



Simultaneous determination of fermented milk aroma compounds by a potentiometric sensor array

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ABSTRACT

The paper reports on the application of an electronic tongue for simultaneous determination of ethanol, acetaldehyde, diacetyl, lactic acid, acetic acid and citric acid content in probiotic fermented milk. The α Astree electronic tongue by Alpha M.O.S. was employed. The sensor array comprised of seven non-specific, cross-sensitive sensors developed especially for food analysis coupled with a reference Ag/AgCl electrode. Samples of plain, strawberry, apple-pear and forest-fruit flavored probiotic fermented milk were analyzed both by standard methods and by the potentiometric sensor array. The results obtained by these methods were used for the development of neural network models for rapid estimation of aroma compounds content in probiotic fermented milk.

The highest correlation (0.967) and lowest standard deviation of error for the training (0.585), selection (0.503) and testing (0.571) subset was obtained for the estimation of ethanol content. The lowest correlation (0.669) was obtained for the estimation of acetaldehyde content. The model exhibited poor performance in average error and standard deviations of errors in all subsets which could be explained by low sensitivity of the sensor array to the compound. The obtained results indicate that the potentiometric electronic tongue coupled with artificial neural networks can be applied as a rapid method for the determination of aroma compounds in probiotic fermented milk.

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1. Introduction

The development of sensors with broad selectivity and high cross-sensitivity has been the main concern over the last decade [1–4]. The incorporation of these sensors in arrays combined with multivariate data processing produced a new approach in sensor technology. The analyzed samples were chemically fingerprinted, similarly as in human sense of taste where non-specific receptors (sensors) react to dissolved compounds and transfer stimuli via the nervous system to the brain, where a neural network processes the signal pattern. Thus this new approach in sensor technology was named “electronic tongue” [1]. The sensors used in arrays are mainly potentiometric [4–6], voltammetric [7–9] and conductometric [10]. The electronic tongue can be used in both qualitative [5] and quantitative [11] analysis and depending on data processing both methods are achievable from a single measurement. Thus, maybe the greatest advantage of using a sensor array is its ability to generate multivariate analytical data in real time and simultaneously permitting identification of matrix effects [12]. A wide range of traditional methodologies are used in food analysis to detect or

determine the compound characteristics of food products. These techniques are precise, accurate and reliable but at the same time destructive, time-consuming, require expensive instrumentation and are unsuitable for *in situ* or *at site* monitoring [13]. The wide application of electronic tongues [5,6,14] confirms its potential in food and beverage analysis and quality control. Sensor array data processing has an important role in the development of electronic tongues. Due to the rapidly growing computing power and faster processing of huge data sets the application of elaborate and diverse methods for data analysis has been facilitated [15]. There are many papers that report on pattern recognition techniques, multivariate data processing and the application of artificial neural networks (ANN) in processing sensor outputs [16–18]. ANN provide several advantages over classical statistical pattern recognition methods such as unified approaches for feature extraction and classification and flexible procedures for finding moderately non-linear solutions [15]. Other advantages of ANN's are the ability to handle noisy or missing data, equations are not involved, a network can deal with previously unseen data once training is completed, large numbers of variables can be included and provide general solutions with good accuracy [19]. The main characteristics of ANN is the ability to learn complex non-linear relationships between inputs and outputs, use sequential training procedures and adapt themselves to the data. ANN models attempt to use some organizational princi-

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ples in a network of weighted directed graphs in which the nodes are artificial neurons and directed edges are connections between neuron inputs and neuron outputs. It can be viewed as massively parallel computing systems consisting of an extremely large number of simple processors with many interconnections [15].

Fermented milks are accepted by the consumer because of their characteristic properties: flavor, odor, color, aroma, appearance and texture. All these sensory qualities are products of multiple fermentations. When designing a dairy product the desired sensory characteristics must be taken into account [20]. The first hours are crucial for the aroma development in freshly fermented dairy products. Lactose is metabolized by microorganisms to lactic acid and the aroma depends on the developed lactate and the byproducts of glycolysis [21]. More than 100 chemical compounds have been isolated from fermented milk and related milk products [22] but only a few (acetaldehyde, ethanol, diacetyl and acetoin) have a high impact on the desired product flavor [23].

