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Multicomponent analysis of fermentation growth media using the electronic tongue (ET)

A. Legin [a], D. Kirsanov [a,*], A. Rudnitskaya [a], J.J.L. Iversen [b], B. Seleznev [a], K.H. Esbensen [c], J. Mortensen [d], L.P. Houmøller [c], Yu. Vlasov [a]

[a] *Laboratory of Chemical Sensors, Chemistry Department, St. Petersburg University, Universitetskaya nab. 79, Mendeleev Center, 199034 St. Petersburg, Russia*
[b] *University of Southern Denmark, Denmark*
[c] *Applied Chemometrics, Analytical Chemistry, and Sampling Research Group (ACACSRG), Aalborg University Esbjerg, Esbjerg, Denmark*
[d] *Department of Life Science and Chemistry, Roskilde University Centre, Denmark*

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Abstract

A potentiometric electronic tongue (ET) consisting of eight cross-sensitive chemical sensors and a standard pH electrode has been applied for analysis of simulated fermentation solutions typical for fermentation processes with *Aspergillus niger*. The electronic tongue has been found capable of simultaneous determination of ammonium, citrate and oxalate in complex media with good precision (typical error within 8%). The system preserved high sensitivity to the targeted substances also in the presence of sodium azide, which is commonly used for suppressing microbial activity in real-world fermentation samples. Sensor performance was fast and reproducible which promises well for routine application of the electronic tongue for fermentation process monitoring.
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1. Introduction

Different kinds of fermentations are widely used in industrial scale in production of food, beverages, enzymes, organic acids, pharmaceuticals, biogas, etc. Various types of living organisms are utilized for fermentation processes with filamentous fungi or moulds of special importance. These organisms are widespread in nature, and some species of filamentous fungi have been used for thousands of years to give taste and durability to the food products, e.g. for production of Asian fermented foods, such as soy sauce and sake, and various mould cheeses.

Filamentous fungi are interesting for the biotechnological industry due to their special properties such as a complex life cycle, cell differentiation, and an efficient system for secretion of proteins. Nowadays filamentous fungi are widely used in biotechnological production of enzymes, organic acids and pharmaceuticals. Some of the most commonly used species are *Aspergillus niger* for production of citric acid [1], *Aspergillus oryzae* for production of heterologous α-amylases [2] and *Penicillium Chrysogenum* for production of penicillin [3].

Continuous monitoring of biotechnological processes is urgently needed for improving efficient management and optimization as well as for quality control with the aim of high quality final products. However, despite the current rapid development of the biotechnology industry, cost effective, real-time and convenient process monitoring means are still lacking. Currently used analytical equipment, such as HPLC, GC, IR, or UV instruments, is expensive. Analyses by these methods are often time-consuming, demand experienced operators and, as a rule, do not provide a comprehensive picture of a process.

* Corresponding author. Tel.: +7-812-328-28-35; fax: +7-812-328-28-35.
E-mail address: dk@mail333.com (D. Kirsanov).
URL: http://www.electronictongue.com.

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Of the promising analytical tools for such applications are the 'electronic tongue' multisensor systems (ET). The idea of the electronic tongue is based on utilization of *arrays* of cross-sensitive chemical sensors, particularly potentiometric, combined with multivariate data analysis. This approach gives an opportunity of performing simultaneous quantitative determination of various substances in multi-component media as well as of detailed monitoring (follow-up) of industrial processes. The key advantages of the electronic tongue are rapid and relatively simple operation, low cost, and an option of simultaneous determination of several components and/or parameters of the media. An electronic tongue system based on potentiometric chemical sensors already proved to be useful in numerous applications including food analysis [4] and monitoring of fermentation processes, namely model batch fermentation of *E. coli* [5] and fermentation of cheese starter culture [6].

In the present work, an electronic tongue based on potentiometric cross-sensitive sensors was applied for the analysis of model growth media closely resembling real-world fermentation samples. Measurements were made in solutions modeling the composition of media where *A. niger* are typically grown. Primary attention was paid to determination of the content of ammonium and organic acids in the media. During fungi growth, different changes of medium content and concentrations of nutrients take place. Fungi consume ammonium and glucose and produce organic acids, e.g. citrate, oxalate, and other substances such as polyols. Monitoring of the presence and changing content of key compounds may be important in practice for identifying the current state of the fermentation, realistic yields, and specific growth rates, and will also be useful for correct determination of optimal harvest times. The present study is a step forward to applying the electronic tongue multisensor system for monitoring of industrial biotechnological processes.

2. Experimental

The ET sensor array in this experiment comprised eight potentiometric chemical sensors based on PVC-plasticized membranes with enhanced cross-sensitivity, particularly to inorganic cations and anions of organic acids. Details of the sensors' compositions and preparation were published elsewhere [7–11]. A set of cationic sensors based on different cation-exchangers was employed. Standard ammonium-selective electrode based on nonactine (e.g. [15] and references therein) was used throughout the experiments for comparison. Several sensors based on different quaternary ammonium salts [10] exhibiting sensitivity to various anions and also to organic acids' anions were included into the sensor array. A standard pH glass electrode was constantly used in all measurements to control the pH in the solutions. Sensors were washed with distilled water between measurements until they reached steady potential that was constant within 10 mV for each sensor. Potentiometric measurements with the sensor array were performed with a custom-made multi-channel digital voltmeter with high input impedance connected to a PC for data acquisition. Responses of sensors of the array were measured versus a Ag/AgCl reference electrode with double liquid junction.

The compositions of the model solutions closely matched averaged results of independent analysis of broths from real batch fermentations of *A. niger* in a minimal medium. Broths were sampled and analyzed during fungi growth from inoculation to stationary phase (approximately 22 h) and analyzed by standard analytical methods such as ion chromatography, HPLC, etc. Then, these data were used for preparation of the set of current *model solutions* with very similar content for all components. The component concentrations are listed in the Table 1. The chemicals used for solutions preparation were of analytical grade. All simulated solutions contained a background of $0.5\,g\,l^{-1}$ KCl, $1.5\,g\,l^{-1}$ KH_2PO_4, $0.5\,g\,l^{-1}$ $MgSO_4$ and 1 ml of Vishniac trace element solution [12] that contains trace elements such as copper, zinc, manganese, etc. that are essential for the microorganisms' growth. It is routinely added to the growth media in different biotechnological processes. Solutions also contained varying quantities of ammonium ions and glucose, which are considered as feed for fungi, and citric, oxalic and pyruvic acids. Furthermore, the solutions contained glycerol, mannitol and erythriol which are typical metabolites produced by fungi during their growth. pH of all the solutions was adjusted to 6 by the addition of NaOH.

Three experimental sessions were carried out. In the first session, which served as a feasibility study, 22 model solutions were prepared containing all components except pyruvate, mannitol and erythritol. The solutions of the second session contained all substances listed in Table 1. In the third experimental session even more complicated samples were prepared. They again contained all components (Table 1) and also 10 mM of *sodium azide*, which is commonly used for stopping microbial activity in growth media after sampling. Sodium azide was added to all solutions to verify its interference on the results produced by the electronic tongue. Three replica measurements in each solution within each experimental session were made. All samples were stored in the laboratory refrigerator between measurements and warmed up to room temperature just before analysis.

The electronic tongue was calibrated with respect to ammonium, oxalate and citrate prior to measurements in model growth media. This was done in order to evaluate the sensitivity of ET to each of these substances in the presence of typical ionic and organic background. Calibration measurements were made in the concentration range from 0.01 to 100 mM for each substance on the background of minimal medium solution (2 mM $MgSO_4$, 7 mM KCl, 11 mM KH_2PO_4) and also 30 mM of glucose. pH of all calibration solutions was adjusted to 6.

Data processing consisted of *multivariate calibration* with respect to organic acids and ammonium concentrations using the sensors responses (potential values versus

Table 1
Full set of solutions simulating growth media of real fermentation processes involving *A. niger*

Sample no.	Time	Citrate (mM)	Pyruvate (mM)	Oxalate (mM)	Glucose (mM)	Glycerol (mM)	Mannitol (mM)	Erythritol (mM)	NH_4Cl (mM)
1	0	0	0	0	45.3	0.14	0.05	0	14.0
2	1.8	0	0	0	45.3	0.17	0.07	0	14.0
3	5.3	0	0	0	44.0	0.24	0.05	0	13.6
4	7.9	0.5	0	0	42.7	0.35	0.05	0.02	13.2
5	10.5	1.4	0	2.6	41.4	0.41	0.05	0.03	12.8
6	11.6	1.7	0	7.8	38.9	0.52	0.03	0.05	12.0
7	12.6	2.2	0	10.4	36.3	0.59	0.03	0.07	11.2
8	13.7	2.6	0	13.0	33.7	0.69	0.03	0.10	10.4
9	14.7	3.3	0	18.1	29.8	0.76	0.05	0.14	9.2
10	15.3	3.6	0	20.7	27.2	0.86	0.05	0.16	8.4
11	15.8	3.8	0	23.3	25.9	0.93	0.07	0.19	8.0
12	16.3	4.0	0	25.9	23.3	1.04	0.09	0.24	7.2
13	16.8	3.8	0	28.5	20.7	1.10	0.10	0.26	6.4
14	17.1	3.8	0	28.5	19.4	1.17	0.10	0.28	6.0
15	17.4	3.8	0	31.1	18.1	1.28	0.12	0.29	5.6
16	17.9	3.8	0	33.7	15.5	1.38	0.13	0.31	4.8
17	18.4	4.0	0	38.9	13.0	1.48	0.17	0.40	4.0
18	18.9	4.3	0	44.0	9.1	1.59	0.21	0.45	2.8
19	19.5	4.7	0.3	46.6	6.5	1.66	0.22	0.47	2.0
20	20	5.0	1.6	49.2	5.2	1.73	0.28	0.52	1.6
21	20.5	5.3	2.6	59.6	1.3	1.90	0.40	0.60	0.4
22	21.1	5.5	2.4	62.2	0	1.90	0.47	0.62	0

The time column shows real time, corresponding to sampling points of real growth media, which composition was simulated in this work.

reference electrode). Two methods were employed for this purpose: Partial Least Squares (PLS) regression and Back-Propagation Artificial Neural Network (ANN). ANN with six input, two hidden and one output neurons was used for the ammonium determination. ANNs with eight input, two hidden and one output neurons were used for citrate and oxalate determination and growth time prediction. A hyperbolic tangent transfer function was used in all cases. Sensor responses were used directly as input data to ANN. Calibration models were made separately for each component. All calibration models were validated using independent test set validation [13]. All data were centred before modelling (both in PLS and ANN). Data were also scaled to lay in the [−1, 1] interval before ANN calculations.

The experimental data sets were separated into calibration and test sets (and also monitoring set in the case of ANN) in two different ways. For ANN, the first and second replica measurements in each of three experiments were randomly divided into calibration and monitoring data sets. For PLS regression, the first and second replicas were used for calibration, while the third measurement data were used as the independent test set for both ANN and PLS. In the framework of the second approach, all replicas of seven randomly selected samples were collected as the test set, while all other data were used for calibration (for PLS) or split randomly into calibration and monitoring data sets (for ANN training). The results obtained for the two test data sets were very similar.

The commercial software Unscrambler v. 7.8 by CAMO and NeuroSolutions by NeuroDimensions Inc. was used for PLS regression and ANN modeling, respectively.

3. Results and discussion

Initially, the sensitivity and selectivity of ET to ammonium and organic acids was evaluated in the presence of typical background inorganic compounds for chosen type of fermentation. The method of evaluation of detection limits and selectivity for the sensor arrays was suggested and described in [14]. Also, a comparison of selectivity of the sensor array with that of discrete sensors was performed. For this purpose calibrations of the sensor array with respect to ammonium, oxalate and citrate were made. The “minimal medium” containing inorganic salts and 30 mM of glucose with pH adjusted to 6 was used as background. It was observed earlier [10] that sensors based on quaternary ammonium salts display sensitivity to both oxalic and citric acids with detection limit of 5 μM. On the minimal medium background detection limit of these sensors increased up to 0.1 mM towards oxalic and to 0.25 mM towards citric acids. This is likely due to the interference of chloride, which is present in the minimal medium in concentration of 7 mM. Detection limit of the sensor array on the minimal medium background was found being 0.08 mM for oxalate and 0.12 mM for citrate. Detection limit of discrete sensors based on quaternary ammonium salts in the minimal medium are low enough to enable determination of oxalate and citrate. It must be noted that all studied anion-sensitive sensors respond to both organic acids with comparable selectivity and, hence, will produce mixed response in solutions containing these two acids simultaneously. Thus, they cannot be used for detection of these acids as discrete sensors. Nevertheless, being incorporated into the array, these sensors

can be used successfully for simultaneous organic acids' determination.

The detection limit of both sensor array and discrete ammonium-selective sensor to ammonium in water was estimated to be lower than 10 μM. The situation in simulated fermentation media was very different—the detection limit of the sensor array to ammonium was found being 0.2 mM whilst it increased to 0.8 mM for discrete ammonium-selective electrodes. The observed ammonium detection limit increase might be related to noticeable interference of potassium, which was present in the minimal medium at the concentration level of 18 mM. According to the literature data [15], logarithm of a selectivity coefficient of the ammonium-selective electrode to potassium determined by separate solutions method (SSM) is about −1. However, in the multi-component solutions containing also sodium and magnesium, besides potassium, selectivity to ammonium of the discrete sensor does not fit with SSM results. Experimentally measured ammonium detection limit for the discrete sensor was twice higher than lowest concentration of ammonium to be detected. Moreover, the response of this sensor to ammonium in the model growth medium is non-linear almost in the whole range of ammonium concentrations as shown in Fig. 1. Thus, a discrete standard ammonium-selective sensor cannot be used for quantitative ammonium determination in the growth medium.

On the other hand, the lowest concentration of ammonium in the growth medium (Table 1) was comparable to the detection limit of the sensor array. Furthermore, it is not a problem for the sensor array if the response is non-linear. Therefore, the electronic tongue could be reliably used for ammonium determination under these conditions. It is important to note that utilization of array of cross-sensitive sensors enables significant improving of selectivity and detection limit in comparison with discrete sensors that is in full agreement with previously published results [14].

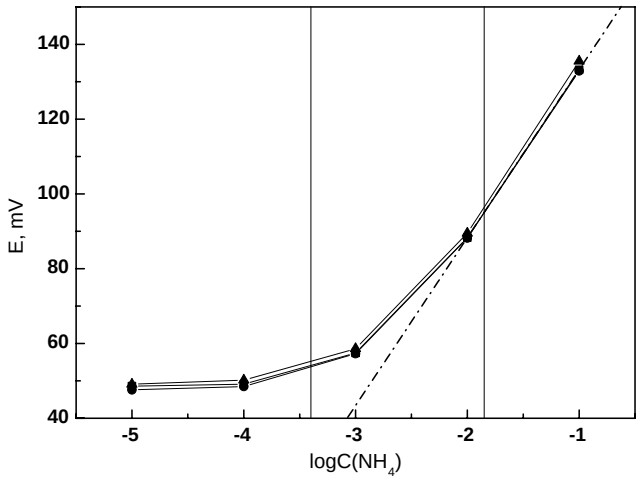

Fig. 1. Response of nonactine-based ammonium-selective electrode to ammonium ions on the minimal medium background. Theoretical linear response of the electrode is shown by the dashed line. Targeted range of ammonium in the growth medium is delineated by the vertical lines.

Table 2
Average relative errors of prediction (%) of ammonium, oxalate and citrate content in simulated fermentation solutions of the first and second experimental sessions

Calibration method	Ammonium	Oxalate	Citrate	Experimental sessions
PLS	12	6	11	First
	10	6	10	Second
ANN	5	5	8	First
	6	6	8	Second

Data were processed using PLS-regression and back-propagation ANN. Average relative error of prediction was calculated according to the formula: $\mathrm{AVR} = \sum_n |C_{\mathrm{real}} - C_{\mathrm{pred}}/C_{\mathrm{real}}|/n$, where n is a number of samples in the test set.

The sensors of the array produced reproducible responses despite being permanently subjected to significant interferences from various components of background solutions at rather high concentrations. Typical sensor potential reproducibility in replicated measurements was in the range of 1–4 mV depending on the sensor type.

After sensitivity study, the first experimental session with simulated solutions containing all components except pyruvate, mannitol and erythritol was performed. During the second experimental session measurements in simulated fermentation solutions containing all substances listed in Table 1 were carried out. The average relative errors of determination of ammonium, oxalate and citrate for the first and the second experimental sessions are shown in Table 2. Comparable results were obtained for both sessions. Thus, no significant interference from pyruvate, mannitol and erythritol on the sensors' response was observed, which is explained by low sensitivity of currently used sensing materials of electronic tongue to polyols.

Two multivariate techniques were employed for sensor array calibration: PLS-regression and back-propagation ANN. No difference between the two methods for oxalate content prediction was observed. However, ANN performed somewhat better in predicting concentrations of ammonium and citrate in comparison with PLS-regression (Table 2). This may mean that the sensors' responses to ammonium and citrate were non-linear. Non-linearity of the data can be evaluated using plots of hidden neurons' activations or outputs versus their inputs. Inputs in this case were summed outputs of neurons of the input layer. The hyperbolic tangent, which was used as a transfer function, has a strongly non-linear part and a nearly linear part. It is possible to evaluate data non-linearity by studying which portion of a transfer function gets activated during training of the ANN with a particular data set [16]. Activities or outputs of two hidden neurons of trained back-propagation ANN for determination of ammonium are shown in Fig. 2. The whole range of the transfer function, including non-linear parts, is getting

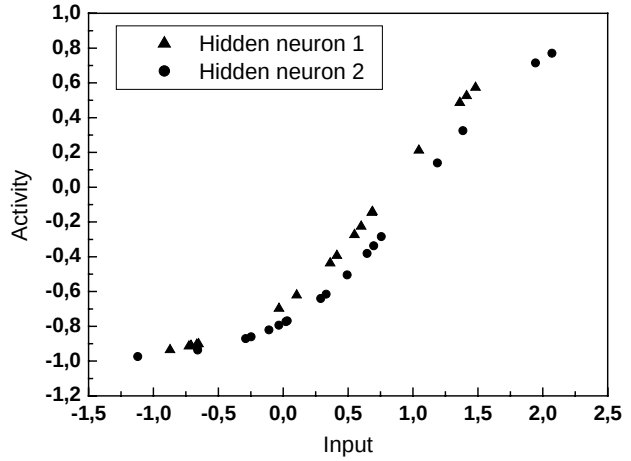

Fig. 2. Input vs. activity (output) of two hidden neurons of back-propagation neural network calibrated for ammonium determination. Activation of the whole range (both linear and non-linear) of the transfer function gives an evidence of significant non-linear dependence of sensor responses to changing ammonium content. Similar results were obtained for citrate.

activated in case of ammonium (and citrate), suggesting that dependences between the sensors' potentials and component concentrations are strongly non-linear. Non-linearity of the sensors' responses can result from significant interferences from other compounds present in the medium, particularly at low contents of ammonium and citrate. In particular, potassium should interfere with response to ammonium whilst citrate and oxalate should interfere with responses to each other. Based on these considerations the results obtained using ANN rather than PLS are reported below.

Results of determination of ammonium, oxalate and citrate content in test samples from the first experimental sessions are shown in Table 3. Calibrations were made using ANN. The test set was comprised of selected samples that were not used in calibration. It can be concluded that the sensor array is capable of measuring simultaneously both the contents of ammonium and two organic acids in the fermentation solutions with a good precision (the average relative errors are shown in Table 2).

In the third experimental session, sodium azide in a concentration of 10 mM was added to all 22 simulated fermentation solutions to see if this substance would interfere with the sensors' responses to ammonium and organic acids. It was found that sodium azide did change the responses of some sensors and, hence, can be detected and quantified itself, if necessary. The influence of sodium azide on the sensor responses is illustrated in Fig. 3 where samples with and without sodium azide form two well-separated clusters on a PCA-score plot. However, in spite of this influence, it was still possible to make valid calibration models for determining ammonium, oxalate and citrate without significant loss of precision compared to azide-free solutions. Average relative errors of component's determination in the test sets are shown in Table 4 (rows 1 and 2) for both cases.

Table 3
Results of determination of ammonium, oxalate and citrate content in the test solutions of the first measurement session

Sample no.	Added (mM)	Predicted (mM)	S.D.
	Ammonium		
2	2.0	2.1	0.4
4	2.8	2.5	0.3
6	4.8	4.9	0.1
14	6.0	6.0	0.04
16	12.0	11.8	0.3
18	13.2	13.7	0.05
19	14.0	13.6	0.02
	Oxalate		
6	7.8	7	1
8	13.0	14	1
9	20.7	21.0	0.3
10	28.5	28	1
16	33.7	33.8	0.6
18	44.0	43.9	0.4
20	49.2	51	2
	Citrate		
6	1.7	2.1	0.1
8	2.6	2.5	0.2
10	3.3	2.9	0.2
13	3.6	3.1	0.03
16	3.8	4.0	0.1
18	4.3	4.5	0.1
20	5.0	5.0	0.2

Data were processed by ANN.

Furthermore, an attempt to make a joint calibration model using the results of measurements in all solutions, both with and without sodium azide, was performed. The prediction ability of the joint calibration model based on all measurements (Table 4, row 3) was comparable (though slightly worse) to that of models made separately for solutions with and without sodium azide (Table 4, rows 1 and 2). Thus, sodium azide presumably changes mainly the standard potential of the sensors rather than their sensitivity, and the influence of sodium azide on the sensors of the electronic tongue can be effectively taken into account by an appropriate calibration model (Fig. 3). This result suggests an option for reliable analysis of real-world fermentation samples where microbial activity was suppressed by azide after sampling.

One of the parameters that can be of interest in biotechnological applications (e.g. [6]) is the time elapsed from the inoculation of the growth medium, i.e. from the start

Table 4
Average relative errors of prediction (%) of ammonium, oxalate and citrate content in model growth media with and without addition of *sodium azide*

Ammonium	Oxalate	Citrate	Samples
6	6	8	Without azide
7	7	7	With azide
11	6	12	All data

Data were processed using ANN.

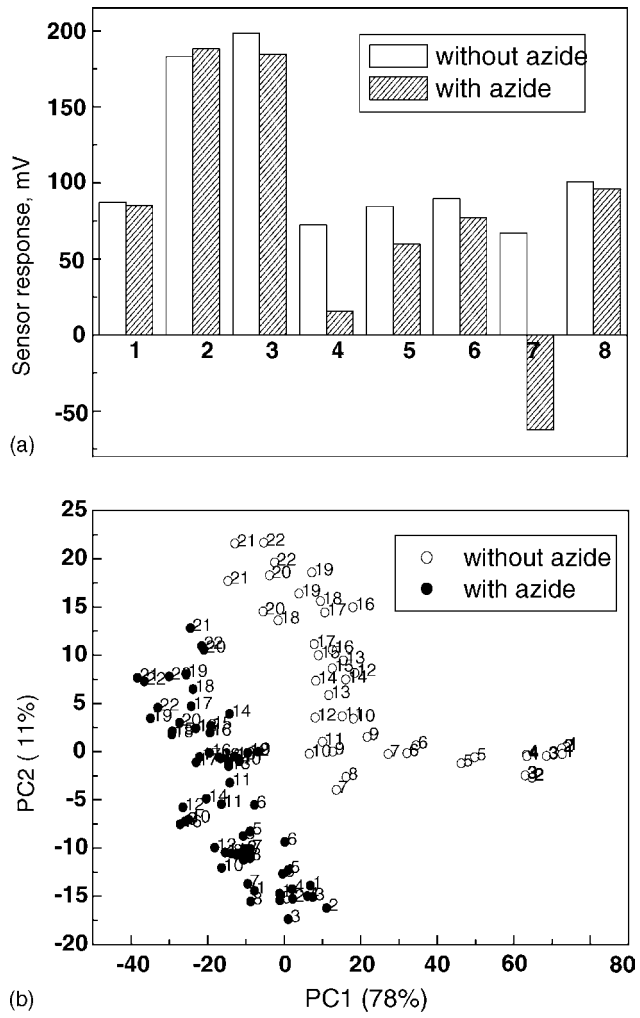

Fig. 3. Difference of sensors' responses in the samples with and without sodium azide. (a) Responses of the sensors of the array (numbers along horizontal axis) in the two samples of the same composition one with sodium azide added and another one without azide. (b) PCA score plot of all data from the third experimental session—the results of all measurements (with and without sodium azide) were considered. All samples containing sodium azide can be clearly distinguished from all azide-free samples.

of the process. The electronic tongue is sensitive enough to track changes of crucial components during bacterial growth. Thus, it is very likely that growth time can be also predicted using the electronic tongue. First three samples were excluded from the data set since no discernible changes in the broth composition occurred during the first 8 h after inoculation. A calibration model was calculated using the rest calibration samples (see Section 2) to fit time dependence of the electronic tongue response. The growth time was predicted for the test samples with average relative error 2%, which is about 17 min. This results is also in good agreement with previous experience, described in [6] where elapsed process time was successfully predicted by the ET for real growth media.

4. Conclusions

An electronic tongue system comprising a sensor array based on eight PVC-plasticized cross-sensitive potentiometric chemical sensors has been successfully applied to simultaneous determination of ammonium, oxalate and citrate content in simulated fermentation media closely resembling real-world samples typical of a process involving *A. niger*. The content of the targeted components in the media were in the range of 0.4–14 mM for ammonium, 0.5–5.5 mM for citrate and 2.6–62.2 mM for oxalate. The average prediction errors in the given ranges did not exceed 8% when a back-propagation artificial neural network was used for data processing. ANN produced somewhat better results than PLS in the data fitting, most likely due to better consideration of significant non-linearity of the dependence of sensor potentials on concentration, particularly for ammonium and citrate at low levels. It was also possible to quantify the content of all three key components in the presence of 10 mM sodium azide, which is commonly used to suppress microbial activity after sampling. Utilization of the electronic tongue for fermentation monitoring shows promise for industrial applications based on good precision and reproducible behavior of the sensor system and due to the possibility of automated measurements and data processing, resulting in inexpensive and rapid analysis (including at-line modalities).

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