Bioelectronic tongue dedicated to the analysis of milk using enzymes linked to carboxylated-PVC membranes modified with gold nanoparticles

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Authors contribution

MR-M, CG-C, and FM-P conceptualized the idea and supervised the work. CP-G and CS-C performed the experiment, curated the data, and wrote the original draft. FM-P involved in software design and development. CP-G and CS-C involved in formal analysis. CG-C and MR-M acquired the funding. CP-G, CS-C, FM-P, MR-M and CG-C reviewed and edited the paper. All authors provided feedback.

Biographies

Clara Perez-Gonzalez obtained the Ms in Nanoscience in 2019 (U. Valladolid. Spain). She is currently working on her PhD Thesis which is dedicated to the development of electrochemical sensors for the analysis of foods. She is author of 5 scientific papers.

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Bioelectronic tongue dedicated to the analysis of milk using enzymes linked to carboxylated-PVC membranes modified with gold nanoparticles

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Highlights

- A potentiometric bioET specifically dedicated to milk analysis was developed.
- Enzymes were covalently linked to membranes combining Carboxilated-PVC and AuNPs.
- The effective enzymatic immobilization helped to retain the enzymatic activity.
- Using SVM and ensemble methods, nine physicochemical parameters can be determined simultaneously.

Abstract

Bioelectronic tongues (bioET) made of sensors combining enzymes and nanomaterials have been shown to be advantageous due to the specificity offered by the biosensors and the enhanced sensitivity provided by the nanomaterials. In this work, an innovative bioET for milk analysis is developed using potentiometric biosensors based on lactic dehydrogenase, galactose oxidase and urease specific for the detection of compounds of interest in milk (lactic acid, galactose and urea). The performance of the biosensors has been fostered by covalently immobilizing the enzymes on membranes of carboxylated polyvinyl chloride combined with gold nanoparticles. The design and composition of the biosensors contributes to preserving the enzymatic activity, allowing limits of detection in the range of 10^{-5} – 10^{-6} M with excellent sensitivity and reproducibility (variation coefficients ranged from 1 to 5.1 %).
The three biosensors, combined in a single device and coupled to a pattern recognition software, can discriminate efficiently twelve classes of milk with different fat content (skimmed, semi-skimmed and whole milk) and nutritional characteristics (calcium enriched, lactose free and folic acid-enriched). The bioET shows an excellent classification capability with an accuracy of up to 99.7%. By applying Support Vector Machine (SVM) analysis, the BioET can perform the simultaneous assessment of eight physicochemical parameters (acidity, fat, proteins, lactose, density, urea, dry matter and nonfat dry matter) with satisfactory correlation coefficients and low residual errors. The results are further improved by implementing ensemble methodologies. The proposed strategy has been demonstrated to be useful for improving the performance of bioETs in the dairy industry.

Keywords

Bioelectronic tongue, milk, biosensor, gold nanoparticles

1. Introduction

In recent years, the field of electronic tongues (ETs) has driven important basic developments (Juzhong, & Jie, 2020; Aouadi et al., 2020; Rodriguez-Mendez, De Saja & González-Antón, 2016; Ha et al. 2015). Much of this progress is related to the design of new sensors which incorporate nanomaterials that improve the sensing characteristics, thanks to their high surface to volume ratio and excellent electrocatalytic properties (Li, Li, Liu, & Chen, 2019; Wang & del Valle, 2021; Sobrino-Gregorio, Bataller, Soto, & Escriche, 2018; Teodoro, Shimizu, Scagion, & Correa, 2019; Americo da Silva et al., 2019). Other advances are related to new approaches to data management, including more efficient data reduction methods and improved pattern recognition algorithms and classification techniques (Tian, Chen, Pan, & Deng, 2013; Prieto et al., 2013).

The emergence of bioelectronic tongues (bioETs) combining classical unspecific sensors with biosensors has been a breakthrough in the field, because these systems simultaneously provide global information about the sample (as in classical ETs) plus information about specific compounds obtained from the biosensors (Wasilewski, Kamysz, & Gebicki, 2020; Skladal, 2020; Ghasemi-Vamankhasti et al., 2012; Yhan et al., 2021; Ha et al., 2017)**.** The performance of electrochemical biosensors can be further improved by combining enzymes or other biological bioreceptors with nanomaterials. Nanomaterials provide an effective platform for the immobilization of biomolecules, inducing unique performance characteristics in terms of sensitivity and specificity. Some examples of voltammetric bioETs based on combinations of enzymes and nanomaterials have recently been reported. For instance, an array formed by phenol oxidases and glucose oxidase combined with nanoparticles has been successfully used to analyze grapes and musts (Garcia-Cabezón et al., 2020; Garcia-Hernandez et al., 2019). Human taste receptors combined with carbon nanotubes (CNTs) or polypyrrole nanotubes have been used to form a field effect transistor with human-tongue-like selectivity (Kim et al., 2011; Song et al., 2012).

Milk is a complex mixture that contains many different compounds, including carbohydrates (mainly lactose), fats, proteins (casein or whey), minerals (such as calcium) and many other miscellaneous constituents. E-tongues have been developed and applied to the dairy industry in quality control, evaluation of taste or freshness, detection of adulterations, origin recognition, etc. (Ciosek, 2016). These previous works have used different types of electrodes and materials (Winquist et al. 1998; Wei, Wang, & Jin, 2013; Pascual et al., 2018; Yu et al., 2015; Li et al., 2015; Ciosek, & Wroblewski, 2015; Tazi et al., 2018; Dias et al., 2009; Pérez-González et al., 2021; Yang et al., 2021; Hruškar et al., 2010; Collier et al. 2003; Valente et al., 2018; Scagiona et al., 2016). Only a few attempts have been made to introduce nanomaterials in ETs applied to the dairy industry. They include an array of voltammetric electrodes modified with nanostructured Layer-by-Layer films (Salvo-Comino et al. 2018), a potentiometric ET using sensors modified with nanoparticles (Mercante et al., 2015) and an impedimetric ET using electrospun nanofibers (Ohlson et al., 2017). However, due to the complexity of milk, the analysis using ETs is not a completely solved problem and new developments in the field are required.

The proposal here is to take a step forward in the field of bioETs by developing novel sensors combining enzymes specific to compounds present in milk (galactose, urea and lactic acid) with nanomaterials. Galactose and its content is an important indicator of milk quality and its content can be measured with individual galactose oxidase (GaOx) biosensors (Ohlson et al., 2017; Kanyong, Krampa, Aniweh, & Awandare, 2019; Mangan et al., 2018; Nguyen et al. 2016). Few examples can be found in the literature, where

GaOx has been combined with nanomaterials such as graphene (Çakıroğlu et al. 2019) or nanoparticles (Migliorini et al., 2018). The detection of urea is also of prime importance to assess the nutritional program of cows and can indicate underlying pathological problems. Few examples of individual nanobiosensors for the detection of urea have been reported. They are based on the combination of urease with nanoparticles (Jakhar & Pundir, 2018) or nanofibers (Jia et al. 2011). Finally, the control of lactic acid is essential to evaluate the fermentation of lactose due to lactic bacteria. Over the last few years, some examples of biosensors based on lactate dehydrogenase (LDH) combined with nanomaterials have been reported (Rahman et al. 2009).