The purpose of this paper was to evaluate the performance of the α Astree electronic tongue (Alpha M.O.S.) for rapid determination of compounds responsible for aroma development in probiotic fermented milk. The evaluation was performed using ANN models for the estimation of the aroma compounds content developed on the basis of sensor array output data. The advantages of the developed method are no sample preparation, no additional costs and short time of analysis. The disadvantage of such a method is extensive initial determination of aroma compounds content by traditional methods. The purpose of such extensive determination is to acquire enough data for reliable ANN model development for the estimation of the aroma compounds content in probiotic fermented milk.

2. Materials and methods

2.1. Samples

The analysis was performed on 40 samples of probiotic fermented milk of different flavor (plain, strawberry flavored, apple-pear flavored and forest-fruit flavored). The samples were obtained from the local market freshly arrived from the producer. The samples were stored for 20 days at two different temperatures (+4 °C and +25 °C) to obtain diverse concentrations of the analyzed aroma compounds. Hydrochloric acid ($w = 37\%$, ISO – For Analysis grade) was purchased from Carlo Erba Reagents.

2.1.1. The α Astree electronic tongue system

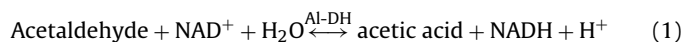
The electronic tongue was obtained from Alpha M.O.S., France. It consists of a 16-position Sample Changer and a 759 Swing Head for sampling (Metrohm, Ltd.), an interface electronic module for signal amplification and analog to digital conversion and a sensor kit, both developed by Alpha M.O.S., France, a reference Ag/AgCl electrode and a mechanical stirrer both from Metrohm, Ltd. The used sensor kit was especially developed for food analysis to ensure good sensitivity and cross-selectivity of each sensor [24]. The active electrode area of the sensors is covered by an organic coating. By variation in composition of this organic coating different sensitivity and selectivity for each sensor to various substances was obtained [25]. The kit comprises of 7 cross-selective, non-specific chemically modified field effect transistors (CHEMFETs).

CHEMFETs are ISFETs with an added membrane to provide selectivity for the desired ions. The ISFET is actually a MOSFET with the gate connection separated from the chip in a form of a reference electrode connected to the gate oxide through the aqueous solution [26]. In this perspective, ISFETs can be seen as electronic devices, as MOSFETs, with the advantage of chemically modifying the threshold voltage through the interface potential at the electrolyte/oxide interface [26].

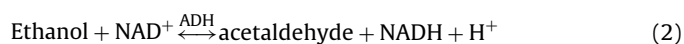
The α Astree electronic tongue was connected to a computer built according to instructions [24] with the Astree II software (Alpha M.O.S., Version 3.0.1., 2003) installed. The Astree II software automatically gathers and stores the sensors output data.

2.2. Quantification of aroma compounds

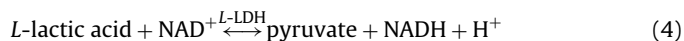
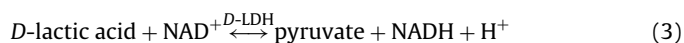
Acetaldehyde, ethanol, lactic acid, citric acid and acetic acid content were determined by enzymatic methods. Acetaldehyde oxidizes into acetic acid in the presence of aldehyde dehydrogenase (Al-DH) by nicotinamide-adenine dinucleotide (NAD) (1).



Acetaldehyde content was determined indirectly through NADH content which was measured spectrophotometrically at 340 nm. Ethanol is oxidized to acetaldehyde in the presence of the enzyme alcohol dehydrogenase (ADH) by NAD in alkaline media (2). Acetaldehyde content was again determined indirectly by NADH content at 340 nm (1).



In the presence of D-lactate dehydrogenase (D-LDH), D-lactic acid is oxidized by NAD to pyruvate. The oxidation of L-lactic acid requires the presence of the enzyme L-lactate dehydrogenase ((3) and (4)).



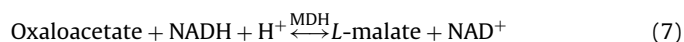
The equilibrium of these reactions lies completely on the side of lactic acid. However, by trapping the pyruvate in a subsequent reaction catalyzed by the enzyme glutamate-pyruvate transaminase (GPT) in the presence of L-glutamate, the equilibrium can be displaced in favor of pyruvate and NADH (5).



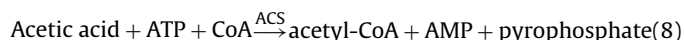
The amount of NADH formed in the above reactions is stoichiometric with the amount of D-lactic acid and L-lactic acid, respectively. NADH was determined by its absorbance at 340 nm. Citric acid is converted to oxaloacetate and acetate in the reaction catalyzed by the enzyme citrate lyase (CL) (6).



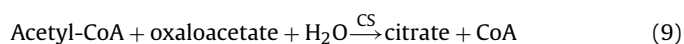
In the presence of the enzymes, malate dehydrogenase (MDH) and L-lactate dehydrogenase (L-LDH), oxaloacetate and its decarboxylation product pyruvate are reduced to L-malate and L-lactate, respectively, by NADH ((7) and (4)).



The amount of NADH oxidized in reactions (4) and (7) is stoichiometric with the amount of citrate. NADH is again determined by its absorbance at 340 nm. Acetic acid is converted in the presence of the enzyme acetyl-CoA synthetase (ACS) with adenosine-5'-triphosphate (ATP) and coenzyme A (CoA) to acetyl-CoA (8).



Acetyl-CoA reacts with oxaloacetate to citrate in the presence of citrate synthetase (9).



The oxaloacetate required for reaction (9) is formed from malate and NAD in the presence of malate dehydrogenase (7). In reaction (7) NAD is reduced to NADH. The determination is based on the increase of NADH absorbance at 340 nm [27]. In the presence of

alkali, creatine and alpha-naphthol, diacetyl forms a compound that results in a red solution, the intensity of which can be measured colorimetrically. Diacetyl content was determined using a calibration curve obtained by applying the same procedure to diacetyl standard solutions ranging from 5 ppm to 25 ppm [28].

2.3. Potentiometric measurements

Ten samples were analyzed by the sensor array every 5th day through a period of 20 days. Every sample was measured 3 times and a total of 120 measurements were performed. Conditioning of the potentiometric sensor array with probiotic fermented milk was performed prior to each analysis session. The conditioning of the sensor array consisted of analyzing plain probiotic fermented milk until the response of each sensor remained unchanged through 3 analysis cycles, each cycle lasting 300 s. The measuring procedure was described elsewhere [29]. The sensor array was rinsed with deionized water for 30 s between measurements. The samples were analyzed at +25 °C. Hydrochloric acid diluted in deionized water (0.01 mol/L) was analyzed as a reference sample together with fermented milk samples to follow and later correct the sensors drift in time.

2.4. Data analysis

The sensor outputs collected by the Astree II software (Alpha M.O.S.) were imported to Microsoft Excel (Microsoft Excel 2002, SP-2) and centered. Centering, a popular data transformation technique, aims to translate the multivariate data cloud to the data center. The operation can be expressed as:

$$x_c = x - \mu(X)$$

where x_c represents a column-wise centered variable of data X [30]. The sensor drift correction was performed using data obtained by the analysis of the reference sample (hydrochloric acid, 0.01 mol/L). The corrected sensor outputs were obtained by subtracting each sample's sensor outputs with a proprietary reference sample sensor outputs. Statistica 7.1 (StatSoft, Inc., 2005) was used for the development of artificial neural network (ANN) models for rapid determination of aroma compounds concentrations in probiotic fermented milk by the potentiometric sensor array. Broadly, a neural network is a collection of interlinked neurons that incrementally learn from the environment (data) to capture essential linear and non-linear trends in complex data so that it provides reliable predictions of new situations, even with partial information [31]. The power of these networks comes from the hidden layer of neurons located between the input and output layer of neurons. The hidden layer may consist of one or many non-linear neurons, and more importantly, it performs continuous, non-linear transformations of the weighted inputs in contrast with the linear mapping used in the linear neuron and step function mapping used in the perceptron [31]. Training algorithms used in the development of the models involve an iterative procedure for minimization of an error function, with adjustments to the weights being made in a sequence of steps. In each step there are two stages. In the first stage, errors are propagated backwards through the network and in the second stage the derivatives are then used to compute the adjustments to be made to the weights. The simplest method for computing the adjustment involves gradient descent [32]. In the development of each ANN model 120 sensor array measurements were used. The measurements were randomly assigned to 3 subsets as follows: 60 measurements for the training subset, 30 measurements for the selection subset and 30 measurements for the testing subset. The training subset was used to train the ANN model, the role of the selection subset was to provide internal val-

idation of the model and the testing subset for external validation of the obtained model. One of the most desirable features of a neural network model is to generalize data well. To avoid overfitting problems, the select and test errors were monitored during training and testing of neural networks with the aim to obtain a model with similar or lower select and test errors than the train error.

An initial screening of ANN models was performed for each fermented milk aroma compound. The screened models employed linear, radial basis function or multi-layer perceptron architectures. Multi-layer perceptrons were selected in all cases for further ANN model development because of their better initial performance in estimating the content of fermented milk aroma compounds. Further training of the models was performed to obtain an optimal number of hidden neurons without exhausting the degree of freedom of the system. Ultimately the ANN models for the estimation of aroma compound content were chosen according to prediction accuracy and minimal number of input and hidden neurons to keep the model as simple as possible. All of the trained ANN models employed a hyperbolic transfer function to produce the output of hidden neurons. The ANN model for the determination of ethanol in probiotic fermented milk had the architecture of a multi-layer perceptron with 6 neurons in the input layer, 4 neurons in the hidden layer and 1 neuron in the output layer. The network was trained by gradient descent algorithm for 100 epochs, followed by 146 epochs of conjugate gradient algorithm. The architecture of the ANN model for the determination of acetic acid concentration in probiotic fermented milk was a multi-layer perceptron with 7 neurons in the input layer, 3 neurons in the hidden layer and 1 neuron in the output layer. The network was trained by gradient descent algorithm for 100 epochs, followed by 58 epochs of conjugate gradient algorithm. The ANN model for rapid estimation of citric acid concentration in probiotic fermented milk had the architecture of a multi-layer perceptron, with 7 neurons in the input layer, 5 neurons in the hidden layer and 1 neuron in the output layer. The network was trained by 100 epochs of gradient descent algorithm followed by 33 epochs of conjugate gradient algorithm. The architecture of the ANN model for the prediction of lactic acid concentration values in probiotic fermented milk was a multi-layer perceptron, with 7 neurons in the input layer, 4 neurons in the hidden layer and 1 neuron in the output layer. The model was trained by 100 epochs of gradient descent algorithm followed by 27 epochs of conjugate gradient algorithm. The ANN model for the determination of diacetyl concentration had a profile of a multi-layer perceptron, with 7 neurons in the input layer, 5 neurons in the hidden layer and 1 neuron in the output layer. The model was trained by gradient descent algorithm for 100 epochs, followed by 71 epochs of conjugate gradient algorithm. The architecture of the ANN model for the determination of acetaldehyde concentration values in probiotic fermented milk was a multi-layer perceptron, with 2 neurons in the input layer, 4 neurons in the hidden layer and 1 neuron in the output layer. The model was trained for 100 epochs of gradient descent algorithm followed by 27 epochs of conjugate gradient algorithm.

3. Results and discussion

The results obtained from classical analysis of the samples and the data acquired by the electronic tongue were used to create ANN regression models for simple and rapid determination of aroma components in probiotic fermented milk by the potentiometric sensor array. The plots of observed and predicted concentration values of the aroma compounds of probiotic fermented milk with their respective correlations are shown in Fig. 1. Table 1 shows the average errors, standard deviations of errors and correlations of the training, selection and testing subsets of the models.

