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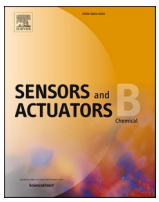

Impact of milk preservation in the classification and prediction capabilities of a voltammetric electronic tongue

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ABSTRACT

Electronic tongues (ETs) are of great interest for the dairy industry. However, still several problems related to the fouling of the electrodes and the preservation conditions of the samples, limit their usage in milk analysis. In this work, the effects of sampling and preservation methods used to analyze raw cow milk in the performance of a portable ET based on serigraphied electrodes have been explored and optimized. Fouling caused by fat and proteins is drastically decreased if milk samples are diluted and sonicated at 20 kHz (pulses of 30 s during 5 min) previous to the analysis. Preservation by freezing at − 20ºC or by addition of azidiol have an important influence in the electrochemical responses, but freezing is a more efficient preservation method for ET analysis than azidiol. Principal Component Analysis of the signals obtained using the optimized conditions allows the discrimination of 180 milk samples with different characteristics. Using Support Vector Machine Regression (SVMR) model, the ET is able to predict the percentage of fat, protein, urea, somatic cell count (SCC) and β-Hydroxybutyric acid (BHB) with good correlation coefficients and low residual errors. The system reported can be thus used for fast in situ analysis of five parameters in a single experiment.

1. Introduction

The dairy industry requires real time and accurate analysis of the composition of their products. The composition varies depending on the species, breed, feed, ambient conditions, the stage of lactation, genetics number of calves, etc. [1]. Techniques currently used for the analysis of milks include FTIR, NIR, or chromatography, among others [2]. Most of them require instruments that must be used by skilled personnel. An alternative approach relies on the use of methods providing a multivariate description of each sample that can be considered as a fingerprint of its chemical composition. The data thus obtained are analyzed by means of statistic pattern recognition methods leading to discrimination and classification of samples.

This multivariate approach has been applied to the analysis of milk by obtaining the non-specific fingerprints from spectroscopic, or chromatographic techniques [3,4]. Complex datasets can also be obtained through the use of multisensor systems. In particular, electrochemical sensors offer major advantages over other classical methods as they are versatile, cheap and portable, they not require expensive equipment and qualified operators, it is possible to operate with low quantity of samples and in comparison with traditional methods, this approach allows to obtain a multiparametric response in one measurement [5]. Electrochemical sensors have been used to develop multisensor systems that combined with a pattern recognition software form the so-called electronic tongues (ET). Such instruments are formed by distinct sensing units functionalized with different materials with cross-selectivity [6–8]. Electrochemical ETs can be purposely developed for a particular application by combining the adequate sensing materials [9,10]. Moreover, miniaturized and disposable electrochemical sensors make the development of portable ETs that can be implemented on line [11].

Several works have explored the benefits of ETs in the dairy industry [12]. These systems consist of potentiometric [13,14] or voltammetric sensors [15–19] and have been mainly used to discriminate and classify milk samples with different characteristics (freshness, nutritional composition, adulterations, etc.). However, milk is an extremely complex solution and the analysis of milk and dairy products using ETs is not

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Table 1
List of cows whose milks were included in the study. Mean physicochemical values.

Cow	Lactation number	DIM (days in milk)	kg of milk	Days of pregnancy	Fat (%)	Protein (%)	SCC (SC/mL)	Urea (ppm)	BHB (mmol/l)
S1	6	309	39	76	4.14	3.63	33	156	0.04
S2	5	67	53	0	**5.95**	3.17	193	96	0.23
S3	5	21	44	0	**4.69**	3.37	26	144	0.05
S4	3	231	37	160	**4.96**	3.68	148	195	0.06
S5	3	84	38	0	3.70	3.20	219	103	0.05
S6	2	400	20	183	4.14	3.83	316	158	0.06
S7	3	28	43	0	4.43	3.36	47	80	0.13
S8	2	24	50	0	4.05	3.07	140	133	0.08
S9	2	62	57	0	**4.54**	3.25	8	143	0.02
S10	1	240	41	34	3.09	3.09	39	150	0.05
S11	1	134	36	0	3.30	3.58	865	116	0.02
S12	1	109	37	0	**5.18**	3.20	71	145	0.05
S13	5	104	52	28	3.05	2.89	65	153	0.01
S14	4	63	53	0	3.05	2.92	271	148	0.06
S15	4	70	41	4	3.41	2.77	42	122	0.02
S16	4	116	51	22	3.50	3.03	16	155	0.04
S17	3	197	45	15	3.32	3.29	26	141	0.08
S18	3	228	43	152	**6.28**	3.46	5	125	0.01
S19	3	162	48	99	3.70	3.81	26	224	0.04
S20	2	75	59	14	3.90	2.99	42	150	0.03
M1	3	110	45	0	4.14	4.14	33	156	0.04
M2	3	85	51	12	5.95	3.95	193	96	0.23
M3	2	62	48	32	4.69	3.69	26	144	0.05
M4	2	75	46	0	4.96	3.68	195	148	0.06
M5	4	115	53	124	3.70	3.70	219	103	0.05

a completely solved problem. Difficulties arise from the presence of fat globules and proteins that cause fouling of the electrodes, reducing the repeatability and lifetime of the sensors. As a consequence, milk has to be pre-treated to eliminate, or at least, substantially diminish the presence of these two main components in the mixture. On the other hand, the microbiologic process that start immediately after milking, can modify the composition of milk. As it is not always possible to analyze milk samples the same day they are collected, it is necessary to develop techniques with the capability of decrease the fouling and methods able to limit the growth of organisms without interfering in the electrochemical measurements.