In enzyme-based biosensors, the use of an adequate method to immobilize the enzymes is crucial to preserve the enzymatic activity and avoid leakages (Nguyen, & Kim, 2017). Covalent immobilization has the advantage of high surface loading and low protein loss (Zucca, & Sanjust, 2014; Lee et al., 2017). Our proposal here is to develop an immobilization membrane using carboxylated PVC (C-PVC) -instead of the classical PVC- where enzymes can be covalently linked using a covalent reaction between carboxyl groups of the C-PVC and the amines on the protein.

In the ETs, it is also important to select the best chemometric methods to process the data. Unsupervised and supervised analysis methods, such as principal component analysis (PCA), linear discrimination analysis (LDA), support vector machines (SVM) or weighed k-nearest neighbor analysis (KKNN), have been extensively applied (Skladal, 2020). One of the emerging trends in data analysis is the combined use of statistical algorithms through ensemble methodologies, where the outputs of the different algorithms are combined in a decision fusion strategy to create a single response for a given problem (Zhou, 2012). However, this strategy has barely been applied in the field of ETs, where they could represent a great advance in complex media analysis such as milk.

In summary, the aim of this work was to develop a potentiometric bioET based on membranes made of carboxylate PVC modified with nanoparticles. The carboxylate PVC is used to covalently link the enzymes able to detect compounds in milks: GaOx, LDH and Ure, which have been selected for their ability to detect important components in milk. Once prepared and characterized, the sensing units are combined in a single device to obtain a bioET that is used to analyze and classify 12 classes of milk with different nutritional characteristics and to predict the eight physicochemical parameters most commonly used in the dairy industry for quality control. In this work, a first approach to an ensemble methodology routine is also proposed for the correlation of data obtained with the bioET with physicochemical parameters.

2. Material and methods

All the reactants were of analytical grade and were used without further purification. They were purchased form Sigma-Aldrich (St.Louis, USA). All the solutions were prepared in MilliQ deionized water (Merck, KGaA, Darmstadt, Germany).

2.1 Milk samples

A set of 120 milk samples corresponding to 12 types of commercial milk types (ten replicas from each milk) were included in the study. This set was formed by milks with different fat content (skimmed, semi-skimmed and whole milk) and nutritional content (lactose-free, calcium-enriched, and folic acid-enriched milk). The milks were analyzed using traditional standard chemical methods: the titratrion method for acidity (ISO 22113:2012), the Hydrometer method for density (ISO 2449:1974), the Gravimetry Röse-

Gottlieb method for fat content (ISO 1211:2010), the Kjeldahl method for protein content (ISO 8968-1:2014), HPLC to determine the lactose content (ISO 22662:2007), and Infrared spectroscopy for the urea content (ISO 9622:2013). Total dry matter (DM) and non-fat dry matter (NFDM) were also analyzed (ISO 6731:2010) (International Organization For Standardization, 2021). The physicochemical data are summarized in Table 1.

Table 1. Milk samples and physicochemical parameters established by traditional standard methods

Sample	Fat content	Nutritional description	Acidity (ºD)	Density (g/ml)	Fat (%m)	Proteins (%m)	Lactose (%m)	NFDM (%m)	DM (%m)	Urea (mg/ml)
S1	Skimmed	Classic	12.55	1031.55	0.31	3.3	5	9.02	9.33	387
S2	Skimmed	Calcium	15.82	1039.47	0.29	3.93	5.59	10.51	10.8	724
S3	Skimmed	Lactose Free	12.66	1033.57	0.32	3.29	0.36	9.02	9.33	<10
S4	Skimmed	Folic Acid	12.57	1033.7	0.40	3.29	4.95	9.04	9.43	586
S5	Semi-Skimmed	Classic	12.55	1031.6	1.56	3.27	4.91	8.91	10.47	355
S6	Semi-Skimmed	Calcium	16.06	1037.29	1.55	3.9	5.49	10.40	11.95	597
S7	Semi-Skimmed	Lactose Free	12.19	1032.09	1.59	3.31	0.42	8.99	10.57	<10
S8	Semi-Skimmed	Folic Acid	12.95	1032.38	1.64	3.21	4.93	8.94	10.58	638
S9	Whole	Classic	12.17	1029.38	3.56	3.21	4.85	8.78	12.33	388
S10	Whole	Calcium	15.86	1035.71	3.55	3.91	5.54	10.45	14.0	769
S11	Whole	Lactose Free	11.98	1029.4	3.59	3.23	0.31	8.82	12.41	<10
S12	Whole	Folic Acid	12.72	1030.55	3.1	3.18	4.94	8.92	12.02	792

2.2 Sensors and biosensors

Gold nanoparticles were synthetized by reduction of tetrachloroauric in the presence of trisodium citrate as the reducing agent, using the classical Turkevich method (Kimling et al., 2006). The colloid obtained was characterized by UV-Vis, showing a maximum at 537 nm. The concentration of AuNPs was calculated by Beer's law, with a particle concentration result of 5.98 x 10^{-11} M and a diameter of 52.1 nm (Haiss, Nguyen, Aveyard, & Fernig, 2007).

Sensors were based on polymeric membranes made of carboxylated PVC [poly (vinyl chloride) carboxylate] (C-PVC) as the polymeric matrix. The C-PVC was mixed with an additive (oleyl alcohol) and a plasticizer [(bis(1-butylpentyl) adipate (named plasticizer A), tris(2-ethylhexyl) phosphate (named plasticizer B) or 2-nitrophenyl-octylether (named plastizicer C)] using tetrahydrofurane as the solvent. A second set of sensors was prepared by introducing gold nanoparticles in the membrane.
The membranes described above were modified with galactose oxidase (GaOx) from *Dactylium dendroides* (Sigma-Aldrich, St. Louis, USA), lactate dehydrogenase (LDH) from *Mus musculus* (Roche diagnostics, Indianapolis, USA), and urease (Ure) from

Canavalia ensiformis (Sigma-Aldrich, St. Louis, USA). The enzymes were covalently linked to the surface of the polymeric membrane using the carbodiimide method (Kazenwadel, Wagner, Rapp, & Franzreb, 2015). The reaction was carried out in two steps. First, the carboxylic groups of the C-PVC were activated by means of EDC (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide. Then, the enzyme was added and a peptide bond was formed between the carboxylic groups on the C-PVC and the superficial amino side chains of the enzyme. As a result of the combination of the six membranes with each of the three enzymes (GaOx, Ure and LDH) a set of 24 membranes were obtained (Table 2).

Table 2. Composition of the sensors.

Sensor	C-PVC (w/w%)	Additive (w/w %)	Plasticizer (P) Type (w/w%)	AuNPs (w/w%)	Enzyme
A				-	-
A-GaOx	32	3	A		GaOx
A-Ure			65		Ure
A-LDH					LDH
B				-	-
B-GaOx	32	3	B		GaOx
B-Ure			65		Ure
B-LDH					LDH
C				-	-
C-GaOx	32	3	C		GaOx
C-Ure			65		Ure
C-LDH					LDH
A-AuNP					-
A-AuNP-GaOx	32	3	A	10	GaOx
A-AuNP-Ure			55		Ure
A-AuNP-LDH					LDH
B-AuNP					-
B-AuNP-GaOx	32	3	B	10	GaOx
B-AuNP-Ure			55		Ure
B-AuNP-LDH					LDH
C-AuNP					-
C-AuNP-GaOx	32	3	C	10	GaOx
C-AuNP-Ure			55		Ure
C-AuNP-LDH					LDH

The bioET was designed using a methacrylate tube, in which 24 holes (0.3 cm diameter) were drilled. The holes were half-filled with an epoxy silver resin (EPO-TEK, Billerica, USA) and the resin was covered with one of the 24 membranes. The inner part of the silver epoxy resin was connected to a data acquisition system (Agilent Data Acquisition Switch Unit 34970A). In all measurements, the Ag/AgCl electrode was used as the reference electrode. Figure 1 shows the scheme of the designed bioET system.

