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Salt release monitoring with specific sensors in "*in vitro*" oral and digestive environments from soft cheeses

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ABSTRACT

The objective of the present work is to demonstrate the interest and the feasibility of the measurement of NaCl concentrations in soft cheeses and in particular an *in vitro* digestion process by the use of chemical sensors. The analyzed matrices were the commercial Italian mozzarella cheeses and domestic cheese base models. The classification of mozzarellas was performed according to their salinity, while the breakdown of cheese base models has been followed both at initial steps of digestion in artificial mouth dispositive mimicking the oral sphere and in a gut-imitating digester (TIM-1). During the breakdown of soft cheese in the digester, the estimated values for Na^+ concentration using mono-Na-ISEs showed correlation coefficients values about 0.907 and 0.832 compared to Ionic Chromatography (IC) reference values, with an important relative error (about 30–40%). The use of ISE array system combining several electrodes, in particular electrodes showing more selectivity to Cl^- and Na^+ ions, showed the best results for Na^+ concentration estimation, with good correlations both in calibration (R=0.962) and in validation (R=0.952) steps. For cheese digestion in the artificial mouth, good correlations for Na^+ concentration were observed using single Na-ISE compared to IC with coefficients ranking between 0.93 and 0.96 for both the calibration and validation steps. Moreover, a fair correlation between chloride ions measured with Cl-ISE2 and Na^+ (R=0.96) was found. The best results were obtained with the use of ISEs array combining, in particular, Cl^- and Na^+ detections. The salinity of commercial mozzarella cheese samples, as far as originally utilized milk type (cow or buffalo), were also satisfactory determined with developed ISE array.

1. Introduction

Food industry is facing continuous changes in public policies, in particular concerning nutritional public recommendations or allegations. In this context, the development of *in vitro* systems to study food product breakdown and nutrients release has gained more and more interest in the last few years. These systems are able to mimic the oral sphere [1–3] as well as the gut [4–8].

In humans the digestion process starts in the oral sphere, where the food is chewed, mixed with saliva and treated with enzymes such as salivary amylase and lingual lipase [9]. Simultaneously the food taste is evaluated by the tongue gustatory receptors. In the case of positive gustatory event, or in other words, when a food sample is recognized tasty, it is forwarded to the gut, composed of stomach, small intestine and large intestine. In stomach the food physical disassembly continues together with protein denaturation into polypeptides and amino acids, while carbohydrates are broken down into sugars under the action of gastric acid, and enzymes: pepsin and amylase correspondingly. The majority of digestion and absorption occurs in small intestine, where the food arrives in the form of semi-liquid chyme. Here it is mixed with bile, pancreatic juice and intestinal enzymes such as

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trypsin, chymotrypsin and sugars enzymes (maltase, lactase and sucrase). The breakdown of fat globules and polysaccharides occurs here. In large intestine the gut bacteria induce fermentation processes which permit to break down some complex saccharides and reabsorb fluid from bolus [10]. Food products such as dietary fibers cannot be absorbed by gastrointestinal tract and mixed with other waste products are excreted from the body.

The very similar processes occur in artificial systems imitating digestion process. Thus, an artificial mouth reproduces the human chewing process by replication of the environment, teeth and application of the same pressure efforts; the food samples are subjected to the real or artificial saliva treatment and then analyzed with specific methods, like Ionic Chromatography (IC) or multisensory systems [11]. For the gut, one of the most used systems is the TIM-1 or TNO-gastrointestinal tract model [6]. TIM-1 is a dynamic computer-controlled *in vitro* system that closely mimics the upper part of the human gastrointestinal tract (stomach, and small intestine composed of duodenum, jejunum and ileum). Its main characteristics are simulation of peristaltic movements, controlled squeezing, body temperature, absorption of nutrients and water in the largest compartments of the small intestine (jejunum and ileum). Moreover, intestine secretions are implemented in order to mimic the pH and concentrations of electrolytes, enzymes, and bile that are observed *in vivo* [7,8]. This system tends to reproduce the different events occurring in the digestive tract leading to results reliable in *in vivo* observations. For instance, it has been used to investigate probiotic survival, food component stability and fatty acid delivery [6,7,12].

In context to be predictive tools, the drawbacks of the system are the lack of online measurement of some essential parameters and nutrients release by the use of appropriate sensors. Among the different parameters to be measured during digestion, NaCl content has gained particular attention. This is because NaCl is a common additive in food industry, where it is used in small amounts to improve significantly the product taste, but the decrease of its delivery and consumption is an important challenge, since health authorities recommend decreasing progressively salt content in food products to prevent health problems [13,14]. Excess intake of sodium induced by the overconsumption of salt is linked to the development of hypertension, cardiovascular disease and other health problems [15,16]. The main sodium source is the sodium chloride (salt) added during the food processes or the preparation of meal. Considered as a flavor enhancer, salt not only acts on salty perception but also on the overall flavor perception. Consequently, a decrease in salt content may reduce the global acceptance of a food, particularly in increasing blandness that could result in a decrease of interest and a negative economical impact. Different strategies can thus be developed to modify the consumption of Na^+, i.e. changing the perception or changing the composition of the foodstuffs. On this last point, it is however necessary to have efficient systems, in particular specific sensors, able to predict Na^+ release during food breakdown and digestion. Another important role of NaCl in food industry is its effectiveness in the preservation of fresh products for longer period.

Recently chemical sensors, and among them ISEs, have been shown to be very useful for the analysis of several characteristics of foodstuffs [17], and the salinity [18] (in terms of sodium chloride content) in particular. In this context, the purpose of the current study is to demonstrate the interest and the feasibility of Na^+ concentration measurements with specific sensors during digestion of a cheese matrix in artificial mouth and TIM-1 digester, as far as to monitor the NaCl amount in fresh Italian mozzarella cheeses produced from different types of milk, from cow and buffalo in particular. Specifically, the different sensors and multisensory system were developed and compared for Na^+ amount evaluation in fresh soft cheeses and in cheeses in different breakdown stages.

2. Material and methods

2.1. Reagents

Poly(vinyl chloride) (PVC) high molecular weight; plasticizers bis(2-ethylhexyl) sebacate (DOS), o-nitrophenyl octyl ether (o-NPOE), dioctylphenylphosphonate (DOPP); ionophores monensin dodecyl ester, valinomycin, nonactin, ion exchangers bis[4-(1,1,3,3-tetramethylbutyl)phenyl]phosphate calcium salt ($Ca(TMBPP)_2$), tetradecylammonium nitrate ($TDACNO_3$) and lipophilic anionic additive potassium tetrakis-(4-chlorophenyl)borate (TpClPBK) were purchased from Fluka. 5,10,15,20-tetraphenylporphyrinato-manganese (III) chloride (MnTPPCl) was synthesized in "Tor Vergata" university laboratories according to the literature reported method [19]. Tetrahydrofurane (THF) solvent was purchased from Sigma-Aldrich and freshly distilled prior to use. All other chemicals were of analytical grade and used without further purification. The standard solutions for potentiometric measurements were prepared dissolving known amount of sodium salts of given anions in distilled water or background solution with adjusted ionic strength (NH_4Cl 0.5 M and NaOH 0.5 M).