This makes necessary to optimize the measurement protocols and to evaluate the effect of practices such as dilution, sonication, freezing or addition of preservatives such as azidiol on the responses of electrochemical sensors. In this work, these effects have been evaluated and optimized using a home-made portable electronic tongue, based on three Screen Printed Electrodes (SPE), modified with different materials, including graphite (C-SPE), nickel oxide nanoparticles (NiO-SPE) and Prussian Blue (PB-SPE). After the optimization of the measurement conditions and the evaluation of the cross-selectivity of the sensors, the array was used to analyze twelve milk samples obtained from individual cows with different characteristics (days of pregnancy, number of pregnancies, lactation days …). The discriminatory capability of the sensor array was investigated using Principal Component Analysis (PCA). Correlations with chemical parameters were also established using SVM mathematical models.

2. Experimental

2.1. Chemicals

Trisodium citrate 5,5-hydrate (VWR International, Radnor, Pennsylvanie, USA), sodium azide (VWR International), chloramphenicol (Millipore. Sigma, Burlington, Massachusetts, USA), bromophenol blue (Millipore. Sigma), ethanol (Greenfield Global, Toronto, Canada). Urea, tributyrin, casein and β-hydroxybutyric acid were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Deionized water from MilliQ (Millipore-Sigma Aldrich, Darmstadt, Germany) (resistivity 18.2 MΩ·cm) was used in all experiments.

2.2. Milk samples

Exploratory experiments for the method optimization were carried out using bulk stainless steel tank raw milk obtained from 20 healthy cows. 30 mL aliquots were used to apply different preservation methods. All samples were received vacuum packed. The effect of dilution in the apparition of fouling of the electrodes was studied by diluting bulk raw milk preserved at 4ºC with water in proportions 1:2; 1:5 and 1:10. Dilutions were made in deionized water to develop a methodology easily applied in the dairy industry without the need of chemical pretreatments.

The impact of the treatment of ultrasound was evaluated as an homogenization method using an Ultrasonic Vibra-Cell Processor: VC 505 (SONICS & MATERIALS, Newtown, USA) by applying 500 watts and a frequency 20 kHz (10 pulses of 30 s during 5 min). Moreover, ultrasounds haven used in the last years to improve the antioxidants stability in food matrixes by inactivating their degradation enzymes [20,21].

The effect of preservation was analyzed using 36 aliquots of tank raw milk where different preservation methods were applied: preservative-free milk stored at 4ºC, preservative-free milk stored at − 20ºC, milk treated with azidiol stored at 4 ºC and milk treated with azidiol stored at 4ºC seven days after milking. Before measurements, preservative-free milk stored at − 20ºC was defrosted at room temperature after seven days while being shaken continuously using orbital motion at 100 rpm. Azidiol (AZ) was prepared as described previously [22] by dissolving 4.5 g of trisodium citrate 5,5-hydrate (VWR International, Radnor, Pennsylvanie, USA), 1.8 g of sodium azide (VWR International), 0.075 g of chloramphenicol (Millipore. Sigma, Burlington, Massachusetts, USA), 0.035 g of bromophenol blue (Millipore. Sigma), and 10 mL of ethanol (Greenfield Global, Toronto, Canada) in 100 mL of sterile distilled water. 75 mL of this mixture were added to 250 mL of milk.

ET capabilities were evaluated once the measurement protocol was optimized from raw milk from the tank. For this purpose, a set of milk corresponding to 20 individual cows milked under veterinary supervision were analyzed. (Table 1). From these samples, 9 aliquots have been taken during the quality control process by pumping from cow udder (10 mL/ 5 min milking) to assess the homogeneity of the samples due to the high complexity of the matrix. Aliquots were measured by alternating the samples.

Physicochemical parameters were obtained by classical standard

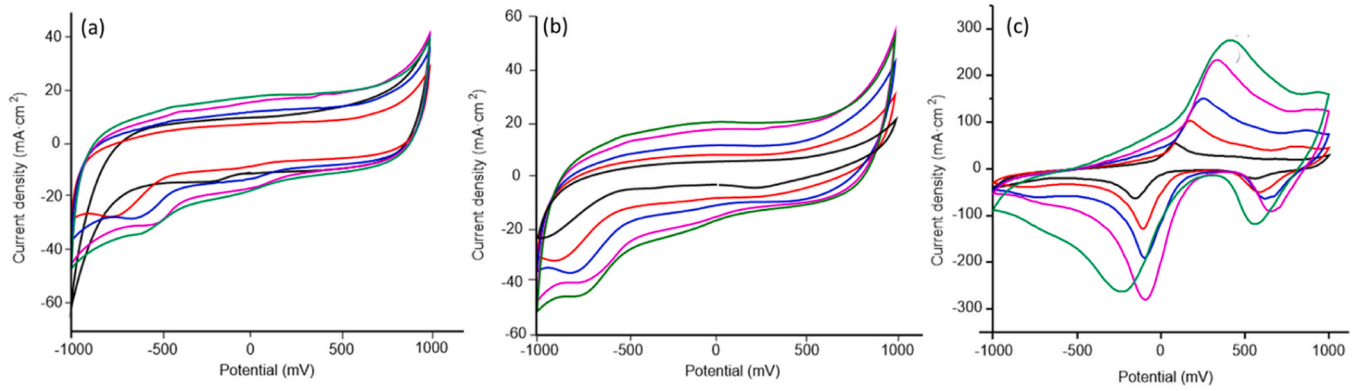

Fig. 1. Cyclic Voltammograms obtained with electrode of (a) C-SPE. (b) NiO-SPE and (c) PB-SPE sensors immersed in raw milk (pink); milk diluted in water: 1:2 (blue); milk diluted 1:4 (red); milk diluted 1:10 (black) and milk diluted 1:2 and sonicated (green).

methods: fat was measured by the Gravimetry Röse-Gottlieb method (ISO 1211:2010). proteins were measured by the Kjeldahl method (ISO 8968–1:2014) and urea by Infrared spectroscopy (ISO 9622:2013) [23]. Sanitary controls were also carried out. Somatic Cells Count (SCC) was measured using the Scepter™ 2.0 Cell Counter to ensure udder health and β-Hydroxybutyric acid (BHB) was measured by FTIR as a control of ketosis processes [24].

2.3. Electronic tongue

The ET was formed by three screen printed electrodes purchased from DropSens (Dropsens-Methrom. Oviedo. Spain). Commercial sensor devices contained a serigraphied pseudo-reference Ag/AgCl electrode (RE) and a carbon counter electrode (CE). Each electrode also has a working electrode made of carbon (electrode C-SPE), Prussian blue (PB-SPE) or nickel oxide nanoparticles (NiO-SPE). Electrochemical measurements were carried out in a portable potentiostat Multi Palmsens4 (Palmsens BV. The Netherlands). Cyclic voltammograms were registered from −1.0 V to + 1.0 V (the scan started at 0 V) at a sweep rate of 0.1 V/s. The applied potential was measured versus the screen-printed internal silver pseudo-reference electrode.

The multivariate data analysis was performed by using MATLAB R2021a (The Mathworks Inc. Natick. MA. USA). A feature extraction method based on "bell-shaped-windowing" curves called "kernels" was used to reduce the number of variables to 10 coefficients per voltammogram [25]. These parameters were used as the input variables for Principal Component Analysis (PCA) used for recognition of sample patterns and dis(similarities) between milk samples. Mathematical correlations between the results obtained using the voltammograms registered by the ET and the physicochemical analysis were established using Support Vector Machines Regression (SVMR) models. Regression models used the following parameters: Radial Basis Function as the core function C= 1 and with a cross-validation segment size of 15. All the mathematical models were subjected to cross-validation.

3. Results

An exploratory study was carried out to evaluate the extent of the matrix interferences. Fig. 1 shows the voltammograms obtained for the three types transducers immersed in raw and diluted milk. The commercial non-enzymatic SPE electrodes have been selected to evaluate their different responses to guarantee cross selectivity. It has been chosen a simple carbon electrode (C-SPE), a modified SPE with Prussian Blue (PB-SPE) due to its redox activity and a modified SPE with nickel oxide nanoparticles (NiO-SPE) to improve the electrocatalytic activity. The response of electrode C-SPE shows a broad background current that is the result of the non-Faradaic current inherent to the complex composition of the matrix. In addition, a small cathodic peak at E_c= −495 mV was observed that, according to the literature, is associated to oxygen-based reactions whose intensity is related to the amount of bulk-dissolved oxygen (DO). This is an important parameter in milk quality since DO is produced by the aerobic respiration of live bacteria. and the intensity of this peak can be a measure of spoilage or contamination of milk [26]. NiO-SPE shows a similar behavior, but in this case the cathodic peak appeared at − 650 mV and shows a higher intensity, probably due to the more efficient electrocatalytic behavior of the NiO nanoparticles modifier. Finally, the PB-SPE electrode showed the expected redox peak of the Prussian Blue electrocatalysts (Ea=250 mV and Ec −50 mV). In addition. voltammograms showed a second redox pair at E_a= 800 mV and Ec= 650 mV associated to the oxidation of antioxidants naturally present in milk (typically conjugated linoleic acid, α-tocopherol, β-carotene, vitamins A and D3, coenzyme Q10, etc.) [27]. These antioxidants could be observed thanks to the electrocatalytic effect of the PB modifier that diminished the oxidation potential of the antioxidant species [28].

In spite of the interesting responses, the performance of electrochemical sensors was limited by the fouling of electrodes caused by adhesion of fats and proteins that gave rise to quite high variation coefficients (CVs) (in the range of 10% in ten consecutive measurements). CVs have been calculated from the mean and standard deviation obtained from the response of the anodic peak of three sensors prepared under the same conditions, for each sample. The short time available from milking to analysis before spoilage of milk takes place is also a problem. In order to decrease the fouling of the electrodes and to preserve milk from spoilage, several methods could be used. These include dilution, sonication, freezing and addition of preservatives such as azidiol. Nonetheless, the effects of these methods on the response of electrochemical sensors of an ET need to be well established.

One easy pretreatment method to reduce the complexity of the matrix could be dilution with water. But dilution can reduce the concentration of other components decreasing the discrimination capability of the ET [26]. As observed in Fig. 1, dilution with water produced a decrease in the intensity of the responses and also a decrease in the background current. The variation coefficient of milk diluted 1:2 improved with respect to the variation coefficient obtained in raw milk (CV < 6%). Further dilution caused irreproducibility probably due to the decrease in the ionic conductivity (CVs <8% in 1:4 and <15% in 1:10).

Ultrasound is a promising technology which produces reduction of viscosity and homogenization of milk fat globules. Moreover. ultrasonication can also be a method for milk preservation preventing spoilage by microorganisms [29]. In our case, ultrasonication resulted in an increase in the intensity of the voltammetric responses probably due to the reduction in viscosity (Fig. 1). The homogenization caused by the ultrasounds improved the repeatability of the responses decreasing the variation coefficients. For instance, the CV of 10 consecutive cycles registered in 1:2 diluted milk, based on the mean and the standard

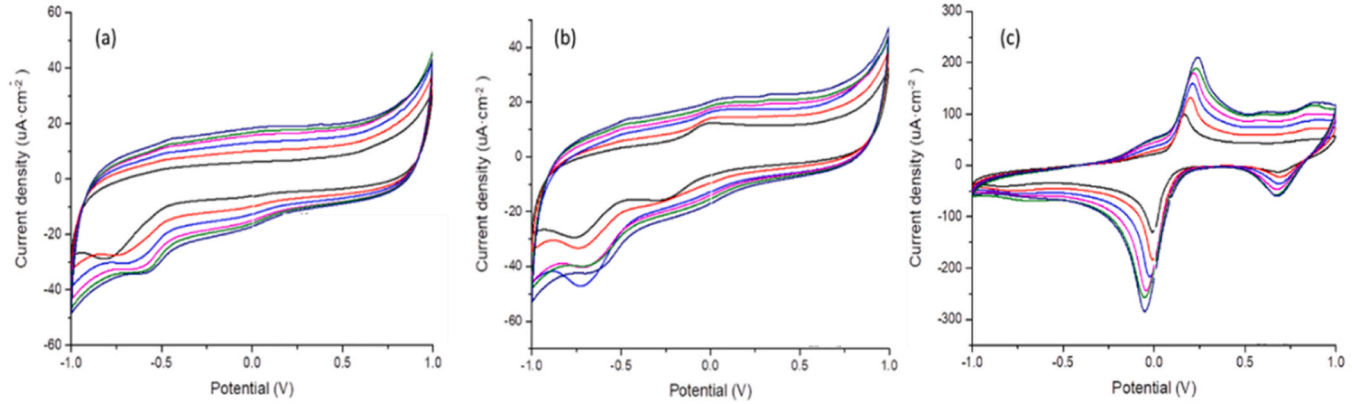

Fig. 2. Cyclic Voltammograms obtained with electrode of (a) C-SPE. (b) NiO-SPE and (c) PB-SPE sensors immersed in raw milk with addition of urea 0 M (black), $1{\cdot}10^{-5}$ M (red), $5{\cdot}10^{-5}$ M (blue), $1{\cdot}10^{-4}$ M (pink), $5{\cdot}10^{-4}$ M (green) and $1{\cdot}10^{-3}$ M (navy blue).

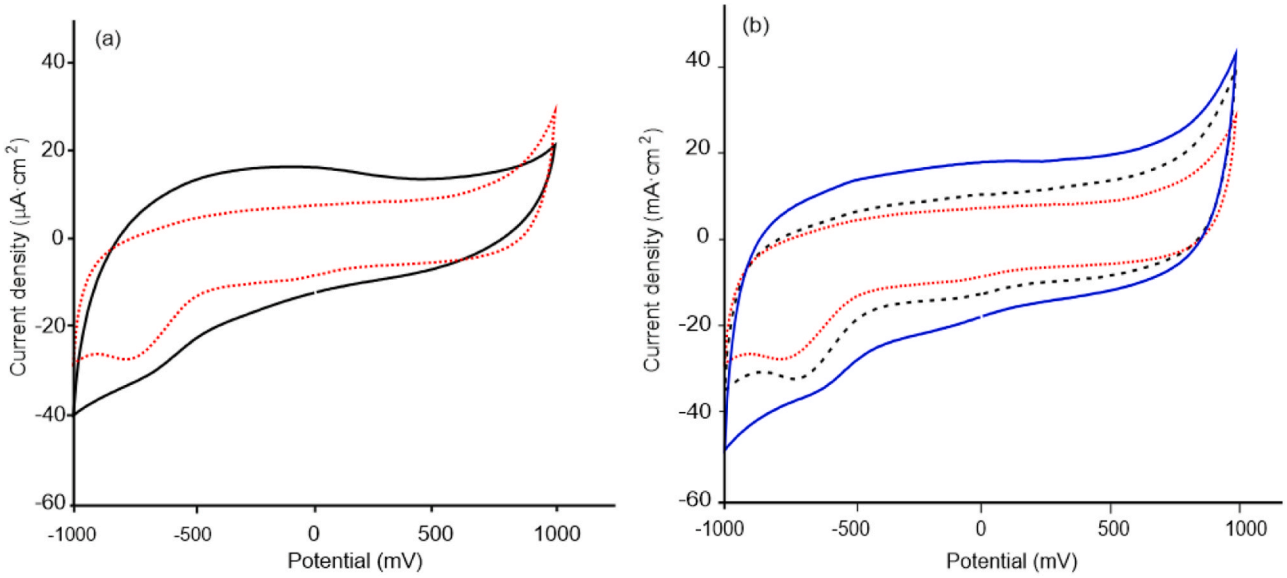

Fig. 3. Cyclic Voltammograms obtained with electrode C-SPE immersed in (a) unpreserved milk stored a 4ºC (red dotted line) and milk stored at − 20 °C (black solid line) (b) unpreserved milk stored a 4ºC (red dotted line), azidiol-preserved milk stored a 4ºC (black dashed line) and azidiol-preserved milk stored a 4ºC after 7 days (blue solid line).

deviation, was 3.5%.

According to the above described results. following experiments were carried out in milk diluted 1:2 in water and sonicated using 10 pulses of 30 s during 5 min.

The Limit of detection (LOD) and the sensitivity of the sensors has been evaluated for all the psychochemical parameters measured from the calibration curves obtained from cyclic voltammograms. The LODs were calculated using the 3σ/m criterion, where σ is the standard deviation for 5 measures of the blank and m is the slope of the calibration plot for the anodic peak. Fig. 2 shows an example of the response of the three different sensors to an increasing concentration of urea in a raw milk (0 M to $1{\cdot}10^{-3}$ M) in the oxidation peak at ~ −0.5 V for C-SPE, ~0.0 V for NiO-SPE and ~0.1 V for PB-SPE. As it can be observed all sensors have demonstrated an increase in the intensity of the response to the analyte, which manifests the sensoŕs sensitivity to the analyzed target molecule. The LOD values of all sensors corresponding to tributyrin, casein and β-Hydroxybutyric acid are listed in Table S1 in the supplementary material. The regression curves of all the sensors for the different analytes are represented in Fig. S1.

Another important issue is to find the best method to preserve milk from spoilage. Freezing is a common method to preserve milk and decrease the spoilage by microorganisms. However. freezing produces the growth of ice crystals and formation of phase separated systems or protein aggregation [30]. Thawing can result in coagulation, degradation of constituents and phase separation. Shaking blending or sonicating defrosted milk can remedy this issue. Another common possibility to inhibit or retarding growth of microorganisms is to use preservatives such as azidiol (AZ) which is widely used to preserve milk samples [31, 32].

Freezing and thawing or the use of preservatives such as azidiol may interfere in the electrochemical evaluation of the quality. In order to evaluate these interferences and to establish the optimal preservation method aliquots of fresh milk were preserved using different methods. The conditions tested were storage at − 20 °C (and thawed after 7 days) and azidiol-preserved milk stored at 4 °C. The azidiol-preserved milk was analyzed just after milking and 7 days later (7 days preserved at 4ºC). As observed in Fig. 3.a. the response offered by milk frozen at − 20ºC (and thawed seven days later) showed a loss of peak definition and an increase in the non-faradaic current, when compared with that obtained with unpreserved milk stored at 4ºC. Lipid droplets are more instable after thawing due to the separation of fats during freezing favoring their aggregation on the sensors surface which explains the

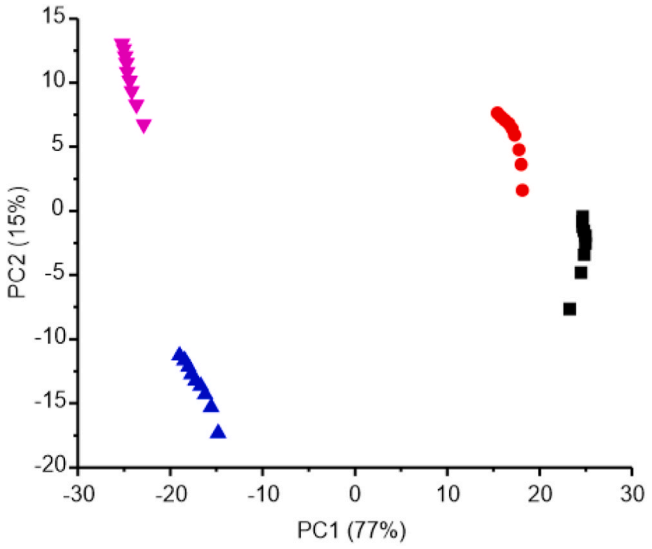

Fig. 4. PCA analysis of preservative-free milk stored at 4ºC (red circle), preservative-free milk stored at − 20ºC (black square); milk treated with azidiol stored at 4 °C (blue triangle) and milk treated with azidiol and measured one week later (pink triangle).

evident increase in the non-faradaic current [31]. This behavior also explains the reduction on the intensity current and the loss of peak definition.

As Fig. 3.b shows, the presence of azidiol was detected by the electrochemical sensors whose responses showed an increase in the intensity with respect to untreated milk, due to an increase in the conductivity. Azidiol is an excellent preservative, however, when voltammograms were recorded one week later, important changes in the response were observed. It can be concluded that azidiol helps to preserve milk, but the microbiological processes are not completely stopped causing an increase in the variation coefficients. In addition, it is well known that enzymatic activity, which is not stopped by the azidiol effect, could produce physic and chemical changes in milk such as: lactose hydrolysis, sugars degradation, decrease of antioxidants content and increment of acidic species.

Principal component analysis (PCA) was applied to evaluate the capability of the three electrode ET to discriminate milk samples according to the preservation method applied (Fig. 4). The scores plot of the PCA obtained showed well-defined and separated clusters for unpreserved milk stored a 4ºC. milk stored at − 20 °C. azidiol-preserved milk stored a 4ºC and azidiol-preserved milk stored a 4ºC during seven days. A total of 92% of the variance of the original data was explained within the first two principal components with 77% in PC1 and 15% in PC2. Unpreserved milk stored a 4ºC and milk stored at − 20 °C were situated in the positive region of PC1 and were clearly separated from milks preserved by azidiol. The similarity between fresh and frozen-thawed milk may be due to the fact that changes induced by freezing the sample. which are mainly related to the destabilization of the micelles of triglycerides in milk. have been reversed using ultrasounds after thawing. Milk preserved with azidiol appeared in the negative region of PC1. It can be observed that samples measured just after the addition of azidiol and milk measured one week later were separated from each other along the PC2. confirming that milk undergoes changes. This can be explained taking into account that azidiol stops the microbiological activity. however other processes such as the enzymatic activity. may continue taking place. producing a change in composition of the sample over time.

Once the discrimination ability of the system was demonstrated. A classification model was developed for the different preservation methods to corroborate the results obtained through the unsupervised methodology previously applied. All milk samples included in this model were diluted 1:2 and submitted to sonication, ensuring that the differences on ionic strength and electric conductivity are only influenced by milk matrices composition. The milk classification analysis was carried out by applying Support Vector Machine classification (SVMC). Which is a widely system used in the development of mathematical classification models based on a non-linear kernel-based supervised pattern recognition technique. Moreover having into account the low number of samples. SVM is one of the more resistant methods to avoid the overfitting [33]. A radial basis function (RBF) was used as the core function of SVMC. This nonlinear kernel approximation is defined by Eq. 1:

$$\boldsymbol{K}(\boldsymbol{x}_i - \boldsymbol{x}_j) = \boldsymbol{exp}((-\boldsymbol{\gamma} \parallel \boldsymbol{x}_i - \boldsymbol{x}_j\parallel^2). \boldsymbol{\gamma} > 0 \quad (1)$$

where x_i and x_j work as the training vectors of the input data while γ is the kernel parameter.

During the validation stage the kernel function penalty parameter (C) and the kernel parameter γ in the SVMC was optimized by means of the grid search method, using a $\log_2$C and $\log_2\gamma$ approach with one interval varying from [10. 10]. The validation accuracies were optimal when C= 0.1 and $\gamma = 0.01$. Full cross-validation method was used to evaluate the success rate attained with the SVMC due to the limited amount of samples. The results of the SVMC are summarized in the confusion matrix shown in Table 2.

Table 2
SVMC validation confusion matrix for four preservation methods.

		Actual			
		Fresh milk (4ºC)	Frozen milk (−20ºC) seven days later	Milk-azidiol (4 °C)	Milk-azidiol (4ºC) seven days later
Predicted	Fresh milk (4ºC)	9 (100%)	0	0	0
	Frozen milk (−20ºC) seven days later	0	9 (100%)	0	0
	Milk-azidiol (4 °C)	0	0	9 (100%)	1 (11.12%)
	Milk-azidiol (4ºC) seven days later	0	0	0	8 (88.88%)

The results obtained on the SVMC model showed a 100% accuracy for the calibration and 97.22% accuracy for the validation. During validation one sample of milk preserved by azidiol stored at 4ºC for seven days was incorrectly classified as a sample preserved using azidiol without aging. One possible explanation of this loss in specificity (false positives) could be attributed to different microbiological content in the samples which implies a different evolution of the matrixes. This result confirms how samples preserved with azidiol show different evolutions over time which in terms of preservation would mean uncontrolled changes on the nutritional characteristics of the samples. Moreover, the classification model was able to perfectly classify all the other samples reflecting the significant differences between the three groups.

Taking into account the results obtained from the electrochemical responses using different pre-treatments. freezing and thawing has been proposed as an optimal milk preservation procedure (based on electrochemical sensors) in cow milks with different characteristics due to the fact that this method guarantees biological inactivity during sample storage. providing greater reproducibility in the measurements. However. this procedure could induce complications such as protein aggregation or fat coagulation and precipitation. For these reason. it has been concluded the neccessity of rehomogeinize the samples after thawing. For that purpose. all samples were diluted 1:2 and submitted to sonication.

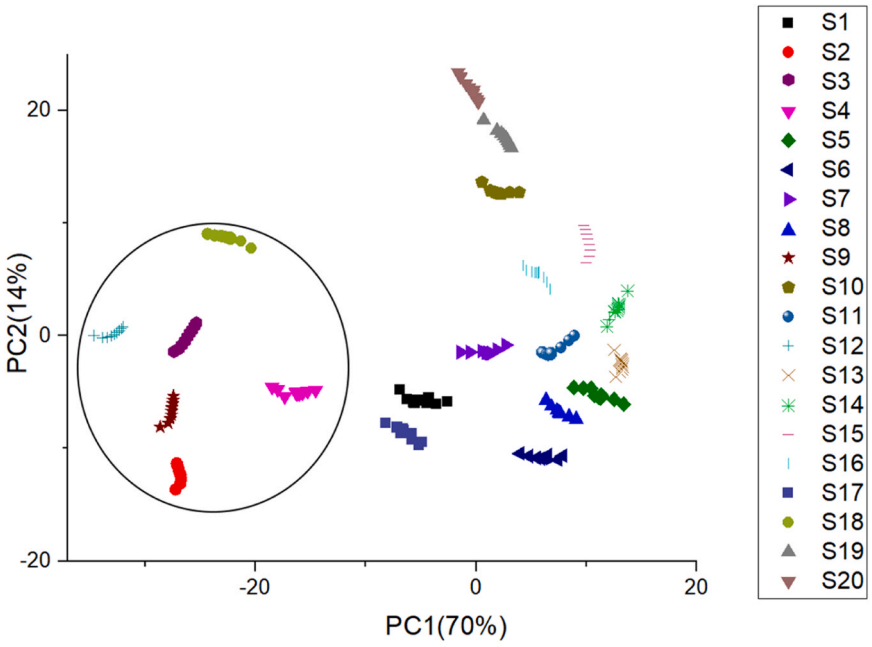

Fig. 5. PCA from the analysis of 180 milk samples. Samples remarked in a black circle correspond with fat contents above 4.5%.

The aim of using this method is the possibility to develop a sensitive device with the capability of discriminate between different milk samples obtained directly from the milking processes without applying an aggressive method of samples pretreatment.

The selected method has been applied in the further works in this paper.

3.1. Analysis of milk with the ET: Quimiometric analysis

The voltammetric ET was used to analyze 180 milk samples obtained from 20 individual cows with different characteristics including fat, protein and urea content, CCS and BHB. The sensors forming the ET provided electrochemical distinct responses towards each milk the quimiometric analysis by PCA was performed to evaluate the discrimination ability of the system. Fig. 5 shows the scores plot resulting from the PCA analysis in which the first two variables were able to explain 84% of the data variance. It can be seen how the ET developed was able to discriminate between the 20 cows included in this essay. The results obtained from each of the nine milk aliquots are distributed on clear clusters corresponding to each cow. Moreover, cows with fat percentages over 4.5 can be identified in as a group located on the left side of the scores diagram, while cows with very low values of fat and proteins (S13 and S14) appear on the opposite side of the diagram, confirming that the developed system was able to determine significant differences between cows based on the nutritional composition of their milks.

One of the main interests of ETs is the possibility of stablishing mathematical models between the data obtained by the array of sensors and the chemical data obtained by classical chemical techniques. Using these models, ETs can be used to obtain several chemical parameters in a single measurement.

In this work, SVMR was used to predict the fat, protein, urea, SCC and BHB content in samples obtained from 20 individual cows. Nine different aliquots from each cow milk were included in the study making a total of one hundred and eight samples. The calibration set was comprised of 75% of the samples randomly selected ensuring that there was enough representation of each one of the cows included in the study. Then the remaining 25% of the samples were used for validation purposes. The radial basis function which could handle the non-linear relationships between the voltammetric signals and the target attributes was chosen as the core function to predict the physicochemical parameters.

Table 3
Correlation parameters from the SVMR analysis.

Parameters		% Fat	% Proteins	Urea (pmm)	BHB (μM)	SCC (SC/mL)
SVM	R_c^2	0.986	0.937	0.927	0.913	0.906
	$RMSE_c$	0.094	0.084	0.356	0.116	0.375
	R_P^2	0.971	0.907	0.908	0.909	0.900
	$RMSE_P$	0.136	0.098	0.410	0.236	0.421
	Range	2–7	2–5	20–272	30–210	90–230

$RMSE_c$: "root mean square of error of calibration". $R^2{}_c$ correlation coefficient in calibration $RMSE_p$: "root means square of error of prediction". $R^2{}_p$ correlation coefficient in prediction

Two data matrices were built: the "X" matrix (predictors) contained the data obtained from the ET for each milk sample (in this case corresponded to the kernels obtained from the voltammetric signals. The "Y" matrix (responses) contained the data of physicochemical data (fat, protein, urea, BHB and RCS). Regression models used the following parameters: RBF core function C= 1 and with a cross-validation segment size of 15.

The correlation coefficients and the errors of calibration and prediction are shown in Table 3. In all cases the developed models achieved acceptable correlation values R^2 (ranging from 0.98 to 0.91) for calibration, with low residual errors (RMSE) between 0.084 and 0.375

Table 4
Predicted and real values obtained for five milk samples through the SVM prediction models.

		Parameters	% Fat	%Proteins	Urea	BHB	SCC
Samples	M1	Predicted	4.52	4.06	161	0.04	43
		Real	4.14	4.14	156	0.04	33
	M2	Predicted	6.01	5.25	100	0.24	182
		Real	5.95	5.95	96	0.23	193
	M3	Predicted	4.58	4.41	149	0.04	32
		Real	4.69	4.69	144	0.05	26
	M4	Predicted	4.84	3.46	185	0.07	168
		Real	4.96	3.68	148	0.06	195
	M5	Predicted	3.79	3.22	106	0.06	228
		Real	3.7	3.7	103	0.05	219

representing less than a 3.5% of error in each case for the variable range measured. As expected, prediction achieved lower R^2 values (ranging from 0.97 to 0.90). Fat content was the parameter with the highest correlation rates. This is clearly due to the influence of fatty acids and viscosity in the response of the sensors. In the case of urea, SCC and BHB, the R^2 for prediction values were lower with values around 0.9 on the validation stage. This could be due to an absence of a significant influence of their content on the sensors performance since urea, SCC and BHB are parameters mostly related to the health state of cows with a lower effect on the global composition of the milk.

Examples of the correlation curves are presented in the supplementary data as Figs. S2–S6.

To study the accuracy of the prediction models developed, a prediction of the physicochemical values of 5 samples was carried out (Table 4).

According to these quantitative results it may be concluded that using SVMR it is possible to establish good correlations between data provided by the ET and the analytical values measured by traditional methods. Thus taking into account the use of appropriate sampling and preservation techniques the use of portable ET presented here could be a method to assess five physicochemical parameters in one single experiment.

4. Conclusions

The results obtained in this work demonstrate that knowledge of the influence of storage and preservation of milk is important to standardize sampling protocols and to ensure accuracy in ET results. Dilution followed by sonication significantly contributes to decrease the fouling improving the repeatability of the electrochemical signals. The use of preservation methods such as freezing or the use of azidiol modify the electrochemical responses. However, freezing and thawing is a reproducible process allowing to store milk for long periods of time. In contrast, azidiol does not stop completely the bacterial growth and the electrochemical responses change with time. Consequently, freezing at − 20ºC is a more efficient preservation method for ET analysis.

Using the optimized conditions the ET is able to discriminate milk from individual cows with different compositions. Moreover, using Support Vector Machine Regression (SVMR) model, the ET is able to predict the percentage of fat, protein urea. RCS and BHB with good correlation coefficients and low residual errors. In summary, after an adequate optimization of the test conditions the portable voltammetric ET reported can be used for in situ analysis or applied in laboratories being a low cost rapid an efficient tool for rapid assessment of the quality of milk.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

CRediT authorship contribution statement

Coral Salvo Comino: Conceptualization, Methodology, Investigation, Writing – review & editing. **Clara Perez Gonzalez:** Data curation, Writing, Software, Formal analysis. **Patricia Martin Bartolome:** Investigation, Sofware, Formal analysis. **Fernando Martin Pedrosa**: Software, Validation. **Cristina Garcia Cabezon:** Supervision, Funding acquisition. **Maria Luz Rodriguez Mendez:** Project administration, Supervision, Resources, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.snb.2023.134138.

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