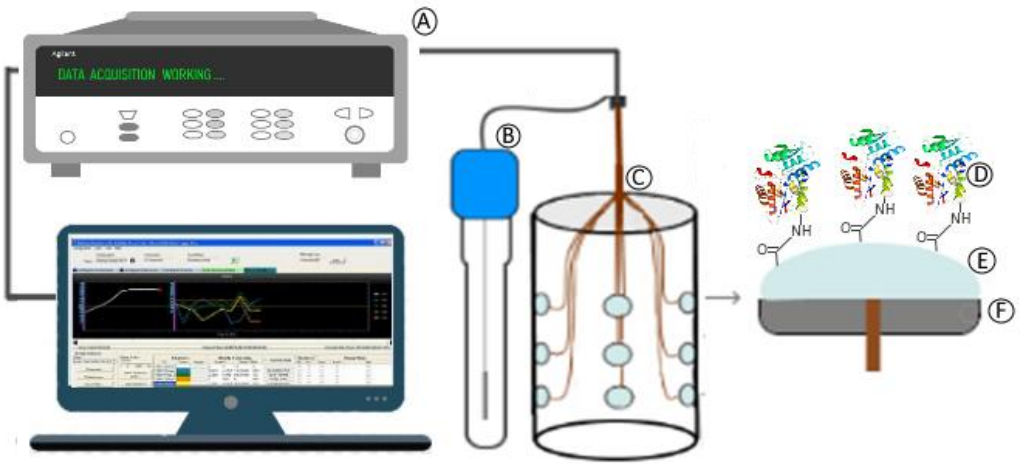

Figure 1. Scheme of the bioET designed in this work. A) Data Acquisition Switch; B) Reference electrode; C) Electronic tongue body; D) Enzyme covalently linked; E) C-PVC membrane; F) Silver epoxy resin and copper wire.

The potentiometric measurements were carried out by immersing the sensor array in a 100 ml glass cell containing the standard solutions or the milk samples. Standard solutions of compounds usually found in milk (KCl, $CaCl_2$, galactose, urea and lactic acid) were prepared in a phosphate buffer (0.1M, pH 7) with concentrations ranging from 1×10^{-4} to 1×10^{-2} M. The milks were diluted 1:1 in phosphate buffer and measured without further modification. In addition, nicotinamide adenine (NAD+) (Roche diagnostics, Indianapolis, USA) was added to the standard solutions in order to simulate the levels usually present in milk (final concentration 12 mM) (Fox, & McSweeney,1998). After immersing the electrodes in the corresponding sample, the membrane potentials were registered every three seconds. The signals were stabilized after 5 minutes (average variation of 1.6 mV/decade between each reading).

The potentials obtained from the sensor array were used as the input variables for multivariate analysis. Principal Component Analysis (PCA) was used to estimate the discrimination ability of the multisensory system. A Support Vector Machine (SVM) was applied to establish correlations with the physicochemical parameters obtained using traditional methods (Theodore, & Robin, 2006; Cortes, & Vapnik, 1995). Additionally, the SVM was applied to elaborate classification models. Finally, an approach towards ensemble methods was implemented by applying Stochastic Gradient Bosting for regression (Friedman, 2002). The statistical analysis was performed by using Matlab R2020b (The Mathworks Inc., Natick, USA), RKWard 0.7.1, and the Caret package (Kuhn, 2008).

3. Results and discussion

3.1 Development and optimization of the sensor array

In order to obtain efficient potentiometric biosensors, the immobilization of the enzymes on the polymeric membrane was accomplished using carboxylated PVC (C-PVC) instead of the bare PVC classically used to fabricate potentiometric sensors (Tazi et al., 2018; Dias et al., 2009). Using C-PVC, the enzymes can be immobilized by establishing a covalent link between the carboxylate groups of the membrane and the amine groups of

the enzymes. In addition, membranes were doped with AuNPs to further increase the intensity of the signals. As observed in Figure 2, the membrane potential increased with the content of AuNPs in the membrane. For instance, the sensitivity values obtained from the slopes of the calibration curves towards galactose were 17.23 mV for sensor A (without AuNPs), 19.13 mV for sensor A-Au containing 5% of AuNPs, and 32.22 mV for sensor A-Au containing 10% of AuNPs. Higher concentrations of AuNPs did not produce any further improvement in the sensitivity values. Based on these findings, the decision was taken to set the AuNPs content at 10%.

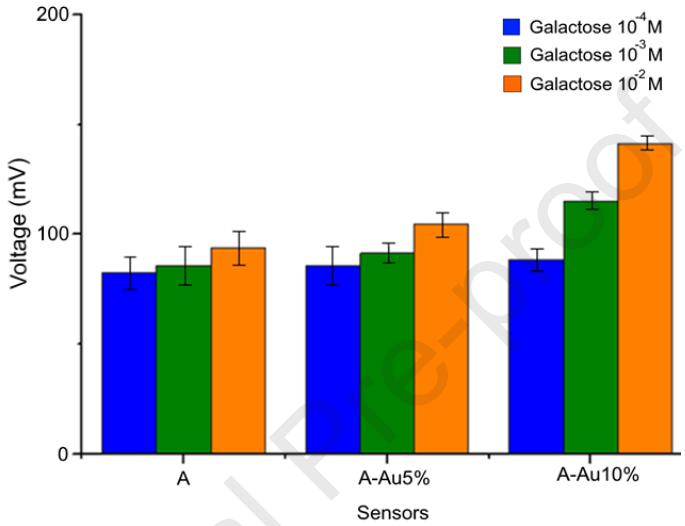

Figure 2. Response of sensor A (without AuNPs), A-AuNPs5% and A-AuNPs10% to increasing concentrations of galactose.

Once the composition of the membranes had been optimized, the enzymes GaOx, Ure and LDH were immobilized at the membrane surface and the responses of the obtained biosensors were analyzed. As observed in Figure 3, the intensity of the responses produced by a bare C-PVC membrane were lower than those obtained when the enzymes were covalently linked to the membrane. Taking the case of urea as an example, the measured voltage increased from 0.057 V in the bare C-PVC sensor (sensor A) to 0.119 V in the AuNP modified sensor (A-AuNP). The enzyme addition increased the intensity of the responses (0.156 V in A-Ure); and they increased even further to 0.276 V in A-AuNP-Ure when the enzyme was combined with AuNPs. Similar results were obtained for LDH or GaOx.

These results indicate that the enzymes are properly immobilized at the surface of the membrane and the enzymatic activity is retained. The synergistic effect observed when C-PVC and AuNPs are combined in the support membrane is also worth noting.