2.2. Cheese samples

2.2.1. Artificial gastric juice composition

Compositions of the different gastric secretions used during the artificial digestion of cheese matrix are given in Table 1.

2.2.2. Domestic cheese matrix composition

Cheese matrix was made by action of Rennet solution (520 mg/L of chymosin, Sanofi Bio-Industries, France) on a mixture of milk protein (Eurial Poitouraine, France), anhydrous milk fat (Cormans, Belgium), NaCl (Jerafrance, France), pure water (MilliQ system, Millipore, USA), and, in some cases, cysteine (Sigma-Aldrich, France). All ingredients were food grade.

In order to prepare 500 g of liquid phase meal, LPM, pure water (275 g), milk fat (110 g), milk powder (110 g), NaCl (10 g) and, if added, cysteine (1 g) were vigorously mixed with a Blender® (Waring, USA) at medium speed (graduation 7) for 12 min at 67 °C. The mixture was cooled in a glass beaker then placed in a thermostated bath at 32 °C. The pH (6.2) was measured using a potentiometric pH glass electrode (Mettler-Toledo, France). After 30 min resting, the Rennet solution (12 mL of Rennet extract diluted in 9 volumes of pure water/Kg of collected mixture from the blender) was added and mixed vigorously during 1 min. Prior to the coagulation, the mixture was immediately poured into a plastic bag, vacuum sealed, then completely immersed in a controlled-temperature bath at 32 °C for 3 h. Products were then stored at 4 °C until use.

2.2.3. Mozzarella samples

24 samples of mozzarella cheese were analyzed; among them 6 mozzarella samples were prepared from buffalo milk and the remaining 18 samples were from cow milk. All samples were purchased from the local stores of Rome, Italy. The measurements included two phases: in the first stage the NaCl content in mozzarella samples was determined and compared with the IC results, while in the second step the PLS-DA discrimination of mozzarellas produced from buffalo or cow milk was performed.

Table 1
Parameters of gastrointestinal digestion in the TIM-1 when simulating digestive conditions of a healthy adult after intake of a solid meal.

Compartment	Stomach	Duodenum	Jejunum	Ileum
Initial residue (mL)	10	40	30	65
Maximal volume (mL)	310	55	150	65
Deliveries parameters				
$t_{1/2}$ (min)	85			250
ß	1.8			2.5
pH/Time (min)	6.5/0 6.0/15 4.5/45 2.9/90 2.3/120 1.8/240 1.7/300	6.0*	6.8*	7.2*
Secretion	0.25 mL/min of pepsin (2080 IU/mL) 0.25 mL/min of lipase (133.2 IU/mL) 0.25 mL/min of HCl 2 M if necessary	0.5 mL/min of bile extract (4% during the first 30 min of digestion then 2%) 0.25 mL/min of pancreatin 4USP (21%) 0.25 mL/min of intestinal electrolyte solution 0.25 mL/min of $NaHCO_3$ 0.5 M if necessary 23600 IU of trypsin (at the beginning of digestion)	0.25 mL/min of $NaHCO_3$ 0.5 M if necessary	

* pH maintained at constant value.

2.2.4. *Cheese samples preparation for Na^+ determination and classification*

2 g of fresh mozzarella cheese or of partially digested cheese sampled from artificial mouth or different compartments of TIM system was reduced in fragments (if needed) and extracted with 10 mL of distilled water. The solid part was centrifuged and 1 mL of liquid extract was collected and utilized for further IC and sensory analysis. In total 49 samples were analyzed, among them 24 commercially available fresh mozzarella cheeses, and home-made soft cheeses treated correspondingly with artificial mouth (8 samples) and TIM-1 digester (17 samples).

2.3. *Artificial mouth system*

Artificial mouth system is composed of a 150 mL closed double jacket vessel with a usable volume of 100 mL, Fig. 1A. The temperature was regulated with a circulating fluid at 35 °C. The top was closed with a cap lined with a Teflon seal. A shear rate of 200 S^{-1} was applied to the cheese matrix using a marine propeller driven by a viscosimeter (RM180s DIN 2:2, Rheometric Scientific, France) at 21 °C. The choice of shear rate was based on previous studies [20].

The following treatment of domestic cheese samples was performed in the artificial mouth dispositive described above: cheese samples were treated with deionized water or human averaged saliva collected from 30 healthy individuals and disintegrated for one minute period. Among the eight tested cheese samples, four samples were mixed with only distilled water, while the other four were mixed with averaged human saliva in the subsequent cheese to water/saliva ratios: 4:1 (80 g cheese/20 mL liquid), 2.7:1 (80 g cheese/30 mL liquid), 2:1 (60 g cheese/30 mL liquid), 0.8:1 (50 g cheese/62.5 mL of liquid). The ratios were fixed with regard to the human variability observed in the amounts of saliva incorporated in cheese matrix [21]. After mixing samples were filtered and the supernatant was diluted 100 times by distilled water and analyzed by IC and specific sensors. Nine measurement replicates were performed resulting in 72 measurements in total.

2.4. *Gastric-small intestinal system (TIM-1)*

The gastrointestinal tract model TIM-1 (TNO, The Netherlands) consists of four successive compartments simulating the stomach, duodenum, jejunum and ileum, Fig. 1B. This system has already been described elsewhere [6]. Briefly, each compartment is composed of glass units with a flexible inside wall. The system is kept at body temperature by pumping water into the space