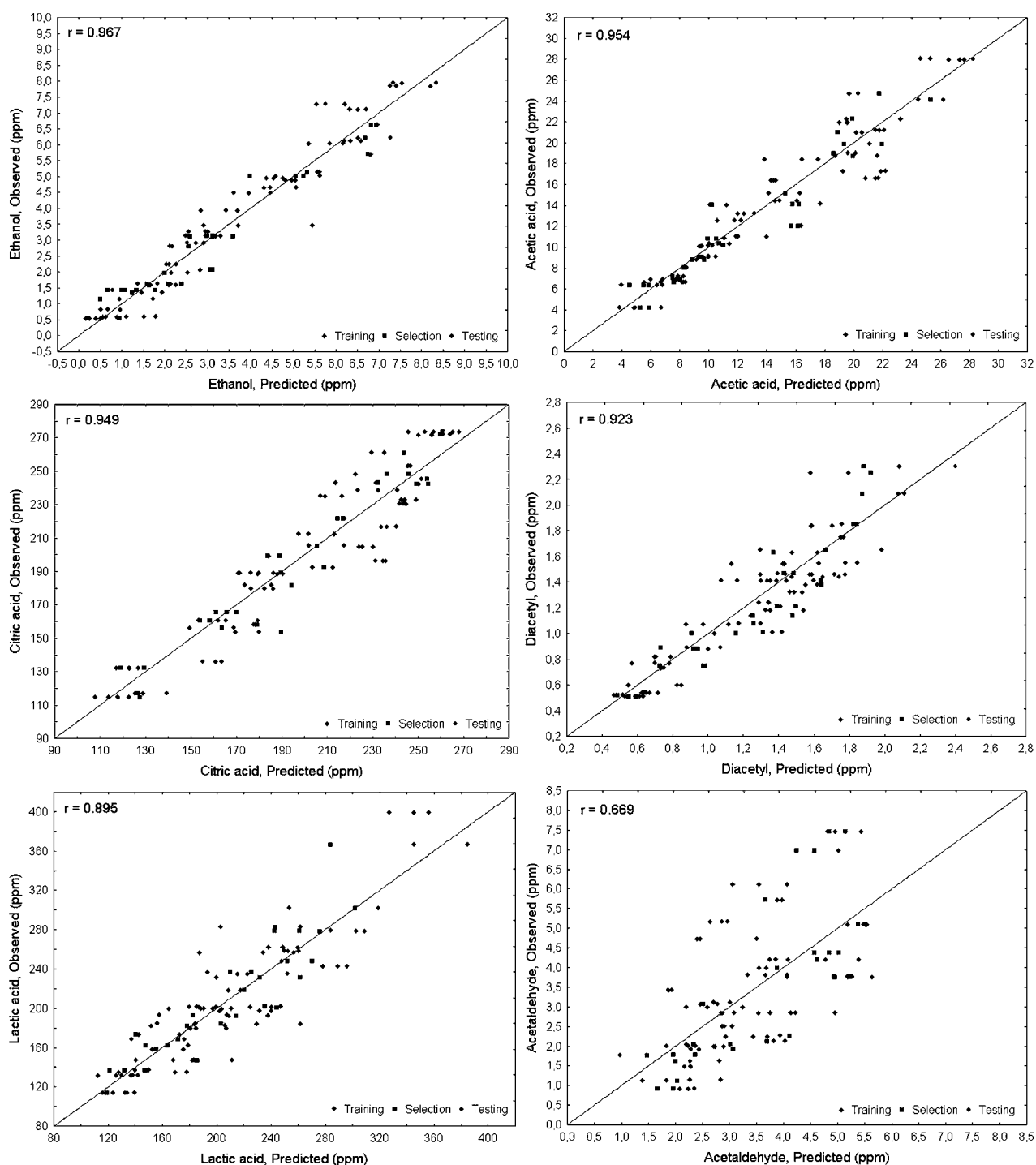


Fig. 1. ANN regression between the potentiometric sensor array and aroma compound content in probiotic fermented milk, number of replicas $n=3$, number of probiotic fermented milk samples $N=40$.

The obtained ANN model for the determination of ethanol in probiotic fermented milk had a train error of 0.079, select error of 0.069 and test error of 0.078. The correlation between the observed and predicted concentration values of ethanol was 0.967 (Fig. 1), with correlations of 0.966, 0.968 and 0.970 for the training, selection and testing subsets, respectively (Table 1). The average errors were -0.003 , 0.084 and -0.092 and the standard deviations of errors were 0.585, 0.503 and 0.571 for the training, selection and testing subsets, respectively (Table 1). The model exhibited great generalization capability with excellent correla-

tion between the observed and predicted concentration values of ethanol in probiotic fermented milk samples with low average errors and low standard deviation of errors. The results imply that the potentiometric sensor array can be used to successfully predict ethanol content in probiotic fermented milk (from 0.5 ppm to 8 ppm). Legin et al. assessed the quality of ethanol, vodka and eau-de-vie. The sensor array used in the research was capable of distinguishing between synthetic and alimentary grain ethanol as well as alimentary ethanol with different degrees of purification [33].

Table 1
Descriptive statistics of the ANN models for rapid determination of aroma compounds content in probiotic fermented milk.

	Data mean (ppm)	Data S.D. (ppm)	Error mean (ppm)	Error S.D. (ppm)	Abs E. mean (ppm)	S.D. ratio	Correlation
Ethanol							
Training	3.761	2.275	−0.003	0.585	0.427	0.257	0.966
Selection	2.900	1.838	0.084	0.503	0.393	0.274	0.968
Testing	3.719	2.359	−0.092	0.571	0.476	0.242	0.970
Acetic acid							
Training	15.492	6.525	−0.004	2.117	1.631	0.324	0.946
Selection	12.537	5.991	0.255	1.733	1.358	0.289	0.957
Testing	12.055	6.295	0.373	1.871	1.272	0.297	0.958
Citric acid							
Training	196.472	50.841	−0.025	16.479	13.729	0.324	0.946
Selection	196.574	46.315	1.014	12.119	9.828	0.262	0.966
Testing	201.940	45.582	2.639	16.007	13.643	0.351	0.942
Diacetyl							
Training	1.204	0.505	−0.002	0.195	0.147	0.387	0.922
Selection	1.188	0.514	0.041	0.185	0.150	0.360	0.935
Testing	1.276	0.411	0.082	0.159	0.144	0.387	0.931
Lactic acid							
Training	215.811	67.844	2.369	32.950	26.080	0.486	0.874
Selection	207.449	59.055	−1.782	25.503	18.259	0.432	0.902
Testing	187.439	54.389	1.742	20.147	15.167	0.370	0.930
Acetaldehyde							
Training	3.248	1.778	0.015	1.320	1.079	0.742	0.672
Selection	3.462	1.990	0.185	1.295	1.056	0.651	0.767
Testing	3.492	1.342	−0.001	1.233	0.973	0.919	0.538

The train error of the ANN model for the determination of acetic acid concentration in probiotic fermented was 0.089, the select error 0.073 and the test error 0.080. The correlation between the observed and predicted concentration values of acetic acid in probiotic fermented milk was 0.954 (Fig. 1). The training subset achieved a correlation of 0.946, the selection subset 0.957 and the testing subset 0.958 (Table 1). The average errors were −0.004, 0.255 and 0.373 and the standard deviations of errors were 2.117, 1.733 and 1.871 for the training, selection and testing subsets, respectively (Table 1). The ANN model for the determination of acetic acid concentration in probiotic fermented milk by the potentiometric sensor array had excellent generalization capability which is represented by the low select and test errors (0.073 and 0.080, respectively). The correlation between the observed and predicted acetic acid concentration values (0.954), the average errors and standard deviations of errors of the ANN model show that the potentiometric sensor array is capable of successfully detecting and quantifying small concentrations of acetic acid (from 4 ppm to 29 ppm). Turner et al. combined an electronic tongue with multivariate data processing to monitor batch *Escherichia coli* fermentation [34]. The electronic tongue (comprising of 23 potentiometric sensors) was able to detect and successfully quantify low acetic acid concentrations (from 0.1 g/L to 6.4 g/L) with an average error of 11%. Verelli et al. investigated the sensitivity of potentiometric sensors to small concentrations of acetic acid developed in white dry Italian wines [35]. The Authors achieved excellent correlation of 0.9947 for the training set and 0.9799 for the validation set between the observed and predicted concentration values of acetic acid in the calibration model (the concentration ranged from 0.025 g/100 mL to 0.060 g/100 mL of acetic acid). The obtained relative errors (from 3.0% to 17.5%) for the wine samples showed that the potentiometric sensors used are very sensitive even to small concentrations of acetic acid.

The obtained train, select and test errors of the ANN model for rapid estimation of citric acid concentration in probiotic fermented milk were 0.104, 0.077 and 0.102, respectively. The correlation between the observed and predicted citric acid concentration values was 0.949 (Fig. 1). The training, selection and testing subsets achieved correlations of 0.946, 0.966 and 0.942, respectively (Table 1). The average errors were −0.025, 1.014 and 2.639, while the standard deviations of errors were 16.479, 12.119 and 16.007

for the training, selection and testing subset (Table 1). Buellens et al. evaluated a potentiometric electronic tongue with 27 sensors for the determination of acid and sugar profiles of tomato juices from different cultivars [11]. Among acids, the authors investigated the performance of the electronic tongue for the determination of citric acid concentration in artificial juice and tomato juice. The obtained correlations with PLS regression for the artificial juice (0.99 for the calibration set and 0.98 for the validation set) and tomato juice (slope of 0.60) indicate that the applied electronic tongue is capable of quantifying citric acid content to a satisfying degree [11]. The developed ANN model for the prediction of citric acid concentration values in probiotic fermented milk showed excellent correlation between the observed and predicted concentration values, low average errors and standard deviations of errors which indicate excellent sensitivity of the potentiometric sensor array to citric acid content in the investigated concentration range (from 110 ppm to 270 ppm).

The train error of the ANN model for the determination of diacetyl concentration was 0.109, the select error 0.106 and the test error 0.100. The ANN model obtained a correlation of 0.923 between the observed and predicted diacetyl concentration values in probiotic fermented milk (Fig. 1). The correlation of the training subset was 0.922, of the selection subset 0.935 and of the testing subset 0.931 (Table 1). The average errors of the models training, selection and testing subsets were −0.002, 0.041 and 0.082, respectively (Table 1). The standard deviations of errors were 0.195, 0.185 and 0.159 for the training, selection and testing subsets, respectively (Table 1). The ANN model for the prediction of diacetyl concentration values had low average errors and standard deviations of errors and obtained a good correlation between the observed and predicted diacetyl concentration values in probiotic fermented milk. This implies that the potentiometric sensor array could be used to predict diacetyl content in probiotic fermented milk in the given range (from 0.4 ppm to 2.4 ppm).

The train, select and test errors of the ANN model for the prediction of lactic acid concentration values in probiotic fermented milk were 0.116, 0.090 and 0.071, respectively. The correlation between the observed and predicted lactic acid concentration values was 0.895 (Fig. 1). The correlation of the training subset was 0.874, of the selection subset 0.902 and of the testing subset 0.930 (Table 1). The average errors were 2.369, −1.782 and 1.742 with the standard

deviations of errors being 32.950, 25.503 and 20.147 for the training, selection and testing subsets, respectively (Table 1). Esbensen et al. reported on the performance of an electronic tongue with 30 non-specific chemical sensors for monitoring batch fermentation process of starting culture for light cheese production [36]. The electronic tongue predicted the lactic acid concentration with a relative error of 10% during the training of the ANN model. The test samples obtained relative errors ranging from 2% to 25%, while the concentration values ranged from 0.99 g/L to 11.18 g/L [36]. According to the developed ANN model for the determination of lactic acid concentration in probiotic fermented milk the potentiometric sensor array exhibited good sensitivity and quantification capability for the given concentration range of lactic acid content (from 100 ppm to 400 ppm).

The train, select and test errors of the ANN model for the determination of acetaldehyde concentration values in probiotic fermented milk were 0.201, 0.200 and 0.189, respectively. The correlation between the observed and predicted acetaldehyde concentration values in probiotic fermented milk was 0.669 (Fig. 1). The obtained correlations for the training, selection and testing subsets were 0.672, 0.767 and 0.538 (Table 1). The models average errors were 0.015, 0.185 and -0.001 with standard deviations of errors of 1.320, 1.295 and 1.233 for the training, selection and testing subsets, respectively (Table 1). This model had significant standard deviations of errors and low correlation between the observed and predicted values of acetaldehyde concentration values in probiotic fermented milk. This could be due to low sensitivity of the sensors to the compound.

4. Conclusions

The developed model for ethanol concentration determination in probiotic fermented milk showed the best prediction capability with low error of prediction. The ANN models for acetic acid, citric acid, lactic acid and diacetyl concentration determination also exhibited good prediction capability with slightly higher prediction errors. The model for acetaldehyde determination exhibited low accuracy of prediction which was most likely caused by low sensitivity of the potentiometric sensor array to acetaldehyde. The potentiometric sensor array exhibited great potential as a tool in rapid determination of aroma compounds in probiotic fermented milk.

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