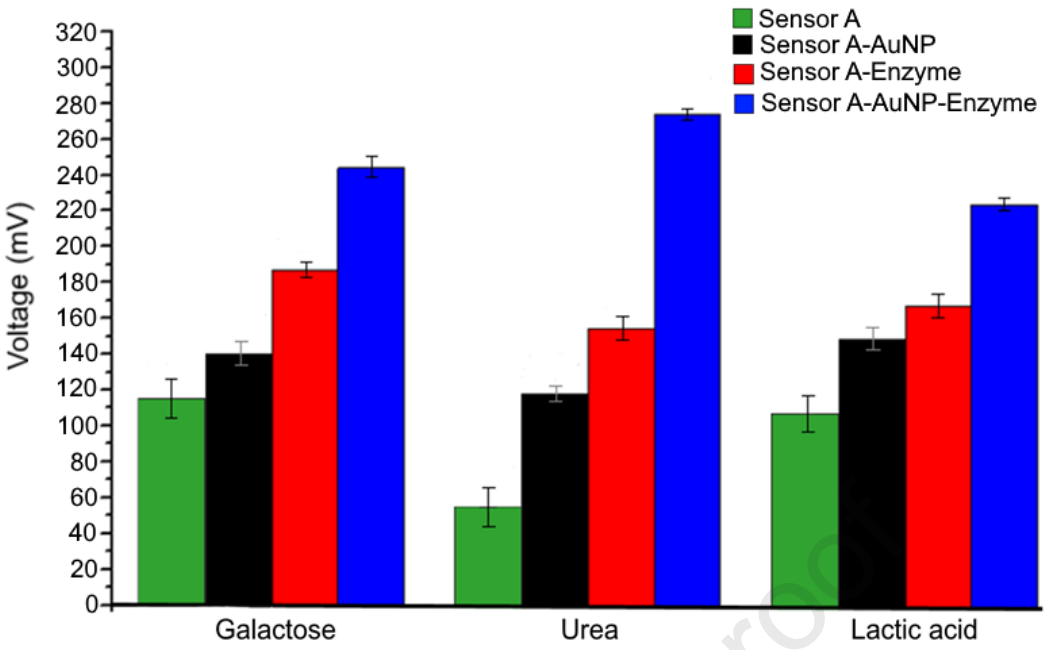

Figure 3: Response of sensors (where the enzyme can be GaOx, Ure or LDH), towards 1 × 10^{-4} M standard solutions of the corresponding target molecule (galactose, urea or lactic acid).

The sensitivity and the LOD of the 24 potentiometric sensors were calculated from the calibration curves registered in standard solutions of compounds usually found in milks (i.e., KCl, $CaCl_2$, galactose, urea and lactic acid), with concentrations ranging from 1 × 10^{-4} to 1 × 10^{-2} M. When the biosensors were immersed in ionic solutions (KCl or $CaCl_2$), LODs and sensitivities were almost constant and independent of the enzyme immobilized in the sensor, with values of 10^{-5} M (Table 3). However, after submerging the biosensors in solutions containing enzyme substrates, the LODs were clearly lower, confirming that the enzymes retain their functionality. In all cases, the biosensors with membranes combining C-PVC and AuNPs showed the lowest LOD (lower concentrations than those found in milk) and the highest sensitivities. For instance, the sensitivities of the class A sensors immersed in lactic acid were as follows: 24.11 mV (A) < 30.72 mV (A-AuNPs) < 37.16 mV (A-LDH) < 78.2 mV (A-AuNP-LDH) (Table 4). This can be attributed to the excellent immobilization and the synergistic effect obtained by the combination of both components.

Other studies support these results. It has been shown that the use of AuNPs is capable of increasing the sensitivity of enzymatic biosensors in potentiometric sensors. Vaghela et al. developed a potentiometric biosensor based on agarose-guar urease nanoconjugate modified with AuNPs. The conjugation of urease with AuNPs showed improvements in the potentiometric response, with limit of detection at 0.5 ppm to the target analyte and a linear response in concentrations from 0.5ppm-50ppm (Vaghela et al. 2018). Similarly, AuNPs have been used as an amplification platform for high sensitivity detection of glucose biosensors. The results revealed the important role of the nanoparticles in the adsorption of the enzymes allowing lower detection limits (>50 µM) and a wide linear range after the optimization of AuNPs electrodeposition on the sensor surface (Chiang et al. 2019).

The repeatability towards standard solutions was evaluated by calculating the variation coefficients of 10 consecutive measurements. The high repeatability (variation

coefficients between 0.11 and 3.9 %) of the results also confirmed that the enzymes were tightly bound to the membrane and no leakages were produced. The reproducibility was determined by comparing the responses of three identical sensors. Variation coefficients ranged from 1.1 to 6.1 %.

Table 3. Sensitivity and LOD values obtained from the slopes of the calibration curves for KCl and $CaCl_2$.

	KCl		$CaCl_2$	
Membrane	Sensitivity ($mV \cdot M^{-1}$)	LOD (M)	Sensitivity ($mV \cdot M^{-1}$)	LOD (M)
A	26.35	$2.11x10^{-5}$	21.65	$2.57x10^{-5}$
A-AuNPs	33.65	$1.56x10^{-5}$	38.56	$1.36x10^{-5}$
A-GaOX	22.35	$1.59x10^{-5}$	20.56	$1.73x10^{-5}$
A-Ure	27.69	$1.64x10^{-5}$	24.68	$1.84x10^{-5}$
A-LDH	24.12	$2.85x10^{-5}$	22.56	$3.5x10^{-5}$
A-AuNP-GaOX	36.23	$8.99x10^{-6}$	38.56	$8.46x10^{-6}$
A-AuNP-Ure	28.98	$1.48x10^{-5}$	26.54	$1.61x10^{-5}$
A-AuNP-LDH	31.26	$1.47x10^{-5}$	24.68	$1.86x10^{-5}$
B	24.22	$2.51x10^{-5}$	23.21	$2.61x10^{-5}$
B-AuNPs	29.87	$1.43x10^{-5}$	31.23	$1.37x10^{-5}$
B-GaOX	20.59	$2.45x10^{-5}$	28.65	$1.76x10^{-5}$
B-Ure	28.54	$2.01x10^{-5}$	20.33	$2.82x10^{-5}$
B-LDH	27.33	$1.64x10^{-5}$	21.89	$2.5x10^{-5}$
B-AuNP-GaOX	22.58	$2.37x10^{-5}$	35.61	$1.51x10^{-5}$
B-AuNP-Ure	27.68	$2.13x10^{-5}$	30.89	$1.91x10^{-5}$
B-AuNP-LDH	33.21	$1.30x10^{-5}$	38.97	$1.11x10^{-5}$
C	31.5	$1.87x10^{-5}$	28.54	$2.03x10^{-5}$
C-AuNPs	38.19	$1.21x10^{-5}$	35.78	$1.28x10^{-5}$
C-GaOX	33.25	$1.92x10^{-5}$	31.25	$2.04x10^{-5}$
C-Ure	28.75	$2.21x10^{-5}$	28.97	$2.19x10^{-5}$
C-LDH	35.14	$1.60x10^{-5}$	25.21	$1.48x10^{-5}$
C-AuNP-GaOX	37.89	$1.68x10^{-5}$	30.25	$2.11x10^{-5}$
C-AuNP-Ure	39.81	$1.23x10^{-5}$	39.56	$1.24x10^{-5}$
C-AuNP-LDH	32.78	$1.70x10^{-5}$	31.72	$1.76x10^{-5}$

Table 4. Sensitivity and LOD values obtained from the slopes of the calibration curves for galactose, urea and lactic acid respectively.