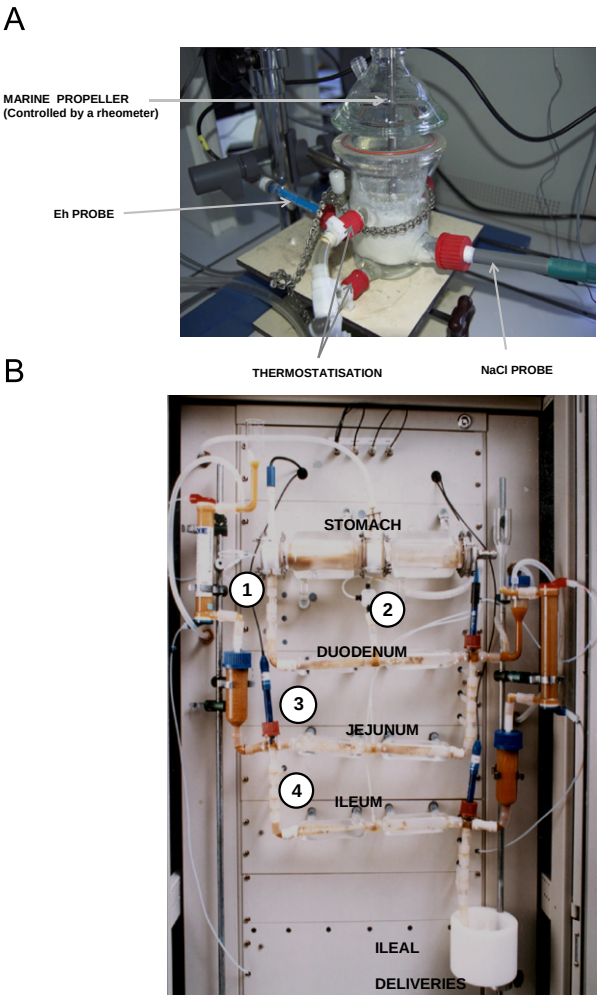

Fig. 1. The schematic presentation of (A) artificial mouth system; (B) TIM-1 digester.

between the glass jacket and the flexible wall. Peristaltic mixing is simulated by alternate compression and relaxation of the flexible walls following changes in the water pressure. The mathematical modeling of gastric and ileal deliveries with power exponential equations of general form $f = 1-2^{-(t/t_{1/2})^{\beta}}$, where f represents the fraction of meal delivered, t and $t_{1/2}$ are the time and the half-time of delivery correspondingly, β is a coefficient describing the shape of the curve, is used for the computer control of chyme transit, as described by Elashoff et al. [22]. Opening and closing the peristaltic valves that connect the compartments regulate chyme transit. The volume and pH are computer-monitored and continuously controlled in each compartment. Simulated gastric, biliary and pancreatic secretions are introduced into the corresponding compartments by computer-controlled pumps. In our study, TIM-1 was programmed to reproduce the digestion of a solid meal in a healthy human adult (Table 1). As the aim of our study was to follow NaCl content during cheese digestion, passive absorption of digestion products and electrolytes was not reproduced (dialysis system was not added). The meal used in our study was prepared as follows: 93 g of cheese was mixed with 217 g of deionized water in a BagMixer (Interscience, France) for 30 s. Then 300 g of meal was introduced in the stomach of the TIM-1 before starting the digestion. Digestions were conducted for 300 min. Samples were taken from each compartment at different time, and from ileal deliveries collected on ice and pooled at 0–60, 60–120, 120–180, 180–240, and 240–300 min. All samples were stored at −20 °C prior to Na^+ analysis.

2.5. Sodium measurements

Sodium amount was estimated either by IC procedure or by the use of ISE sensors as described below.

2.5.1. Ionic Chromatography method

The IC system (Dionex ICS 3000 series, Dionex, USA) consists of a pump, a column oven, an auto injector, and a suppressed conductivity detector. Separation of compounds was achieved using a IonPac CS12A (3 × 150 mm, 5 μm) column with a CG12A (2 × 50 mm) gard-column (Dionex, USA). The column oven was controlled at 30 °C. The elution was done with a solution of sulfuric acid (11 mM) as mobile phase. The chromatograph was operated isocratically with a flow-rate of 0.5 mL/min. The injection volume was 20 μl. The analytical current (40 mA) was used for the suppressor CSRS 300, 2 mm (Dionex, USA). The total run time was 15 min. The identification of ions was performed comparing the IC retention times with those of the authentic standards. 1 mL of the different samples were centrifuged at 1000 *g* during 5 min and filtrated through a 0.22 μm nylon filter (Sartorius, France) before IC analysis.

2.5.2. ISE method

The estimation of Na^+ content was performed by the use of two different ISEs. The first one was a commercially available Na^+ polymer membrane electrode Na-ISE 1 (Metrohm Ion Analysis, Switzerland) working with a reference electrode Dri-RefTM (DRIEF-2, WPI, UK). The Na-ISE 1 and reference electrode were connected to an interface (ELIT 8088 multi-channel pH-meter/redox-meter Computer interface, Bioblock, France) piloted by specific software (Eight channel interface). Another Na-ISE 2 utilized was a homemade PVC-based polymeric membrane electrode containing monensin dodecyl ester ionophore.

In general, for the preparation of polymer membranes 1–10 wt% of ionophore or ion exchanger was distributed in PVC/plasticizer (1:2) polymeric matrix dissolved in THF and varying amount of lipophilic additives was added, if needed. Membrane solution received in such a way was poured into a glass ring, which was tightly fixed on a glass plate. After allowing the solvent to evaporate overnight the homogeneous elastic membrane was obtained. The disks of 8 mm diameter were cut out with a punch and fixed on the top of the hollow PVC tubes and then filled with an inner 0.01 M NaCl solution and supplemented with inner Ag/AgCl reference electrode. Then the freshly prepared electrodes were soaked in 0.01 M NaCl and potentiometric properties of electrodes were evaluated one day after the preparation. Electrochemical potentials were measured with the following galvanic cell:

Ag|AgCl(s)| KCl(3M)|| sample solution | ion selective membrane | NaCl, 0.01 M|AgCl(s)|Ag

Double-junction reference electrode with 3 M potassium chloride bridge was from AMEL, Italy. Electrode potentials were measured at ambient temperature (22 °C) and uniform stirring rate. The calibration of electrodes was performed by stepwise addition of the calculated amounts of freshly prepared standard solutions to the background electrolyte varying in such a way that the concentration of primary ion is in 10^{-6} to 10^{-1} M range.

The amounts of sodium and chloride in analyzed samples were evaluated in two different manners. The first one consisted of the direct calibration of Methrohm Na-ISE done with known concentration of NaCl added to the digestion medium used for the experiments and further calculation of Na^+ content referencing to a calibration curve, while the double known addition method was implemented in the second case [23]. The latter method provides the possibility to determine the amount of primary ion by the addition of two aliquots of NaCl standard in the analyzed sample and to avoid the electrode calibration procedure. The subsequent additions of 30 and 300 μL of 1 M NaCl standard solution whose concentration was about 100 times the sample concentration were performed on 30 mL of cheese sample preparation (0.5 mL of cheese sample prepared for IC analysis (see above) was adjusted to final volume of 30 mL by addition of distilled water and ionic strength adjusting solution). The potentials of Na-ISE and Cl-ISE were registered before and after both additions. The initial amounts of Na^+ and Cl^- in sample were then calculated from the registered ISEs potential values according the standard procedure [24]. Polycrystalline Cl-ISE2 was utilized for Cl^- ions concentrations determination (Sensor System Company, Russia).

