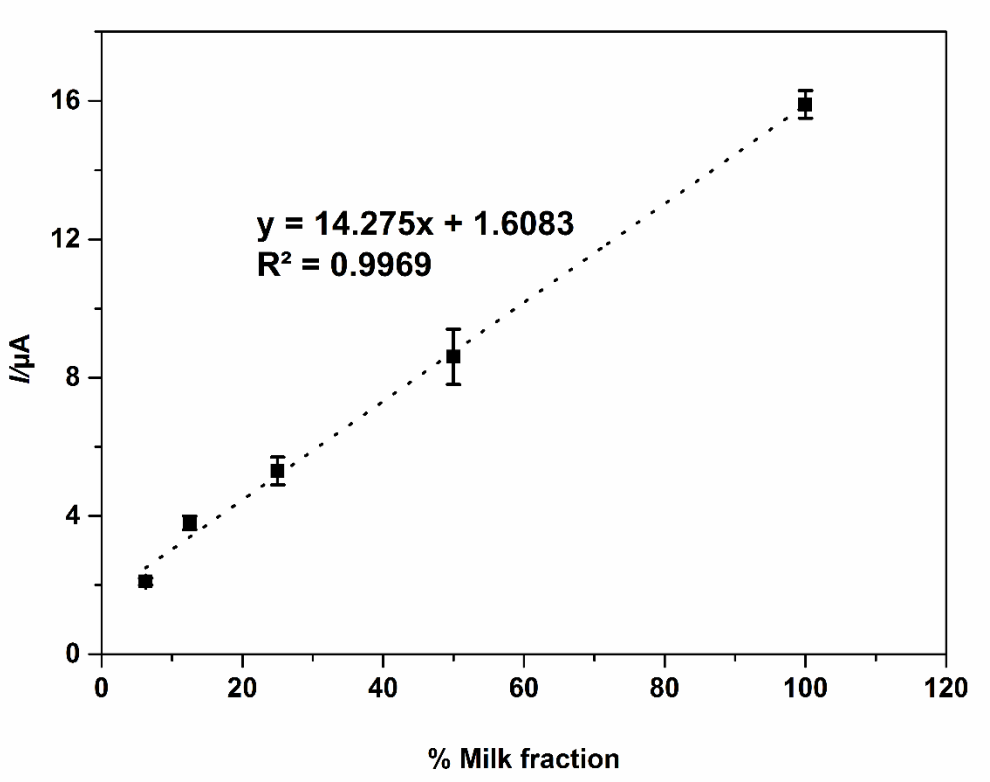

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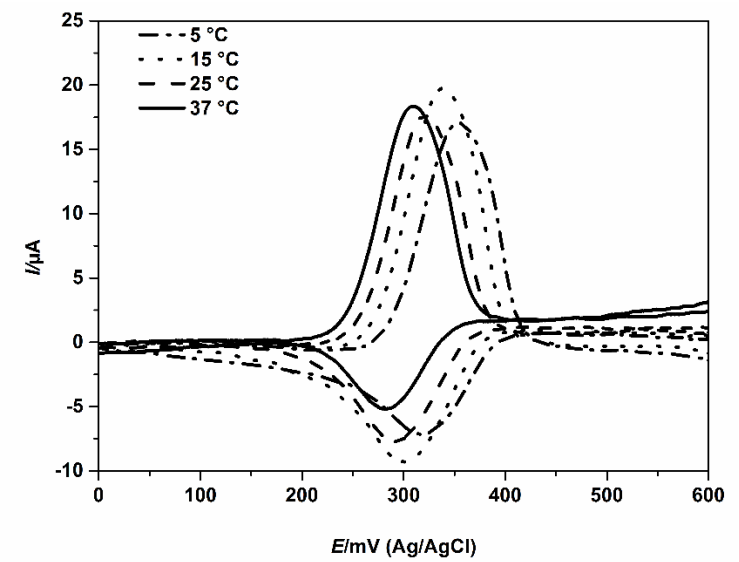
573 Figure 2



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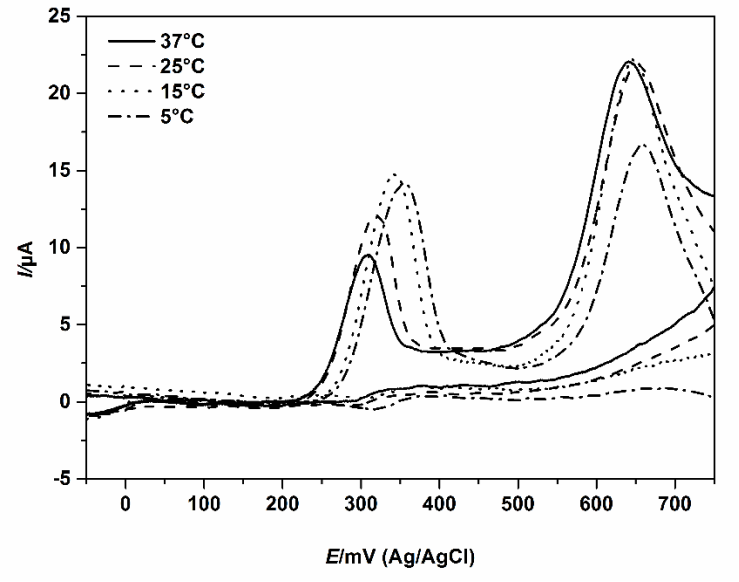

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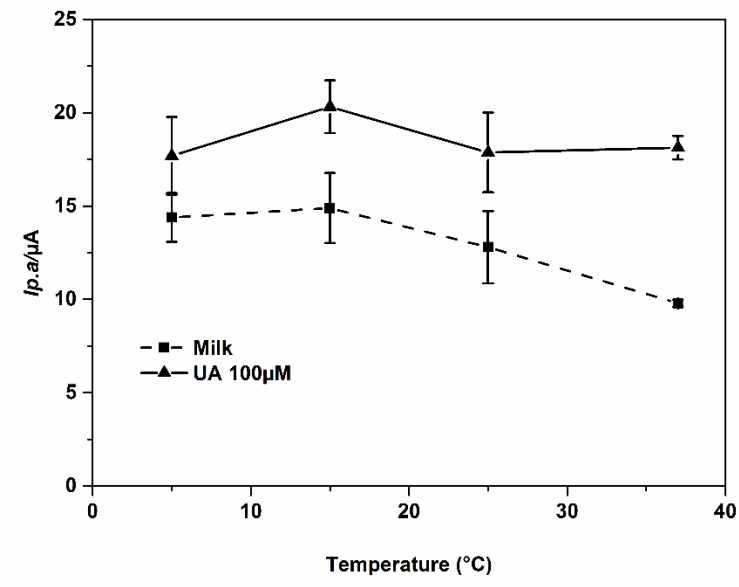

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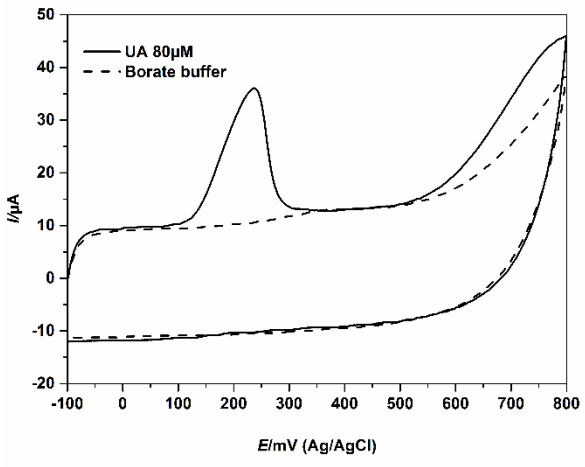
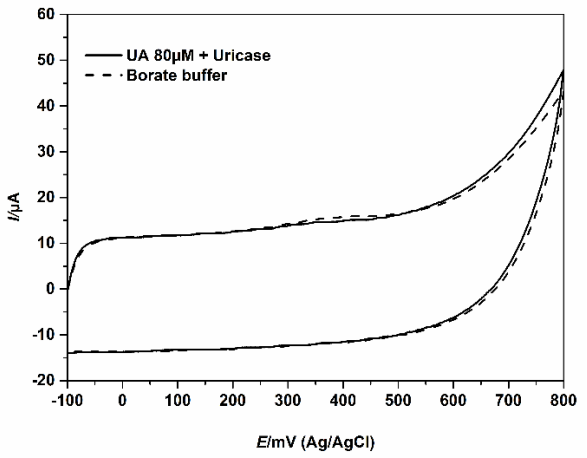
577 Figure 3





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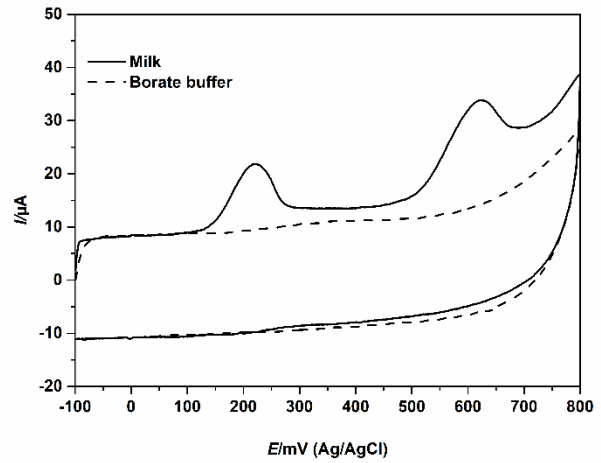

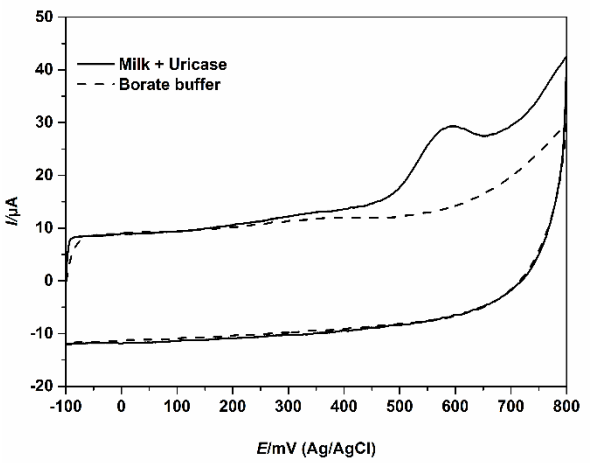

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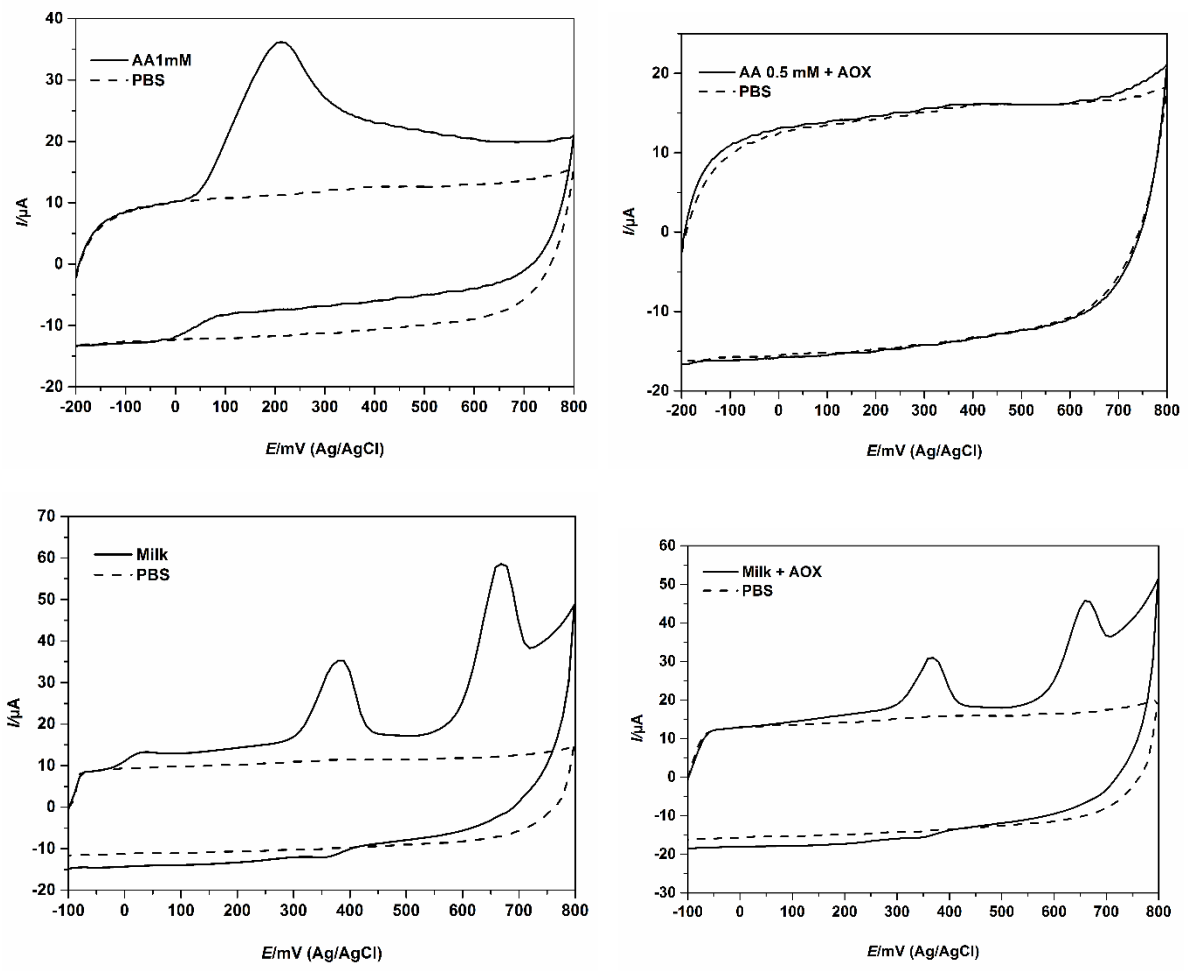
582 Figure 4



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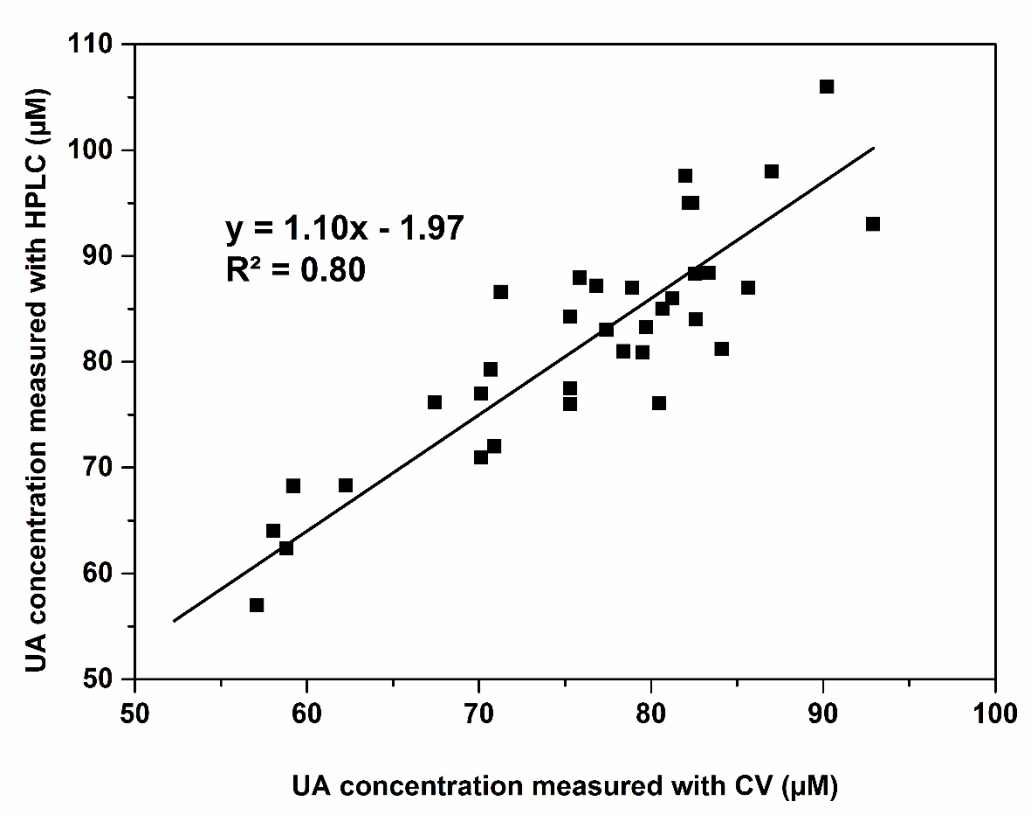
587 Figure 5



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