	Bare		P-AuNPs		P-GaOx		P-AuNP-GaOx	
Plastifier (P)	Sensitivity ($mV \cdot M^{-1}$)	LOD (M)	Sensitivity ($mV \cdot M^{-1}$)	LOD (M)	Sensitivity ($mV \cdot M^{-1}$)	LOD (M)	Sensitivity ($mV \cdot M^{-1}$)	LOD (M)
A	17.36	$2.18x10^{-5}$	32.22	$1.63x10^{-5}$	27.26	$1.44x10^{-5}$	55.78	$8.79x10^{-6}$
B	15.69	$3.14x10^{-5}$	30.3	$1.41x10^{-5}$	28.98	$1.29x10^{-5}$	53.7	$8.07x10^{-6}$
C	21.23	$2.27x10^{-5}$	35.2	$1.30x10^{-5}$	28.75	$1.31x10^{-5}$	56.3	$5.79x10^{-6}$

	Bare		P-AuNPs		P-Ure		P-AuNP-Ure	
Plastifier (P)	Sensitivity ($mV \cdot M^{-1}$)	LOD (M)	Sensitivity ($mV \cdot M^{-1}$)	LOD (M)	Sensitivity ($mV \cdot M^{-1}$)	LOD (M)	Sensitivity ($mV \cdot M^{-1}$)	LOD (M)
A	3.56	$1.06x10^{-4}$	19.6	$2.68x10^{-5}$	21.1	$2.15x10^{-5}$	72.35	$7.73x10^{-6}$
B	8.08	$6.07x10^{-5}$	24.3	$1.76x10^{-5}$	16.2	$3.54x10^{-5}$	76.5	$8.36x10^{-6}$
C	8.88	$5.44x10^{-5}$	25.1	$1.83x10^{-5}$	29.8	$2.13x10^{-5}$	64.32	$8.33x10^{-6}$

	Bare		P-AuNPs		P-LDH		P-AuNP-LDH	
Plastifier (P)	Sensitivity ($mV \cdot M^{-1}$)	LOD (M)	Sensitivity ($mV \cdot M^{-1}$)	LOD (M)	Sensitivity ($mV \cdot M^{-1}$)	LOD (M)	Sensitivity ($mV \cdot M^{-1}$)	LOD (M)
A	24.11	$1.57x10^{-5}$	30.72	$1.71x10^{-5}$	37.16	$1.46x10^{-5}$	78.2	$7.54x10^{-6}$
B	30.57	$1.61x10^{-5}$	38.15	$1.12x10^{-5}$	33.84	$1.89x10^{-5}$	87.34	$4.91x10^{-6}$
C	27.29	$1.77x10^{-5}$	36.81	$1.24x10^{-5}$	35.21	$1.48x10^{-5}$	89.56	$5.13x10^{-6}$

As observed in the results shown in the Tables, the set of 24 sensors showed a variety of responses. This variety was caused by the specificity of the enzymes to their target molecules, but also to the composition of the membrane (presence or absence of AuNPs and nature of the plastifier).

The high level of cross-sensitivity validated the combination of the 24 sensors developed to form a multisensor system coupled to a pattern recognition software to obtain a bioET. As a first approach, the response of the bioET to standard solutions of KCl, $CaCl_2$, galactose, urea and lactic acid at three concentrations (1×10^{-4}, 1×10^{-3} and 1×10^{-2} M in phosphate buffer 0.1M) was analyzed. Data obtained from the sensor array were used as the input for PCA. PC1 explained 59% of the covariance, PC2 23 %, and PC3 14%. The scores plot shown in Figure 4.A shows that the bioET could discriminate between the different compounds according to their chemical nature: ionic salts (KCl and $CaCl_2$) appeared on the right side of the diagram in the positive region of PC1 and in the negative of PC2. Galactose appeared in the upper part, lactic acid appeared in the central region of the diagram, and urea appeared on the left side of the diagram in the negative region of PC1 and PC2. The bioET could also separate clusters of solutions with different concentrations along the first component.