2.5.3. ISEs array method.

Polymeric membrane electrodes for chloride (Cl-ISE1, MnTPPCl based), potassium (K-ISE, valinomycin based), ammonia (NH_4-ISE, nonactin based), calcium (Ca-ISE, $Ca(TMBPP)_2$ based) and nitrate (NO_3-ISE, $TDANO_3$ based) have been also prepared and tested according to the above discussed method. The electrodes were combined in an array and their potentials in 10 artificially prepared gastric juice samples without cheese and 17 samples of cheese mixed with a gastric juice, collected from different fractions of the TIM system during the digestion process, were registered simultaneously versus Ag/AgCl reference electrode by high-impedance analog-to-digital potentiometer (LiquiLab, ECO-SENS srl, Italy). pH was measured for all cheese samples with the help of a glass pH electrode (AMEL, Italy).

2.6. Statistical analysis

All experiments were done at least in triplicates. Whenever necessary, statistical analysis was done by the use of Statistica software (StatSoft, France). Partial Least Squares Regression (PLS), Principal Component Regression (PCR) and Multiple Linear Regression (MLR) methods were applied to correlate ISEs array output with the IC results received for Na^+ content in analyzed

samples [24,25]. Mozzarella cheeses classification was performed with PLS discriminant analysis (PLS-DA).

All the above mentioned regression methods relate the variations in one or several analyte variables (*Y*-variables) to the variations of several sensor response predictors (*X*-variables), with explanatory or predictive purposes. MLR is a linear and parametric method based on ordinary least squares assuming that the analyte content can be represented as a linear combination of individual sensors responses: $R_i = \beta_{1i}p_1 + \beta_{2i}p_2 + \cdots + \beta_{ni}p_n + \varepsilon_i$, where β_{ji} is a coefficient of the i analyte influencing the signal R_i, p_j is the signal obtained only for sensor j, ε_i is the residual error. For n analytes the following data matrix can be constructed: $R = P\beta + \varepsilon$. In this matrix P and R are known values, while β can be evaluated by least square method. This operation involves a matrix inversion, which leads to collinearity problems if the variables are not linearly independent. The ability to vary independently of each other is a crucial requirement for variables used as predictors with this method. MLR also requires more samples than predictors or the matrix cannot be inverted.

PLS regression combines characteristics of principal component analysis (PCA) and MLR. It is mostly useful when there is a need to predict a set Y of variables (analytes) from a large set X of independent predictors (sensor responses). Considering M observations described by K variables which are stored in a matrix denoted as $Y = MK$, and that the values of J predictors collected in these M observations are collected in the matrix $X = MJ$. The goal of PLS regression is to predict Y from X and to describe their common structure. When Y is a vector and X is full rank, this goal is accomplished using ordinary multiple regression. When the number of predictors is large compared to the number of observations, X can be singular and the regression approach is not feasible (i.e., because of multi-collinearity). Several approaches have been developed to resolve this problem. One approach is to eliminate some predictors (trying to avoid the multicollinearity). Another approach is called principal component regression, PCR, and performs a PCA analysis of the X matrix and then fit an MLR model, using the PCs of X as regressors on Y. The orthogonality of the principal components eliminates the multi-collinearity problem. The prediction error of all the regression methods is strongly dependent on the number of variables (sensors) and on the number of measurements. In the case of a small dataset, in order to have realistic error estimation a cross validation technique is applied.

The PLS-DA analysis approach consists of the assignment of a sample to the classes included in the analysis. The most common case is that of a binary discriminant variable: a question with a Yes/No answer. In this case the discriminant variable is coded 0/1 (Yes=1, No=0) as *Y*-variable in the model. By building a PLS model with all indicator variables as *Y*, it is possible to directly predict class membership from the *X*-variables describing the samples. The model is interpreted by viewing comparing the predicted values for each class indicator *Y*-variable, if $Y_{pred} > 0.5$ it means "roughly 1" that is to say "class member"; when $Y_{pred} < 0.5$ means "roughly 0" that is to say "non-member". Once the PLS model has been checked and validated it is ready for new samples classification.

The data were used without any preprocessing and scaling. Due to the restricted number of analyzed samples the validation was performed using a leave-one-out cross-validation procedure. The RMSEC and RMSEP (Root Mean Square Error of Calibration and Prediction correspondingly) and the correlation coefficients of predicted versus measured correlation lines were used to evaluate the efficiency of applied regression model. Data treatment was performed with commercial Unscrambler (v. 9.1, 2004, CAMO PROCESS AS, Norway) and Matlab (v.7.0, 2005, The MathWorks, Inc., Natick, USA).

3. Results and discussion

3.1. Domestic cheeses breakdown in TIM digester

Initially we have monitored the NaCl content in 17 domestically prepared soft cheese samples collected in the stomach, duodenum, jejunum, ileum and ileal delivery compartments of the TIM system during the digestion process. The amount of NaCl has been estimated by the use of an ISE probe or multisensory array and the values were compared with those obtained with IC analysis. The results are presented in Fig. 2. A continuous evolution of Na^+ concentrations in the different compartments corresponding to the release of Na^+ from the cheese matrix was detected either by IC measurement or by single Na-ISE 1 probe and multisensory array composed by Na-, Cl-, K-, Ca- and NO_3-ISEs. However, if the evolution profiles were similar, the highest standard deviations in Na^+ content determination were

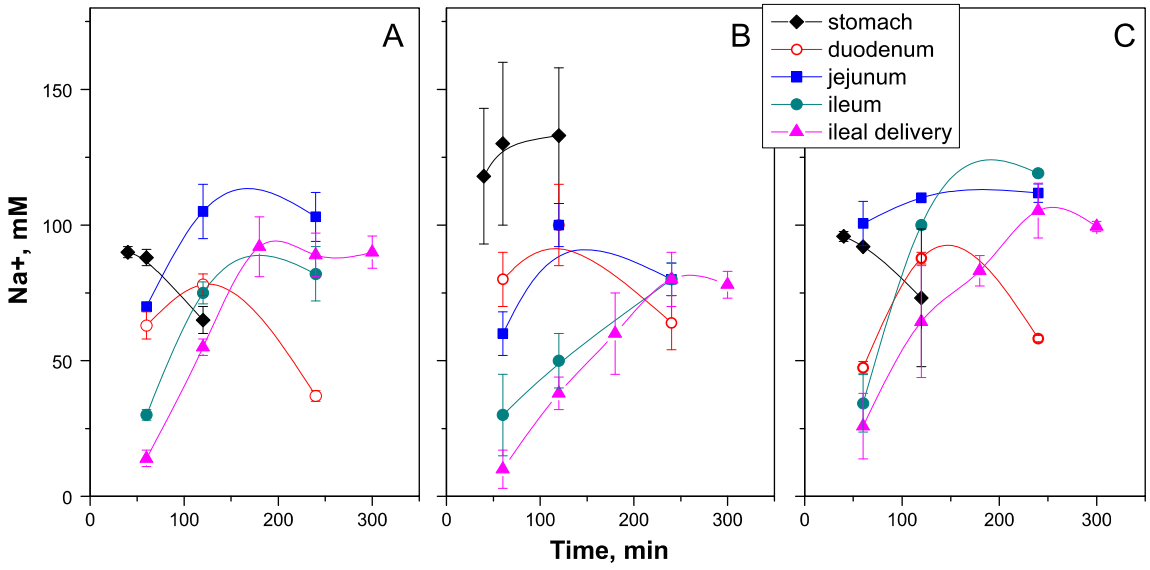

Fig. 2. Time course of Na^+ concentrations measured in the different compartments of TIM-1 system during the digestion of cheese base model. Measurements were performed with (A) IC; (B) Na-ISE 1; (C) ISEs array correspondingly.

obtained with the single Na-ISE 1 in particular for the cheese samples withdrawn from all TIM system compartments during the first 2 h of the digestion process. In comparison to the mono Na-ISE 1, a home-made Na-ISE 2 containing PVC-polymeric membrane doped with monensin dodecyl ester ionophore has shown better performance for the determination of Na^+ content in cheese samples in different degrees of digestion. As emphasized in Fig. 3A, the correlation coefficients, R, between the predictive Na^+ contents obtained with mono Na-ISE 1 and Na-ISE 2 compared to the IC value were about 0.907 and 0.832, and the mean relative errors of determination were 27.2% and 39.3% correspondingly. Such high values of mean relative error indicate the serious drawbacks in the application of single Na-ISEs for the monitoring of cheese digestions process in the TIM-1 system.

On this basis, we have tested other ISEs, as far as sensor array utility in order to improve the predictive capacity of the sensors to monitor TIM-1 system digestion processes. First we have checked the possibility of indirect determination of Na^+ content arising from the salinity (NaCl content) of digested-cheese samples by means of counter Cl-ions detection with Cl-ISE 1. Unfortunately, it was found, that application of Cl-ISE 1 containing polymeric membrane based on Mn(III)TPPCl ionophore did not improved the quality of the measurements performed on the different fractions recovered from the digestion experiment. In particular, the Cl-ISE 1 probe showed the most imprecise relative error values which varied in a very large range (from 6% to 200%), and the correlation coefficient between predicted Na^+ content values obtained with Cl-ISE 1 and those determined by IC was only $R=0.747$. We explain such unsatisfactory result by the obliged variation of Cl-ions concentration in TIM-1 system due to the additions of gastric juice portions and system pH adjustment (by means of HCl) during the digestion experiment. The instable potentiometric behavior and insufficient selectivity impede the application of mono Cl-ISE 1 for the indirect Na^+ detection during the food breakdown in the gut digester.

In the last two decades the problem of insufficient selectivity of most of the single chemical sensors for liquid phase analysis has been satisfactorily solved by sensor arrays application [26–30]. In such an approach, the responses of several cross-sensitive sensors towards the same impulse, like the analyte concentration change for instance, is simultaneously registered and the sophisticated data processing techniques such as regression or pattern recognition methods are then utilized to interpret the received multicomponent output and to correlate it to the searching analyte content [31]. Thus, Darder et al. have reported recently the multisensor prototype comprising commercial ISEs for calcium and nitrate ions, a conductivity cell, a pH half-cell electrode and a homemade anionic sensor based on a biopolymer–clay hybrid material with intercalated chitosan [26]. This array was applied for the recognition of nutrient solutions employed in fertigation. The system was able to discern among hydroponic solutions of close composition and has shown the long-term stability allowing the comparison of new unknown solutions with past cases registered in previous days. Del Valle and colleagues have reported the applications of two different ISE-based arrays (electronic tongues) to the monitoring of the nutrient solution composition used in closed soilless greenhouse systems: one was applied for the simultaneous determination of ammonium, potassium, nitrate, sodium, and chloride and the other for the simultaneous determination of these five elements plus phosphate and were used during the winter and summer periods respectively [27]. The variations in detected ions concentrations obtained by means of sensor arrays were then explained according to the plants metabolism using physiological arguments. Moreover, the developed ISE array was able to detect in real time the anomaly introduced deliberately in the automatic recomposition system, demonstrating clearly its utility in control of closed soilless systems. The micro-sensor array of miniaturized solid-state ISEs for cystic fibrosis diagnosis in humans by simultaneous analysis of sodium, potassium and chloride in sweat was described by Lynch et al. [28]. The utility of such a micro-sensors was shown in the portable instruments capable of rapid multicomponent analysis and diagnosis of cystic fibrosis with a high degree of success. The developed array has proper functioning even after approximately three months of regular use, with no noticeable decrease in performance. The integrated array of microelectrodes fabricated from epoxy-glass laminate that comprised also a miniaturized reference electrode, integrated on the same substrate for the classification of milk has been reported by Ciosek and Wróblewski [29]. The array was

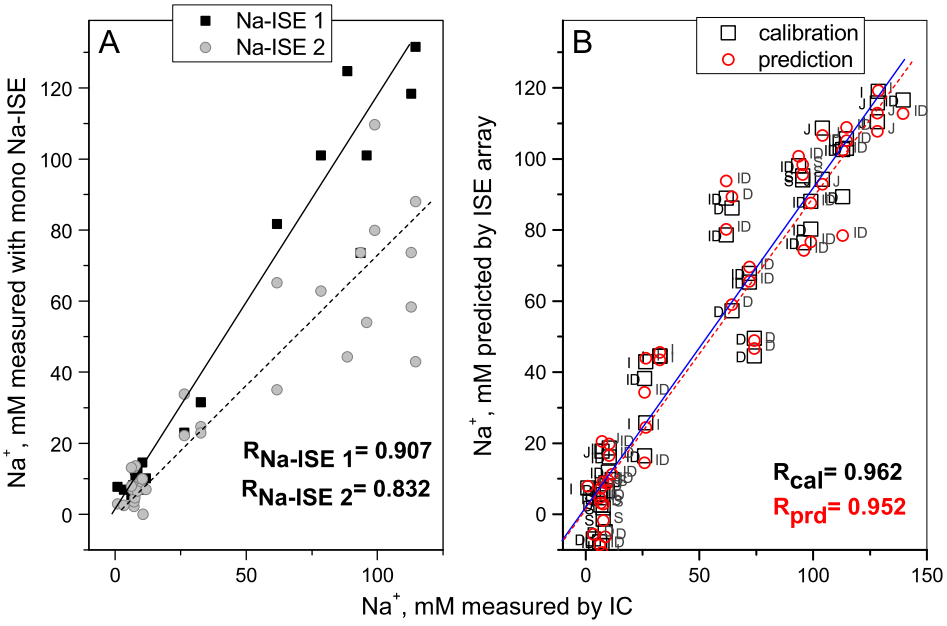

Fig. 3. A correlation between Na^+, mM content determined during domestic cheese digestion process in the TIM system with (A) mono-Na-ISE 1, 2 by double known addition method versus IC measurements; (B) ISEs array and PLS1 prediction. Abbreviations in the figure correspond to S—stomach, D—duodenum, J—jejunum, I—ileum, ID—ileal delivery.

composed from four ISEs selective towards ammonia, chloride, hydrocarbonate and calcium ions and four partially selective sensors with cationic sensitivity. The system exhibited satisfactory classification abilities towards milk originating from various diaries. Miniaturized, integrated array of ISEs for determination of calcium and total hardness in natural waters has been described by Saurina et al. [30]. The array consisted electrodes selective towards calcium, magnesium, ammonia, potassium, sodium, lithium ions and pH. The proposed sensor array could overcome the insufficient selectivity of the calcium and magnesium ISEs taking in advantage the cross-selectivities of cation species towards each ISE. The quantification of calcium and total hardness in the water samples was performed by means of chemometric methods; the obtained results were in concordance with those given by the standard method based on complexometry.

In order to improve the estimation of Na^+ content in the gastric juice and then in the digested-cheese samples obtained from different studies performed on TIM-1 system digestion process, the sensor array composed of Na-ISE 2, Cl-ISE 1, Cl-ISE 2, K-ISE, NH_4-ISE, Ca-ISE and pH glass electrode was utilized in the next step. The potentials of these sensors have been simultaneously registered versus Ag/AgCl reference electrode in all analyzed samples and the regression methods employed to correlate the ISEs array response to the content of Na^+ evaluated by IC.

The Partial Least Squares regression (PLS1) method has been utilized for the multicomponent data analysis. The first 4 Principal Components (PCs), being the linear functions of the original variables, estimated to contain, in decreasing order, the main structured information in the data, represented 90% and 88% of all explained variance for calibration and prediction procedure correspondingly in cheese samples. The PLS1 result of Na^+ content in digested-cheese samples during the *in vitro* gut process determined by ISEs array system and compared to the values evaluated by IC is shown in Fig. 3B. It can be seen that a satisfactory correlation between predicted and measured values of Na^+ was achieved both in calibration (R=0.962, slope 0.899, offset 1.79) and prediction (R=0.952, slope 0.887, offset 2.46) steps. The root mean square error of calibration was RMSEC=13.0 mM, of prediction RMSEP=14.4 mM.

We have evaluated the influence of each ISE in array for the success of Na^+ content determination by created PLS1 model. For this purpose the loadings of ISE variables along first two PCs evaluated in PLS model calibration have been compared. The loadings show how well each ISE variable was taken into account by the model components. The higher is the loading value, the greater is the ISE contribution degree to the meaningful variation in the data. Moreover, the sign of the loadings along the same PC permit to valuate the correlations in the ISEs behavior. The appearance of ISEs and Na^+ variable loadings along the same direction indicates the strong influence of such ISEs on the final prediction result. From the PLS1 model we have found, that the highest loadings along PC1 and, hence, the higher influence on the proper Na^+ content determination have shown Na-ISE 2, and Cl-ISE 2. The loadings of NH_4- and K-ISEs have the same sign of Na^+ loading along PC1, while anion-selective NO_3-ISE and Cl-ISE have had the loading of opposite to determined variable sign.

Concerning cheese-free gastric juice samples, no satisfactory results were achieved for analysis with single Na- and Cl-ISEs probably due to the low and similar content of Na^+ and Cl^-. On the contrary, the application of ISE array for determination of Na^+ amount in cheese-free gastric juices utilized in various TIM digestion system compartments permitted a correct prediction with correlation coefficients of 0.899 and 0.824 for calibration and validation correspondingly (data not shown). This result demonstrates the efficiency and thus, the pertinence to focus on ISE array technology for the measurement of Na^+, particularly in *in vitro* systems used to measure a wide range of ion concentrations.

3.2. Domestic cheeses digestion in artificial mouth

As above-mentioned, in humans a very first impact to food, and in particular, to its gustatory quality, occurs in oral cavity. Simultaneously to disintegration, the food digestion process also starts in mouth. From this point of view the artificial mouth, which mimics the processes occurring in human mouth may be useful in the evaluation of several food characteristics, and among them salinity, mainly determined by NaCl presence in food. In order to evaluate the release of Na^+ in the first steps of soft cheese breakdown we have monitored the NaCl content in 8 domestically prepared soft cheese samples treated with deionized water and/or averaged human saliva and diminished in artificial mouth system. It was found, that after disintegration in

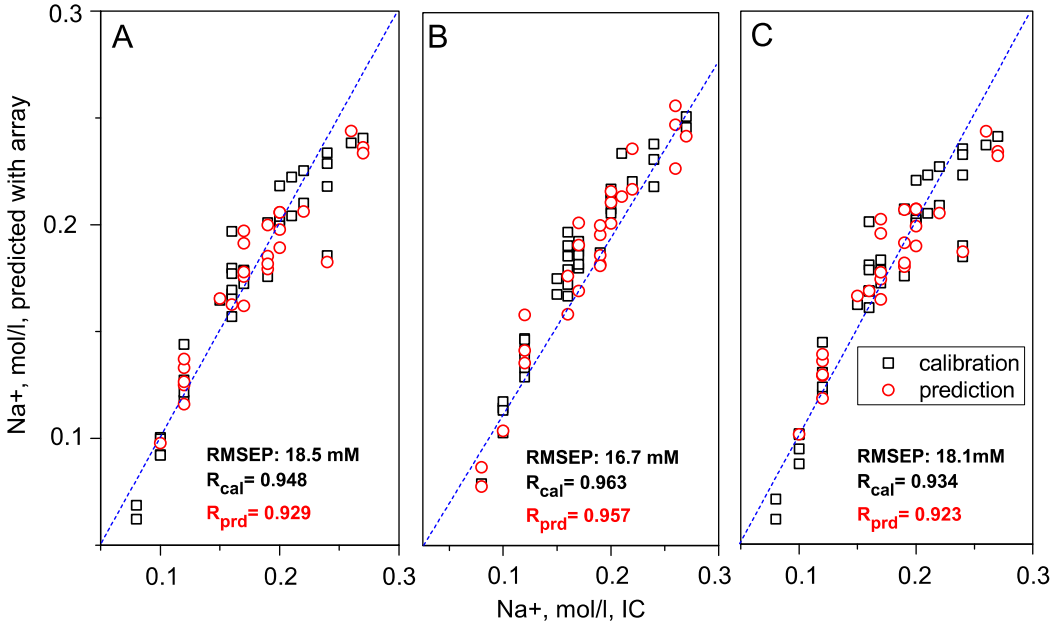

Fig. 4. The amount of Na^+ in domestic cheeses on the first steps of digestion in artificial mouth determined with sensor array and correlated to IC result by (A) PCR, (B) MLR, (C) PLS1 regression methods.

artificial mouth for 1 min of the same cheese samples treated with deionized water and averaged human saliva, the higher amount of sodium was remit from water treated samples, while in general the slower Na^+ release was registered for saliva-treated cheeses (the mean value of Na^+ determined with IC was 0.178 ± 0.054 and 0.172 ± 0.042 mol/L for water and saliva treated samples correspondingly). This slower release can be attributed to the partial absorption of Na^+ by saliva at initial steps of digestion [32].

The multisensor array previously tested for cheese breakdown monitoring in TIM-1 digester, as far as single Na-ISE 1 and Cl-ISE 2 probes were utilized to estimate Na^+ amount. For this the sensor readings were compared to IC data. Different regression methods, namely PLS1, PCR and MLR, were run on the same data set obtained from multisensory array for Na^+ determination in domestic cheese matrices in order to compare their performance and select the best procedure. As can be seen from Fig. 4, the best predictive ability expressed in the highest correlation coefficient values both for calibration and prediction procedures and the lowest RMSEP = 16.7 mM were obtained for MLR method. This indicates that the variables, i.e. the responses of sensors in array, are linearly independent. On the contrary, the difficulties in high Na^+ concentration prediction were detected for PCR and PLS1 methods.

As above mentioned, saltiness is a taste produced primarily by the presence of sodium ions. The possibility of inverse determination of Na^+ (and saltiness) content via measurement of Cl^- ions content with polycrystalline Cl-ISE 2 was checked. On the contrary to the cheese breakdown experiment in TIM-1 digester, where the HCl was added in stomach compartment in order to mimic digestion in humans, the initial digestion in oral cavity occurs at close to neutral media, and the amount of Cl^- is mainly determined by the amount of salt in a food sample, and hence, it can be correlated to the food salinity and Na^+ content. As can be seen from Fig. 5, a good correlation between Na^+ content determined by IC in domestic soft cheeses at initial steps of digestion in artificial mouth and the response of polycrystalline Cl-ISE 2 has been found. The both prediction correlation coefficient and RMSEP were higher for Cl-ISE 2 probe, 0.967 and 15.5 mM correspondingly, confirming the possibility of indirect Na^+ detection in soft cheeses with this sensor. This result is in correspondence to the formerly reported studies of cow milk saltiness undertaken by Jones and Davies [33]. The authors have showed a straight correlation between sodium and chlorine content determined in cow milk from two different breeds by gravimetric method, and, hence the possibility of indirect saltiness determination by chlorine content evaluation.

3.3. Mozzarella cheese saltiness

Inspired by the promising results on sensor array application for Na^+ monitoring in domestic cheese matrix, we then carried out the analysis of commercial samples of semi-soft Italian mozzarella cheeses. Mozzarella is short-time storage fresh cheese, traditionally produced from the cow or domestic water buffalo milk. It has a round shape and prior to the consumption is stored in the salty brine in order to preserve the spoilage as far as to enhance the cheese taste. Hence, the amount of NaCl in mozzarella brine should be high enough to ensure the longevity and proper cheese preservation. On the other hand a very high concentration of salt in brine may negatively influence the mozzarella gustatory characteristics. During the mozzarella production process NaCl is added to the "pasta filata"—a stretched and kneaded cheese mass from which the final round-shaped mozzarella pieces are then formed. According to the standards, the amount of the NaCl in final mozzarella cheese should not exceed 3.5 wt%. The amount of Na^+ arising from the milk itself varies significantly depending on the milk type (cow or buffalo correspondingly). In general, buffalo milk is saltier than cow milk. Thus for mozzarella cheese producers the determination of NaCl amount that should be added to the stretched cheese before the final product fabrication is an important challenge. Another important aspect is the discrimination among cow and buffalo mozzarella cheeses, since many cases of the more precious buffalo milk adulteration with cow milk for the production of mozzarella at high costs have been registered till now [34]. We have utilized multisensory array that recently has demonstrated its utility for Na^+ release monitoring in home-made soft cheese during the digestion process for the analysis of 24 mozzarella

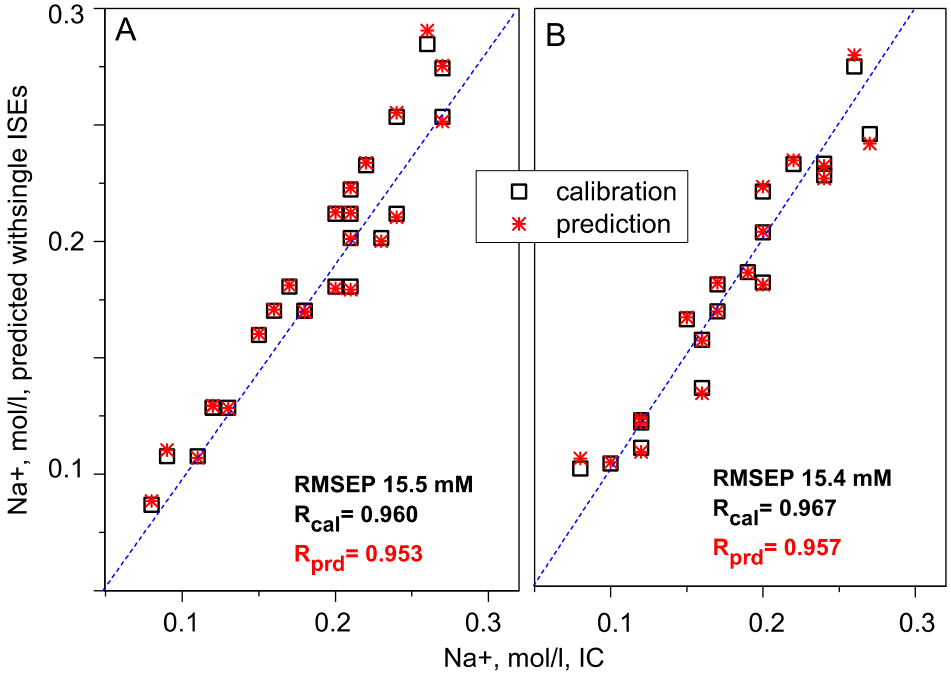

Fig. 5. The amount of Na^+ determined by IC at first steps of domestic soft cheeses digestion in artificial mouth and correlated to (A) Na-ISE1 and (B) Cl-ISE2 responses.

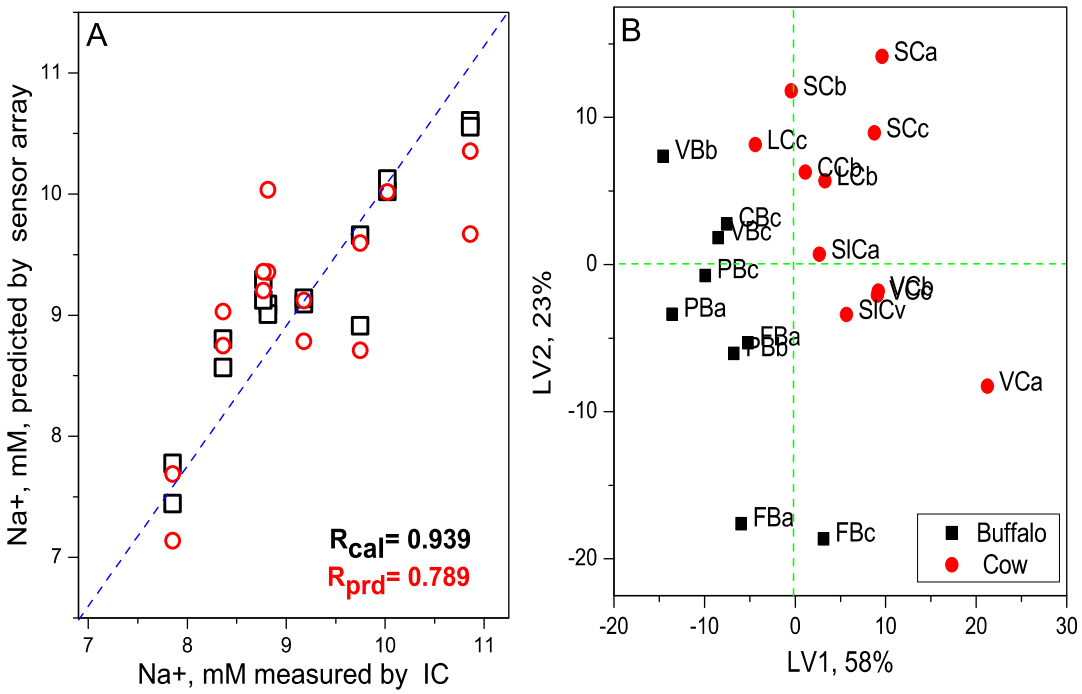

Fig. 6. Na^+ evaluation in mozzarella cheeses (A) PLS1 correlation of ISEs array response versus Na^+ concentration determined with IC; (B) PLS-DA discrimination of mozzarellas made of buffalo and cow milk.

cheeses, which were separated in 2 groups. For the first group, containing 8 mozzarella cheeses from different producers, the amount of Na^+ has been determined by IC method and correlated then to the multisensory array readings via PLS1 method. The measurements were performed in 2 replicates. As can be seen from Fig. 6A, a satisfactory correlation between predicted and measured values of Na^+ was achieved in calibration step (R=0.940, slope 0.883, offset 1.07, 6 PC), while for the leave-one-out validation procedure the lower correlation coefficient (R^2=0.789) and RMSEP=6.9 mM can be explained by the relatively small selection size (8 mozzarella samples made of cow milk were measured in 2 replicates). This preliminary result demonstrates a utility of multisensory system application for the mozzarella cheese salinity determination. The tests for the application of multisensory system for the on-line monitoring of mozzarella cheese saltiness during the industrial production process are now in progress in our laboratories.

The ability of multisensory array to discriminate between cow and buffalo milk mozzarellas was demonstrated via PLS-DA analysis of the second mozzarella cheese group. Among 16 samples, 6 were mozzarella made from the milk of water buffalo, produced in Campania region of Italy and had an official status of a protected destination of origin (PDO), while other 10 mozzarella cheeses were of "fior di latte" quality and produced from the cow milk. Three different representative parts of every mozzarella cheese were tested and utilized for the discrimination procedure, among them (a) the portion of brine solution where mozzarella was stored; (b) the liquid extract squeezed from the cheese body; and (c) directly measured cheese body where the sensors were softly pressed in. Fig. 6B and Table 2 show the results of PLS-DA analysis. The system exhibited satisfactory classification abilities towards mozzarella cheeses originating from various milk types. Thus all 6 buffalo mozzarellas were correctly classified, while among "fior di latte" samples 8 were classified correctly and 2 non-identified. In total 87.5% of mozzarella cheeses were correctly identified by ISE array according to the nature of the source milk.

Table 2
PLSDA validation of 16 mozzarella samples prepared with cow and buffalo milk.

	Real		
	Cow	Non-classified	Buffalo
Predicted			
Cow	8	2	–
Buffalo	–	–	6

4. Conclusions

In this study, we have been able, for the first time, to evaluate the sodium ion concentration with specific sensors, both in dynamic systems such as artificial mouth and gut, as far as in commercial mozzarella cheese samples. The possibility to follow the food breakdown process and the evolution of Na^+ ion concentrations in home-made cheese samples treated with saliva and gastric juice at different steps of digestion was shown. For the first time, a prototype of ISE array has been tested successfully on such samples. On comparison with single Cl^- and Na-ISEs, real better performances have been found for multisensor array application for Na^+ content determination. The possibility of satisfactory discrimination among mozzarella cheeses produced from cow and buffalo milk opens wide perspectives in mozzarella cheese quality control. In complement to the improvement of sodium detection system, we propose to develop systems able to specifically evaluate in similar conditions other characteristic compounds and to adapt the probe systems to industrial conditions in order to be able to follow in real time and on-line the release of taste components.

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