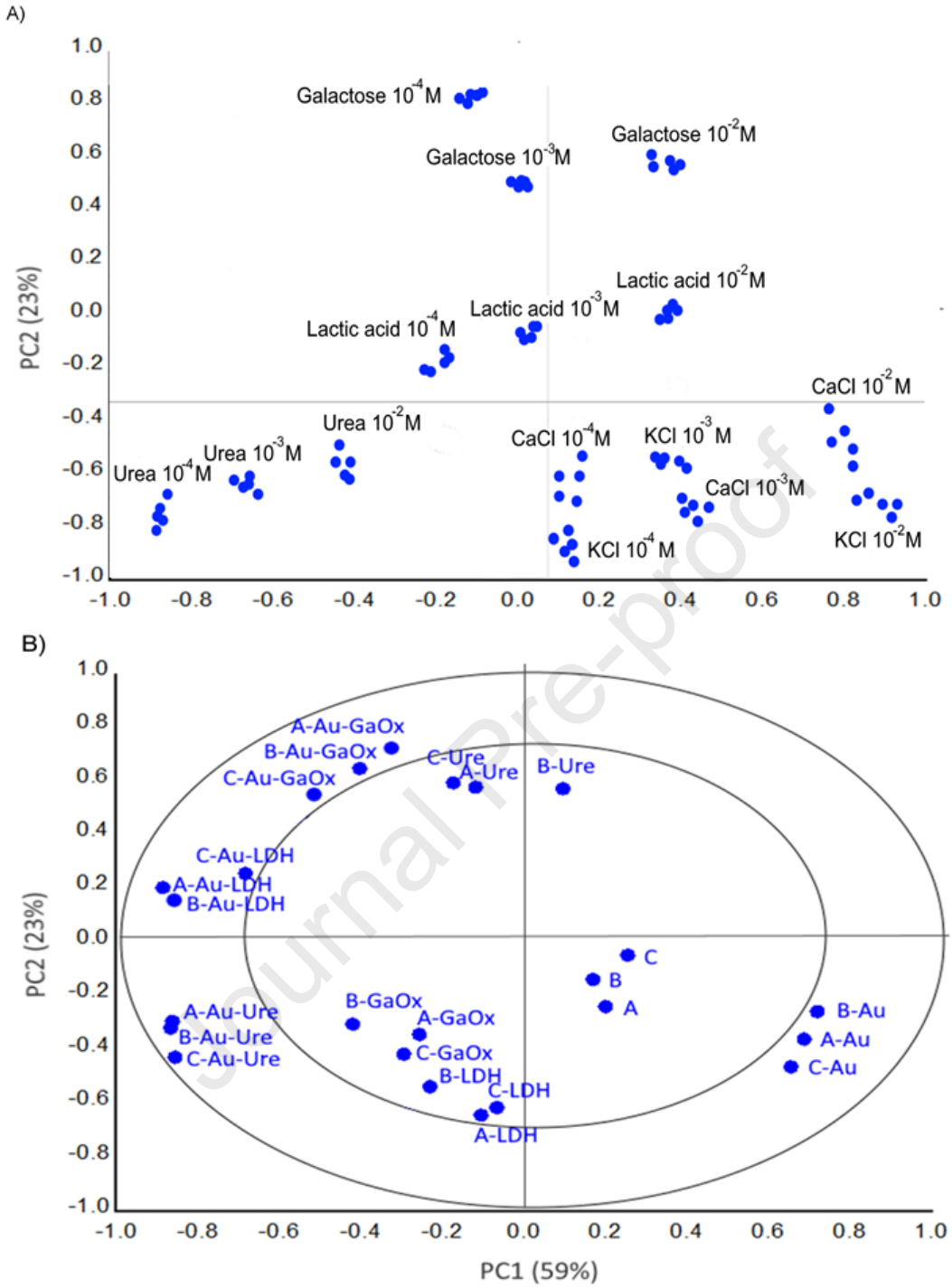

Figure 4. (A) PCA score plot and (B) loading plot obtained using an array of 24 potentiometric sensors immersed in standard solutions of KCl, $CaCl_2$, galactose, urea and lactic acid at three concentrations (1 x 10^{-4}, 1 x 10^{-3} and 1 x 10^{-2} M).

The loading plot of the PCA shown in Figure 4.B shows that sensors appear in different regions of the diagram, confirming the cross-selectivity: the biosensors appear in the negative PC1 region; whereas sensors without the enzyme, influence the positive PC1 component. The most important fact is that the biosensors containing combinations of enzymes and AuNPs have large loading coefficients, indicating that they have a strong influence on the principal component and they play an important role in the discrimination

of compounds present in milk. In contrast, several sensors did not bring relevant information to the system. For instance, the non-enzymatic sensors A, B, C showed loading coefficients close to zero, so their role in the discrimination capability of the array is negligible. In order to simplify the bioET, sensors bringing information below the 70% confidence interval were removed from the array. In addition, sensors fabricated with plasticizers A and B provided redundant information to the mathematical model, while the sensors prepared with plastifier B were removed. The final bioET was made up of 9 sensors, three unspecific sensors that provide global information about the sample (A-AuNp, B-AuNP, C-AuNP), 2 biosensors specific for galactose (A-AuNP-GaOx, C-AuNP-GaOx), 2 for urea (A-AuNP-Ure, C-AuNP-Ure), and 2 for LDH (A-AuNP-LDH, C-AuNP-LDH). This is an important fact, as the reduction from 24 to 9 sensors simplifies the device considerably and makes it more operational.

3.2 Analysis of milk with the bioET: Discrimination

The simplified bioET was used to analyze milk samples with different nutritional compositions. For this purpose, the sensors were immersed in milks (dilution 1:1 in buffer phosphate 0.1M Ph7) and measured ten times. Figure 5 shows an example of the potentiometric profiles obtained when immersing the bioET in milks with different fat content (whole, semi-skimmed and skimmed). The Figure shows that the responses of the sensors depend on the composition of the milk analyzed and illustrates the cross-sensitivity of the sensors included in the array.

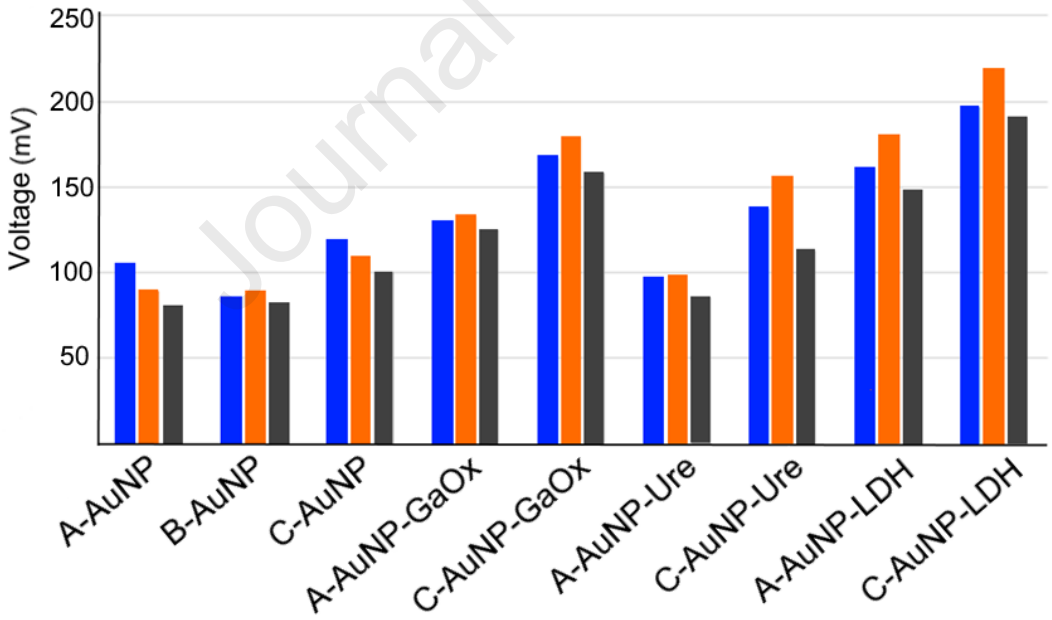

Figure 5. Potentiometric profiles of the sensors included in the bioET immersed in milks with different fat content: whole (orange), semi-skimmed (grey) and skimmed (blue).

PCA was applied to evaluate the capability of the simplified bioET to discriminate milk samples according to their nutritional content. The scores plot of the PCA obtained for the twelve types of milk (Figure 6) showed well-defined and separated clusters for classic milks, lactose free milks and milks enriched in calcium or folic acid. The explained

variance was 43 % for PC1, 21 % for PC2 and 19 % for PC3. A total of 83 % was explained with the first three principal components. Classic milks were situated in the negative region of PC1 and were clearly separated from the rest of the milks. This may be due to the fact that, unlike the other milks analyzed, classic milks have not suffered any modification apart from the UHT process.

Lactose-free milks (obtained by the action of the enzyme β-galactosidase, which breaks down lactose into glucose and galactose) appeared in the first quadrant, far from the other classes, thus confirming the effectiveness of the galactose biosensor. Milks enriched in calcium and folic acid appeared clearly discriminated from each other. Bearing in mind that the addition of calcium or folic acid can modify the pH and the ionic strength, these changes have an effect on the enzymatic activity, thus facilitating discrimination.

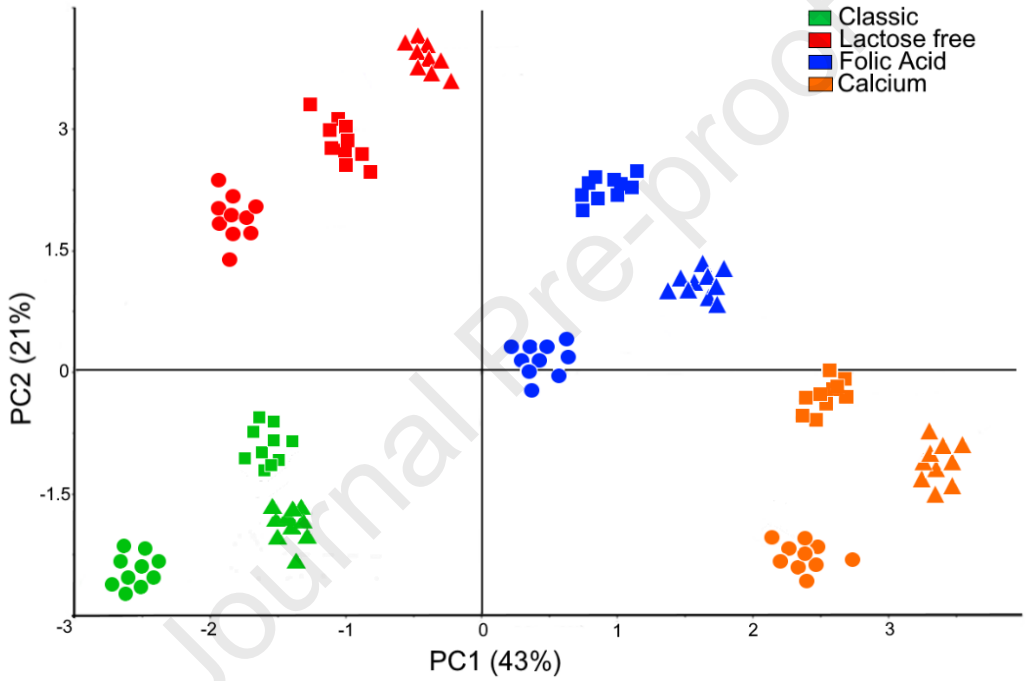

Figure 6. PCA analysis of the milks analyzed with the bioET. Milks with different fat content: whole (circle), semi-skimmed (triangle) and skimmed (square).

The results presented here have a degree of novelty. Different potentiometric ETs have been previously used in the determination of milk adulteration (Dias et al. 2009), in the monitoring of fermentation processes (Tazi et al. 2018) and in the discrimination of milk samples with different fat content (Mercante et al. 2015). However, only few studies have been reported using ETs to discriminate UHT milk samples with different nutritional properties. For instance, Mercante et al. developed an ET based on nanostructured hybrid films that was capable to differentiate between milk samples with large differences in their fat content, but samples with similar fat content appeared mixed on the principal component analysis. Pérez-González et al. developed a simplified potentiometric ET capable of discriminating between commercial milk samples. with different nutritional composition as well as by fat content with higher reproducibility (Pérez-González et al. 2021). The BioET presented here can discriminate between clusters that correspond to semi-skimmed, skimmed and whole milks. These clusters are arranged across the PC2

within each group of milks according to its nutritional content. Therefore, by incorporating enzymes and AuNPs, the discrimination capacity of the system has been increased.

3.3 Analysis of milk with the bioET: Classification models

The milk classification analysis was based on the features from the nine sensors that make up the simplified bioET by applying the Support Vector Machine classification method (SVMC). The Support Vector Machine (SVM) is a kernel-based supervised pattern recognition technique, established by Cortes and Vapnik and based on statistical learning theory (Cortes, & Vapnik, 1995). Compared with other approaches, SVM possesses the advantages of avoiding overfitting, is capable of establishing non-linear correlations between data sets and of dealing with high-dimensional input.

The SVM classification chosen was based on the radial basis function (RBF) as a nonlinear kernel approximation, defined as

$$\boldsymbol{K(x_i - x_j) = exp(\left(-\gamma \parallel x_i - x_j \parallel^2\right), \quad \gamma > 0}$$

where x_i and x_j are the training vectors of the input data, and γ is the kernel parameter.

Before the validation stage, to achieve a better performance, the kernel function penalty parameter (C) and the kernel parameter γ in the SVM were optimized. To optimize these parameters, the grid search method was applied, where approaches were made using $\log_2 C$ and $\log_2 \gamma$, varying from [10, 10] at one interval (Cortes, & Vapnik, 1995). The grid points of (C, γ) were confirmed through the validation accuracy in the [10, 10] grid. The results showed that the best validation accuracy was achieved when C=1 and γ=0.1. Due to the relatively small number of samples available, the leave-one-out cross-validation method was used to better evaluate the true success rate that can be reached with the SVM.

The classification of the samples was carried out in two steps. Initially, a study was proposed aimed at determining whether the milk samples could be classified based on their lactose content (presence or absence of lactose), as well as folic acid and calcium content (samples with or without enrichments in calcium or folic acid). This led to the development of three different classification models.

The results obtained for each of the models were the following: 98.2% calibration accuracy and 97.2% validation accuracy for milk samples with or without lactose; 96.3% calibration accuracy and 95.8% validation accuracy for samples with folic acid enrichments; and finally, 97.8% accuracy for the calibration and 97.1% in the validation was achieved in the classification model to determine which milk samples were enriched in calcium. All the classification models developed in this approach were able to establish mathematical models with high accuracy values.

A second approach was taken as an attempt to classify the analyzed milk samples according to their nutritional composition and their fat content, which resulted in a total of twelve categories. By applying SVMC, the results obtained for the simplified bioET showed 99.7% accuracy in the calibration and 98.4% accuracy in the validation. These results determined that the electronic tongue developed with nine sensors was able to classify milk samples according to their nutritional content as well as for their fat content.

3.4 Prediction of chemical parameters: Correlations between electronic tongue and chemical analysis

One of the main advantages of ETs is the possibility to predict the concentration of several components in a single measurement. For this purpose, mathematical models must be developed to establish correlations between data provided by the sensor array and physicochemical data measured by traditional methods. It is expected that the presence of biosensors could help to achieve good correlations with specific compounds.

The simplified bioET developed here was used to predict parameters commonly used to assess the gross composition of milks, including the total amount of fats, total proteins (casein or whey), carbohydrates (lactose), urea, and total solids (dry matter and non-fat dry matter) which is the residue left when water and gases are removed. Only few attempts to use ETs to evaluate the chemical composition of milk have been reported previously (Hruskar et al. 2010; Salvo-Comino et al. 2018; Pérez-González et al. 2021).

Support Vector Machine regression was used to determine the nature of the relationships between the data collected by the bioET and the physicochemical parameters. To forecast acidity, density, percentage of protein, lactose, fat, DM and NFDM, the Radial Basis Function was chosen as the core function, since it can handle non-linear interactions between the sensor inputs and the target characteristics. The regression models were created using SVM Regression (epsilon SVM, kernel type: radial basis function, C value: 1, cross validation segments size: 15, and standard deviation weighting process in all cases).

As observed in Table 5, the values obtained for the coefficients of correlation and errors for the calibration and the prediction reached values of R^2 above 0.98 for calibration and prediction, with low errors (RMSE) between 0.101 and 0.139. These high correlation coefficients could be due to the specificity induced by the presence of the biosensors. In fact, the biosensor containing galactose oxidase provides data about galactose; LDH can give information on lactic acid, which is in turn related to the acidity of the milk; while urease can account for the levels of urea. The good correlations with acidity, density fat and dry matter can be attributed to the fact that the enzymes contained in the array are sensitive to pH. In addition, potentiometic measurements are sensitive to the percentage of water (which is inversely proportional to density) and to the fat content (directly related to the conductivity and the double layer at the electrode surface). These results show that the reduced bioET is capable of establishing good correlations with the physicochemical parameters thanks to the selection of the suitable sensors in previous steps of this work.

If we compare the results of the regression with the previous work (Pérez-González et al. 2021) we observe an increase of the correlation coefficients as well a reduction in the errors (RMSE). The increase in R^2 for lactose and acidity is especially remarkable. Correlation coefficients have improved from values of 0.96 and 0.90 respectively in the validation, to 0.99 in both cases. These results demonstrate the effectiveness of the use of biosensors in the composition of a ET providing specific information on compounds of interest in milk, such as lactose, without losing global information of the sample.

Table 5. Correlation parameters from the SVM regression analysis.

Parameters		Acidity	Density	%Proteins	%Fat	%Lactose	%DM	%NFDM	Urea
SVM	R^2_C	0.9953	0.9910	0.9941	0.9956	0.9924	0.9933	0.9991	0.9915
	$RMSE_C$	0.1177	0.1376	0.1088	0.1018	0.1093	0.1233	0.1146	0.1187
	R^2_P	0.9946	0.9903	0.9944	0.9951	0.9928	0.9927	0.9982	0.9902
	$RMSE_P$	0.1181	0.1396	0.1092	0.1055	0.1113	0.1281	0.1151	0.1193

493 *3.5 Ensemble method development*

494 Although the SVM was very capable of establishing mathematical models for the correct
495 classification of the milk samples and the prediction of the physicochemical parameters;
496 here, we aimed to go a step further by establishing correlation models using ensemble
497 methodologies.
498 In the context of machine learning, ensemble methods are commonly defined as a
499 machine learning system, designed with a set of independent models working in parallel,
500 whose outputs are combined with a decision fusion strategy to create a single response
501 for a given problem. Therefore, an ensemble method aims to combine several separate
502 models to achieve a better result than each individual method in terms of consistency and
503 accuracy (Zhou, 2012).
504 The first step in developing an ensemble method is to select the individual methods.
505 Different algorithms may lead to different results for the same data by imposing a specific
506 structure for it. Moreover, there is no single algorithm able to perform consistently well
507 for different problems and there are no clear rules to follow while selecting individual
508 algorithms for a given problem.
509 In principle, any individual models could be used as long as they are suitable for the
510 dataset. In this work case, the Caret Package developed for R is used to select the
511 individual algorithms (Kuhn, 2008). The generated models should be as different from
512 each other as possible. A high level of diversity means that they will be able to capture
513 different information about the data and can overcome the weaknesses of single
514 techniques, since each technique handles the error made by the others.
515 Starting with the SVM regression model (svmRadial), five models were selected to ensure
516 their diversity. For this, the "max.dissim" function of the Caret Package was used, in
517 which the Jaccard dissimilarity function was selected as the diversity criterion. The
518 models selected were: Support Vector Machine (svmRadial), Quasi-recurrent Neural
519 Networks (qrnn), Cubist Regression Model (cubist), Weighed k-nearest neighbor (kknn),
520 and Bagged Earth (bagEarth).
521 The support vector machine was chosen as the starting model due to its great performance
522 in the previous section of this work. Furthermore, SVM is a powerful method widely used
523 in the development of ensemble models.
524 Once the models had been selected, the original data were split into two sets: a training
525 set containing 75% of the original data to be used in the calibration process of each
526 algorithm, and a testing set covering the remaining 25% of the data for validation. It is
527 essential to verify that both sets of data are representative of all the recognized categories;
528 consequently, the percentage of each set is computed in relation to the total data as well
529 as the amount of data in each category.
530 Each algorithm was executed individually, but the control parameters for all of them were
531 established beforehand. Validation was performed using repeated 10-fold cross-
532 validation, to establish reasonable values for the tuning parameters and random search
533 was established as the preferred method. After each individual algorithm was applied, the
534 Stochastic Gradient Boosting (gbm) method was used to generate the ensemble through
535 the Caret Ensemble package in R (Kuhn, 2008).
536 Stochastic Gradient Boosting is a machine learning algorithm, able to perform
537 classification and regression problems. Gradient Boosting is especially convenient,
538 because of its computational efficiency and robustness to overfitting, as a simple
539 technique to develop ensemble decision trees by creating training trees on subsamples of
540 the training dataset (Friedman, 2002).

Table 6 shows the values obtained for the correlation and error coefficients for the calibration and prediction obtained by the ensemble. The coefficients of correlation and mean errors for the calibration and prediction reached values of R^2 above 0.9992 for both calibration and prediction, with low errors (RMSE) between 0.0033 and 0.0172.

Table 6: Correlation parameters from the ensemble regression analysis.

Parameters		Acidity	Density	%Proteins	%Fat	%Lactose	%DM	%NFDM	Urea
Ensemble	R^2_C	0.9997	0.9999	0.9999	0.9994	0.9999	0.9999	0.9999	0.9999
	$RMSE_C$	0.0097	0.0053	0.0041	0.0164	0.0042	0.0037	0.0033	0.0040
	R^2_P	0.9994	0.9997	0.9998	0.9992	0.9998	0.9998	0.9998	0.9998
	$RMSE_P$	0.0102	0.0075	0.0056	0.0172	0.0051	0.0045	0.0041	0.0048

Considering the high values of the correlation parameters achieved with SVM regression, it was expected that the result of the regression ensemble would reach nearly 100% precision while establishing correlations, since there is a very reduced number of errors in the original model. However, the intention in this section is not to ensure the capability of the simplified bioET to establish correlations with the studied parameters, but to demonstrate the possibility of combining the developed system with ensemble methodologies that could be applied in the study of future and more complex samples, where the settings may not be as good as they should be.

4. Conclusions

In this work, a bioET with improved characteristics was developed and used to predict the chemical characteristics of milk with unprecedented accuracy. The system incorporates biosensors based on membranes of carboxylated PVC (C-PVC) containing gold nanoparticles (AuNPs), where GaOx, LDH and Ure were effectively immobilized. The developed biosensors and the associated methodology have resulted in a bioET where the enzymes can work simultaneously while also preserving the enzymatic activity. Nanoparticles have proven to have a potential to amplify the electrochemical signals.
The biosensors have shown excellent sensitivity and reproducibility towards standard solutions of compounds usually found in milk ($CaCl_2$, KCl, urea, lactic acid and galactose), with excellent sensitivity and reproducibility, showing LODs of 10^{-6} M.
The bioET was successfully used to discriminate between milks by applying PCA based on their nutritional content. The bioET shows an excellent classification capability and can classify milk with different compositions by applying SVM with accuracies above 95%. The system can predict the acidity, density, %proteins, %lactose, %fat and dry matter with low errors and high correlation coefficients. The results show that the SVM models constructed with the e-tongue and physicochemical parameters have potential for use in simultaneously assessing 8 parameters, thus reducing the time of analysis. Moreover, it has been proved that applying ensemble methodologies can further improve the correlation between the bioET data and the physicochemical parameters. Investigations into the efficiency of the prototype devices can create new application possibilities and suggest successful implementations in real applications.

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.

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Authors contribution
MR-M, CG-C, and FM-P conceptualized the idea and supervised the work. CP-G and CS-C performed the experiment, curated the data, and wrote the original draft. FM-P involved in software design and development. CP-G and CS-C involved in formal analysis. CG-C and MR-M acquired the funding. CP-G, CS-C, FM-P, MR-M and CG-C reviewed and edited the paper. All authors provided feedback.

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Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: