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Microplate Differential Calorimetric Biosensor for Ascorbic Acid Analysis in Food and Pharmaceuticals

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In this paper we report on the development of a label-free low-volume (12.5 μL), high-throughput microplate calorimetric biosensor for fast ascorbic acid quantification in food and pharmaceutical products. The sensor is based on microplate differential calorimetry (MiDiCal) technology in which the heat generation, due to the exothermic reaction between ascorbic acid and ascorbate oxidase, is differentially monitored between two neighboring wells of an IC-built wafer. A severe discrepancy is found between expected and observed sensor readings. To investigate the underlying mechanisms of these findings a mathematical model, taking into account the biochemical reactions and diffusion properties of oxygen, ascorbic acid, and ascorbate oxidase, is developed. This model shows that oxygen depletion in the microliter reaction volumes, immediately after injection of sample (ascorbic acid) into the well, causes the enzymatic reaction to slow down. Calibration experiments show that the sensor's signal is linearly correlated to the area under the output versus time profile for the ascorbic acid concentration range from 2.4 to 350 mM with a limit of detection of 0.8 mM. Validation experiments on fruit juice samples, food supplements, and a pain reliever supplemented with ascorbic acid reveal that the designed method correlates well with HPLC reference measurements. The main advantages of the presented biosensor are the low analysis cost due to the low amounts of enzyme and reagents required and the possibility to integrate the device in fully automated laboratory analysis systems for high-throughput screening and analysis.

The use of calorimetry as a transduction mechanism in enzymatic biosensors has been reported in the literature for the quantification of a whole range of analytes.[1,2] These biosensors measure the heat generation (or dissipation) caused by the specific reaction between substrate and enzyme and correlate it to the concentration of the substrate.[3] Two different types of devices are commonly used for the calorimetric detection of metabolites: thermistor-based devices, which are based on the change in the resistance with changing temperature,[4] and thermopile-based devices, which are based on the Seebeck effect.[5]

Research in the area of calorimetric biosensors nowadays focuses on miniaturization and microfabrication of calorimetric devices[6] to fulfill one or more of the following objectives:[7] (i) increased sample throughput, (ii) decreased reagent volumes, and (iii) the possibility to integrate the device in fully automated lab systems.

These objectives can be realized by implementing the calorimetric transducer into a miniaturized flow injection analysis system (FIA) or by implementing the technology in miniaturized microplate assays allowing the simultaneous measurement of large numbers of samples. Most of the calorimetric biosensors discussed in the literature, and especially the thermistor-based devices, make use of FIA technology. The miniaturization of the devices offers the possibility to create multisensing devices.[8,9] Calorimetric biosensors are also often described in combination with microfluidics. The integration of the thermal transducer in a microfluidics environment results in ideal mixing behavior of the different reagents, leading to fast response and recovery times.[10] In general, thermopile-based systems are considered more suitable for microfabrication since thermal noise can be rejected with a high efficiency and when compared to thermistors they are self-generating offsetless devices, reducing the need for extensive temperature control.[11]

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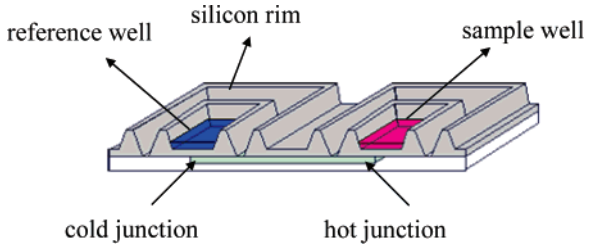

Figure 1. Schematic drawing of the structure of the microplate differential calorimeter. A full microplate consists of 48 combinations of reference wells and sample wells.

Although automated and miniaturized FIA systems increase the sample throughput, and reduce the amount of reagents significantly, a gain in sample throughput can only be obtained when tens or hundreds of samples can be measured simultaneously on one automated calorimetric device. In analogy with the implementation of enzymatic assays in microplate array format (96, 384, 1536 wells) and an optical detection system,[12,13] we report on an innovative concept where the microplate array format is used for the calorimetric detection of low molecular analytes. The calorimeter is based on microplate differential calorimetry (MiDiCal) technology[14] that applies an array of 96 wells (with a maximal volume of 20 μL) allowing the simultaneous quantification of 48 samples. The transduction principle is based on the differential measurement of the heat generated between two wells, located at the cold and the hot junctions of a thermopile (Figure 1). In one well the reaction heat of the biochemical reaction between substrate and enzyme is monitored and compared to that of the reference well where no specific biochemical reaction takes place. This will suppress the thermal noise associated with the dilution of the sample in the buffer. Since total assay volume in the microplate differential calorimeter is low there is no considerable need for immobilization of enzymes to reduce the analysis cost.

Ascorbic acid is chosen as model component for the calorimetric detection because of its importance in the human diet. It has antioxidative properties and plays an important role as a vitamin in a lot of biochemical processes.[15] Common analysis methods for ascorbic acid include electrochemical,[16,17] spectrophotometric,[18,19] as well as chromatographic methods.[20,21] An immobilization-free calorimetric method was described[22] for the quantification of ascorbic acid with the aid of an isothermal batch instrument. Reliable results are obtained with this device, but the described method is coupled to some disadvantages such as the high enzyme load per assay (22 U), the need for extensive manual operations, and a decreased throughput.

The objective of this paper is to develop a microplate differential microcalorimetric biosensor for fast ascorbic acid quantification in food and pharmaceutical products. The reaction kinetics in the biosensor are studied by means of a mathematical model describing the heat generation, the heat fluxes, and the reagents diffusion within the wells of the microcalorimetric device. The use of mathematical models to describe the biochemical reactions as well as the signal transduction in a biosensor has recently become a favorable method to analyze in detail the working principle of a specific biosensor[23,24] and also to improve the sensor performance without time-consuming trial and error experiments.[25]

EXPERIMENTAL SECTION

1. Reagents. The enzymatic oxidation of ascorbic acid into dehydroascorbic acid at pH 5.6 obeys the following stoichiometry:

$$2\ \text{ascorbate} + O_2 \xrightarrow{\text{ascorbate oxidase}} 2\ \text{dehydroascorbate} + 2H_2O \quad (1)$$

The enthalpy change (ΔH) for the oxidation of ascorbate is -128.75 kJ/mol.[26] Ascorbate oxidase (E.C. 1.10.3.3) from *Cucurbita* sp. (product code A0157, 296 U/mg) was purchased from Sigma Aldrich (St. Louis, U.S.A.). Stock solutions of 30U/10 μL acetic acid buffer (0.1 M, pH 5.6, 5 mM EDTA) were stored for maximal of 3 weeks at 4 °C in the dark. All reagents were purchased from Sigma Aldrich and were of analytical grade. Ascorbic acid stock solutions were prepared fresh every day and stored in the dark at 4 °C until analysis was performed.

2. Samples. Fruit samples (oranges, lemons, grapefruits, and limes) were obtained from a local grocery, and 2 mL of the handmade fruit juice was centrifuged for 10 min at 4 °C at 25 000*g*. The supernatant was filtered (Millipore (Billerica, U.S.A.), 0.45 μm) and stored at -20 °C until analysis was performed.

Two vitamin supplements (Redoxon, and UPSA-C) and one pain reliever, enriched with ascorbic acid (aspirin C) were obtained from a local pharmacy and dissolved into 50 mL of a (6%) metaphosphoric acid buffer under continuous stirring in the dark for 10 min. Subsequent sample preparation of the vitamin C products was identical to the sample preparation of the fruit samples. Before the analysis was carried out, the samples were defrosted at 4 °C, shaded from the light.

3. HPLC Analysis. Ascorbic acid reference analyses were carried out on the following high-pressure liquid chromatography device: Agilent Hewlett-Packard device 1100 series (Agilent Technologies, Palo Alto, U.S.A.) equipped with a Prevail "Rocket" column (C18, 53 mm × 7 mm, 3 μm particle diameter) (Alltech Associates Inc., Deerfield, U.S.A.). Analysis took place at room

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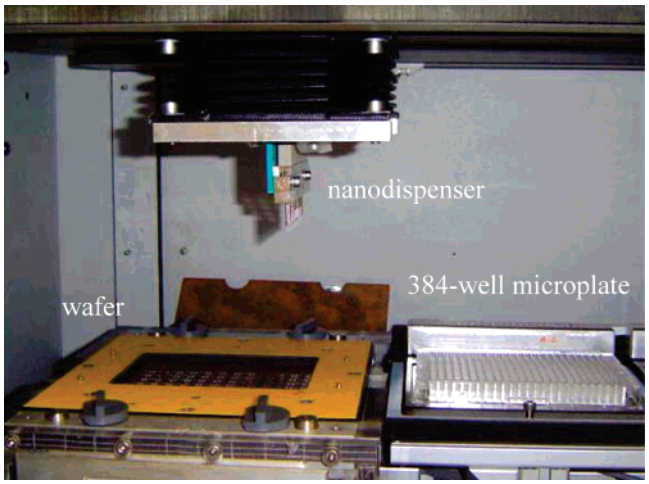

Figure 2. Picture of the microplate differential calorimeter. All the reagents for the analysis are placed in a standard 384-well microplate from where the nanodispenser aspirates the reagents and dispenses them on the wafer of the microplate differential calorimeter.

temperature, but the samples were stored at 4 °C in the autosampling system of the device. Distilled water, adjusted with formic acid to pH 2.5, and acetonitrile were used as mobile phase in gradient elution mode.[27] Ascorbic acid was detected with a diode array detector (DAD) set at a wavelength of 242 nm (with a reference wavelength of 360 nm). The ascorbic acid peak was observed at a retention time of 2.3 min. The total run time for one analysis was 5.5 min. The samples were diluted with distilled water to obtain ascorbic acid concentrations in the linear range of the HPLC analysis (0.031−0.51 mM)

4. Microplate Differential Calorimeter. A picture of the microplate differential calorimeter setup is shown in Figure 2. A nanodispenser (Seyonic, Neuchatel, Switzerland) with eight channels was integrated in the device to dispense the different reagents in the wells of the wafer. The nanodispenser was controlled with home-written software. The wafer of the microplate differential calorimeter has approximately the same dimensions as a standard 96-well microplate ($12\ 10^{-2}$ m × $8\ 10^{-2}$ m). The thermopiles are etched by integrated circuit technology on the top of a polyimide layer and consist of 64 thermocouples, composed of aluminum and p-polysilicon as semiconductor materials,[14] a combination which is frequently found in the literature.[28] The signal is converted on a ratio of 20 mV/K. The thermopile yields a responsivity of 7 V/W which is remarkably better than the device presented by others.[10] They report a responsivity of 0.94 V/W with 16 p-type polysilicon/gold microthermocouples.

5. Optimization of the Microplate Differential Calorimeter Setup. A first set of experiments was executed to determine the optimal filling order of the microplate differential calorimeter. Theoretically, four different filling orders (Table 1) are possible with regard to the order of injection of enzyme and substrate. The experiments were carried out for three different concentrations of ascorbic acid (0.45, 4.50, and 18 mM) and ascorbate oxidase concentration of 0.1 U/well. Concentrations are expressed here as the ascorbic acid concentration in the total volume of the well. A second set of experiments was done to optimize the ascorbate oxidase concentration. Four different concentrations of ascorbate oxidase (0.1, 0.5, 1.0, and 5 U/well) were tested with the three above-mentioned ascorbic acid concentrations to investigate the reaction kinetics and to optimize the analysis with regard to reaction time. Experiments were conducted in a temperature-controlled thermostatic room of 25 °C (±0.1 °C), and four repetitions per filling order and ascorbate oxidase concentration were executed.

Table 1. Overview of the Different Filling Orders with Respect to the Order of Injection of Reagents in the Wells of the Microplate Differential Calorimeter (E = Enzyme; B = Buffer; S = Substrate)

	injection 1		injection 2	
	well 1	well 2	well 1	well 2
(A)	E-10 μL	E-10 μL	S-1 μL	B-1 μL
(B)	E-10 μL	B-10 μL	S-1 μL	S-1 μL
(C)	S-10 μL	S-10 μL	E-1 μL	B-1 μL
(D)	S-10 μL	B-10 μL	E-1 μL	E-1 μL

6. Calibration and Validation. The microplate differential calorimeter was calibrated with three different ranges of ascorbic acid concentration (10−350 mM, 0.5−50 mM, and 0.5−10 mM). Concentrations are expressed here as the ascorbic acid concentration in the injected sample. The analyses were performed with filling order B and with an ascorbate oxidase concentration of 0.5 U/well. Per ascorbic acid concentration four repetitions were taken. Ascorbic acid dilution series were prepared automatically from the freshly prepared stock solution with an automatic liquid handling system (Multiprobe II, Perkin-Elmer, Boston, U.S.A.) in standard 384-well microplates which are placed directly in the microplate differential calorimeter. In validation analysis samples were also transferred first to a standard 384-well microplate with the automatic liquid handling system. The calorimetric measurements were executed with four repetitions per sample.

7. Mathematical Model. 7.1. Model Equations. The time and space dependency of the concentrations of ascorbic acid and oxygen are calculated by means of diffusion−reaction equations of the involved species:

$$\frac{\delta}{\delta t} c_i = \nabla D_i \nabla c_i + r_i \tag{2}$$

In these equations c_i represents the species concentration [mM], D_i the molecular diffusion coefficients [m s^{-1}], and r_i the reaction rate [mM s^{-1}]. The reaction terms are modeled based on the catalytic mechanism of the enzyme ascorbate oxidase:[29]

$$\begin{gathered} AA + E \leftrightarrow AA{-}E \rightarrow P + E_r \\ E_r + O_2 \leftrightarrow E_r{-}O_2 \\ AA + E_r{-}O_2 \leftrightarrow AA{-}E_r{-}O_2 \rightarrow P + E + 2H_2O \end{gathered} \tag{3}$$

In the first step, ascorbate oxidase E is reduced by the substrate AA. The reduced enzyme E_r forms a complex with

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oxygen in the second step. Finally in the third step the complex E_r–O_2 reacts with the substrate to render the original enzyme state. Equations are solved for the seven reacting species of the above catalytic system. The reaction rate for ascorbate reads as follows:

$$r_{AA} = -k_1[AA][E] + k_2[AA{-}E] - k_6[AA][E_r{-}O_2] + k_7[AA{-}E_r{-}O_2] \quad (4)$$

Similar terms are written for the other species, involving a total of eight reaction constants. The Fourier equation for heat conduction is solved to model the sensor response. The heat generation q_r [W m^{-3}] of the enzymatic reaction equals

$$q_r = h_r(k_3[AA{-}E] + k_8[AA{-}E_r{-}O_2]) \quad (5)$$

where h_r (J mol^{-1}) is the change in molar enthalpy due to the oxidation of ascorbic acid. The thermal parameters are thermophysical properties of water and lumped properties of the microplate.

7.2. Model Solution. The finite element method is used to solve the differential equations by employing FEMLAB 3.1 (Comsol, Stockholm, Sweden). The transient solution is obtained in a few minutes of CPU time. Simulations are executed for a single well of the microplate in an equivalent axi-symmetrical 2-D space. The reaction takes place in a 12.5 μL drop between the walls of the well. Initially, the drop is saturated with oxygen in equilibrium with the surrounding air and with uniform initial concentrations of all species. When the reaction starts, the model predicts how oxygen is consumed and the deficit is supplied by means of diffusion from the surrounding air and into the drop. The model also predicts how ascorbic acid diffuses through the drop from regions of high concentration to regions of low concentration. Finally, the model predicts how the generated heat diffuses through the system and is convected away by the surrounding air. The sensor response is linearly proportional to the resulting temperature of the microplate. The values of the reaction constants and reaction heat were calculated using a nonlinear least-square optimization of the predicted temperature response with respect to the measured signals. This procedure was implemented in MATLAB 7.0 (The Mathworks, Natick, MA). The modeled enzyme concentration was estimated to fit the measured response accurately to the proposed reaction scheme. The estimated value should be interpreted as an effective concentration, representing unresolved catalytic biochemical mechanisms.

RESULTS AND DISCUSSION

1. Optimization of the Microplate Differential Calorimeter. Typical microplate differential calorimetric sensor signals for four different ascorbic acid concentrations are depicted in Figure 3. It was found that the output voltage versus time profile is subject to the filling order of the reaction wells and to the enzyme concentrations used. Therefore these two parameters were studied and optimized with respect to the sensor's performance.

1.1. Filling Order of the Microplate Differential Calorimeter. Experiments were performed to find the most ideal filling order for the ascorbic acid measurement with the microplate differential calorimeter. Different signal parameters (peak height, area under the peak, or the ascending slope)[3] are available in calorimetry for the correlation of the response signal with the concentration of the substrate. The area under the output voltage versus time profile[22] was chosen to correlate the sensor response to the ascorbic acid concentration. Figure 4 shows the calculated standardized areas for the four different combinations in relation to the three ascorbic acid concentrations. The standardization was achieved by dividing the area by the initial ascorbic acid concentration. Tukey tests were executed to find significant ($\alpha = 0.05$) differences between the different filling orders for the three different concentrations. Per concentration and filling order four different repetitions were included in the analysis. Assays with filling orders C and D result in significant lower signal outputs for the analysis of 4.5 and 18 mM ascorbic acid compared to assays with filling orders A and B. In addition to the lower signal outputs, stronger baseline shifts and higher injection peaks were noticed for filling orders C and D. These observations are probably due to the suboptimal mixing of the enzyme with the substrate which is caused by the difference in diffusivity between enzyme and substrate. Although the areas under the output voltage versus time profile for the analysis of 4.5 and 18 mM are significantly higher for filling order A than for filling order B, filling order B is preferred above filling order A. In filling order B the amount of enzyme is reduced with a factor 2, and preliminary calibration analysis indicates that filling order B results in better correlation between ascorbic acid concentration and the area under the output voltage versus time profile. Therefore, the remaining experiments were carried out with filling order B in which two adjacent wells were first filled with 10 μL of pure buffer and 10 μL of enzyme solution, followed by the injection of 1 μL of sample. For calibration and validation experiments this last step was preceded by the dispensing of 1.5 μL of buffer to stabilize the microplate differential calorimeter without affecting the sensor performance.

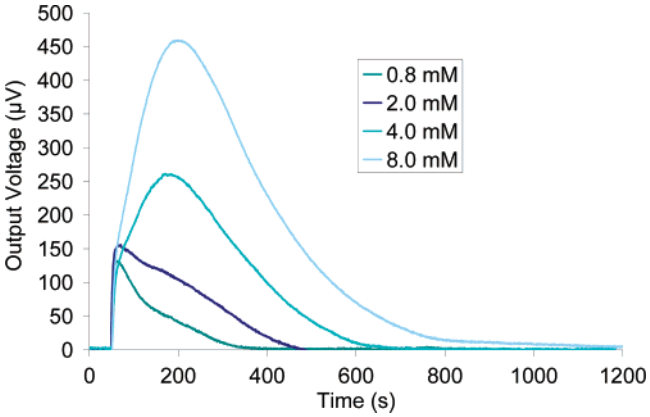

Figure 3. Typical sensor signals for four different ascorbic acid concentrations with the optimal filling order of the microplate differential calorimetric biosensor. Concentrations are expressed as the ascorbic acid concentration in the total volume of the well.

1.2. Ascorbate Oxidase Concentration of the Microplate Differential Calorimeter. To optimize the reaction kinetics of the enzymatic assay, four different concentrations of ascorbate oxidase were tested (0.1, 0.5, 1.0, and 5.0 U/well) in relation to three different concentrations of ascorbic acid (0.45, 4.5, and 18 mM). No difference in sensor response was observed for the different enzyme concentrations when ascorbic acid concentrations of 0.45 and 4.5 mM were analyzed. However, there was an

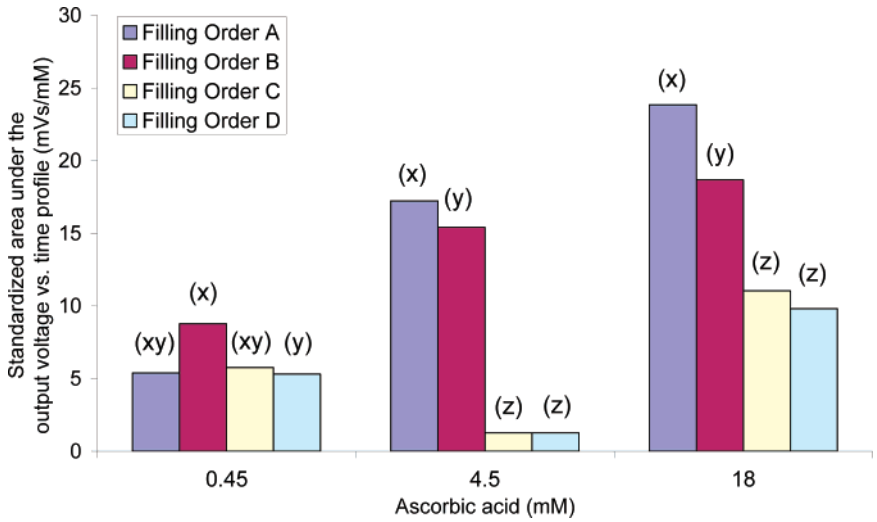

Figure 4. Standardized areas under the output voltage vs time profile for four different filling orders applied in the analysis of three different ascorbic acid concentrations. Standardization was achieved by dividing the area by the initial ascorbic acid concentration. Tukey tests were carried out per concentration level. Bars indicated with a similar letter were not significantly different ($\alpha = 0.05\%$).

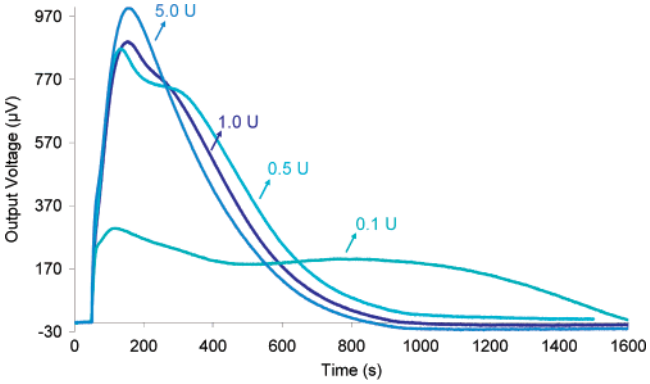

Figure 5. Difference in sensor response signal for four different ascorbate oxidase concentrations (0.1, 0.5, 1.0, and 5.0 U/well) for a fixed ascorbic acid concentration of 18 mM.

Table 2. Parameter Values for the Reaction–Diffusion Model

D_{O2}	3.0×10^{-9} m^2 s^{-1}
D_{AA}	8.0×10^{-10} m^2 s^{-1}
D_{Enzyme}	1.0×10^{-10} m^2 s^{-1}
h_r	1.125×10^{-5} J mol^{-1}
$k_1{}^a$	1 s^{-1} mM^{-1}
$k_2{}^a$	0.0 s^{-1}
$k_3{}^a$	0.0034 s^{-1}
$k_4{}^a$	1.0 s^{-1} mM^{-1}
$k_5{}^a$	0.7 s^{-1}
$k_6{}^a$	0.6 s^{-1} mM^{-1}
$k_7{}^a$	0.0 s^{-1}
$k_8{}^a$	0.0064 s^{-1}

[a] Parameters obtained by nonlinear least-square optimization of the predicted temperature response with respect to the measured signals.

important difference in the shape of the sensor response between the different enzyme concentrations for the analysis of a concentration of 18 mM ascorbic acid (Figure 5). Sensor responses for ascorbate oxidase concentrations of 0.5, 1.0, and 5.0 U/well show a similar pattern. At the moment of the ascorbic acid injection a lot of heat is generated. This sharp increase in output voltage is followed by a long tailing, representing a reaction rate which slows down with time. The reaction signal of the analysis with 0.1 U/well is characterized by a lower peak height, followed by a period of constant heat generation, between 400 and 1000 s, after which the signal output decreases slowly. The steady-state condition was to some extent also observed in the response signal of the analyses with ascorbate oxidase concentrations of 0.5 and 1.0 U/well. This difference in sensor signal for the four investigated ascorbate oxidase concentrations is explained further in section 2 of the Results and Discussion. Despite the difference in shape of the response signals the total area under the curve remains constant ($\pm 3.3 \times 10^3$ mV s) for the four different ascorbate oxidase concentrations with a coefficient of variation of 2.5% for all 16 measurements. From these experiments it was concluded that there is no benefit to use enzyme concentrations higher than 0.5 U/well neither toward the speeding up of the enzymatic reaction (for ascorbic acid concentrations up to 18 mM) nor toward the increase of the area under the output voltage versus time profile. Hence the concentration of the ascorbate oxidase for further experiments was kept fixed at 0.5 U/well.

2. Reaction–Diffusion Model of the Microplate Differential Calorimeter. The results presented above illustrate that the shape of the sensor response depends on the enzyme concentration. For lower enzyme concentrations a condition of steady-state heat generation was observed at a submaximum conversion rate. It is also observed that the ascorbic acid analysis takes about 800 s for the higher enzyme concentrations and up to 1600 s for the lower ones, whereas a simple calculation of the reaction time learns that the reaction, in a perfectly homogeneous reaction medium saturated with oxygen, should last less than 60 s. To study the discrepancies between expected and observed sensor readings a mathematical model was developed that takes into account reaction kinetics (heat generation) and diffusion properties of the reagents within the well of the wafer. The parameter values for the reaction–diffusion model are given in Table 2.

First the model was validated by comparing simulated and measured sensor responses for four different concentrations of ascorbic acid (8.0, 24, and 40 mM) with a fixed ascorbate oxidase

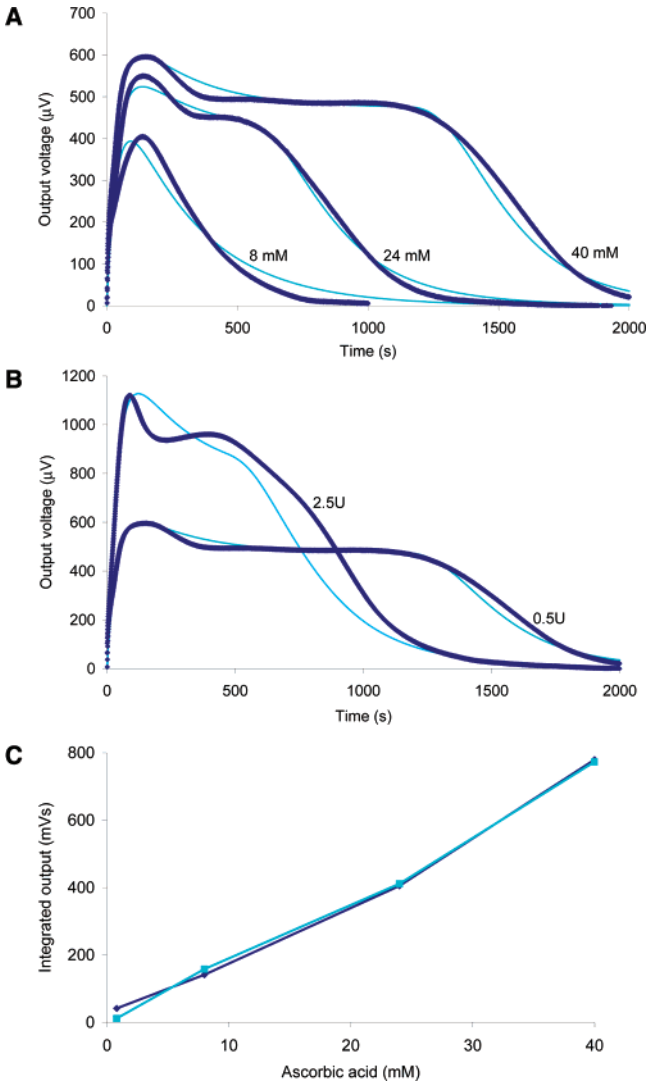

Figure 6. Validation of the mathematical model for (A) four different ascorbic acid concentrations with a fixed concentration of ascorbate oxidase (0.5 U/well) and (B) different concentrations of ascorbate oxidase with a fixed ascorbic acid concentration (40 mM); panel C compares the measured and modeled areas under the voltage vs time profiles of the validation experiments. The dark lines and symbols correspond to the measured signal response, whereas the light lines and symbols correspond to the simulated signal responses.

concentration (0.5 U/well) (Figure 6A). Second, validation experiments were carried out for two different concentrations of ascorbate oxidase (0.5 and 2.5 U/well) with a fixed concentration of ascorbic acid (40 mM) (Figure 6B). The measured sensor responses were taken as the average of four response signals from four different wells. From the similarity between the different curves we conclude that the applied model sufficiently describes the signal response. This is also illustrated by Figure 6C in which theoretical calculations of the output versus time area are compared to the actually measured values.

The model predicts the occurrence of a shoulder, shortly after the start of the measurement (Figure 6A). On the plateau of the shoulder the rate of ascorbic acid transformation is constant. This steady-state phase is probably caused by the limited diffusion of the cosubstrate, oxygen, from the surrounding air into the well. Enzymatic conversion, and as a consequence heat generation, will generally take place in the border region of the microliter drop with the surrounding air where oxygen concentration is abundant. As such the conversion rate of ascorbic acid in this phase will depend on the concentration of ascorbate oxidase, present in the microliter drop. Since ascorbate oxidase is assumed to be uniform in the microliter drop, only the enzymes present in the border region will effectively have catalytic power. This explains the prolonged reaction time of the enzymatic assay. After this phase the curves start to decline. The shoulder effect subsequently followed by the steady-state condition is not observed with the analysis of low concentrations of ascorbic acid. In this setup oxygen is present in relative abundance compared to ascorbic acid. When higher concentrations of enzyme are used in the assay (Figure 6B), there was an increased peak signal and a shortened plateau time. More importantly, the model also captures the first peak at higher ascorbic acid concentrations. The complex stepwise reaction mechanism[29] appears to be the cause of this behavior. Indeed, when a simple oxygen-dependent Michaelis−Menten model is assumed (data not shown), the plateau could also be predicted, but without the prior peak. The first peak is the result of the initial relative abundance of oxygen, which results in fast conversion. After this short period, however, the reaction is controlled by the supply of oxygen from the environment, as explained above.

To support the hypothesis of oxygen deficiency, the concentration profiles of oxygen and ascorbic acid are plotted in the well of the wafer based on the mathematical model for two enzyme concentrations. Figure 7 shows the profile of the oxygen and ascorbate, together with the temperature profile on six different time steps (0, 250, 500, 1000, 1500, and 2000 s) for an assay with an initial concentration of 40 mM ascorbic acid and for both 0.5 and 2.5 U/well of ascorbate oxidase. The concentration plots indicate that after the start of the reaction the available oxygen is consumed immediately, leading to a disappearance of the oxygen in the lower part of the well. This oxygen profile remains constant (600−1400 s) during the analysis whereby an equilibrium is obtained between the consumption of oxygen in the enzymatic reaction and the supply of oxygen from the surrounding air. This shows clearly that the conversion of ascorbic acid will only take place at the surface of the well and not at the bottom of the well. Thereby an ascorbic acid gradient is induced in the well which makes that diffusion of ascorbic acid becomes a second critical parameter in the enzymatic monitoring of the microplate differential calorimeter. When the ascorbic acid content gets depleted, oxygen diffuses further into the well, where the reaction can proceed locally. Simulations (data not shown) with the mathematical model whereby the oxygen concentration is kept constant throughout the entire enzymatic reaction indicate that the reaction time for an assay with an ascorbic acid concentration of 40 mM decreases with a factor 4 compared to assays with variable oxygen concentration.

3. Calibration Curve. The calibration curve was based on 220 measurements, which were collected over 5 different measurement days. A high correlation ($R^2 > 0.99$) was found between the area under the output voltage versus time profile and the ascorbic acid concentration. The calibration curve shows linearity with the ascorbic acid concentration up to 350 mM with a limit of detection (LOD) of 0.8 mM ascorbic acid and a limit of quantification (LOQ) of 2.4 mM ascorbic acid. This LOD and LOQ were

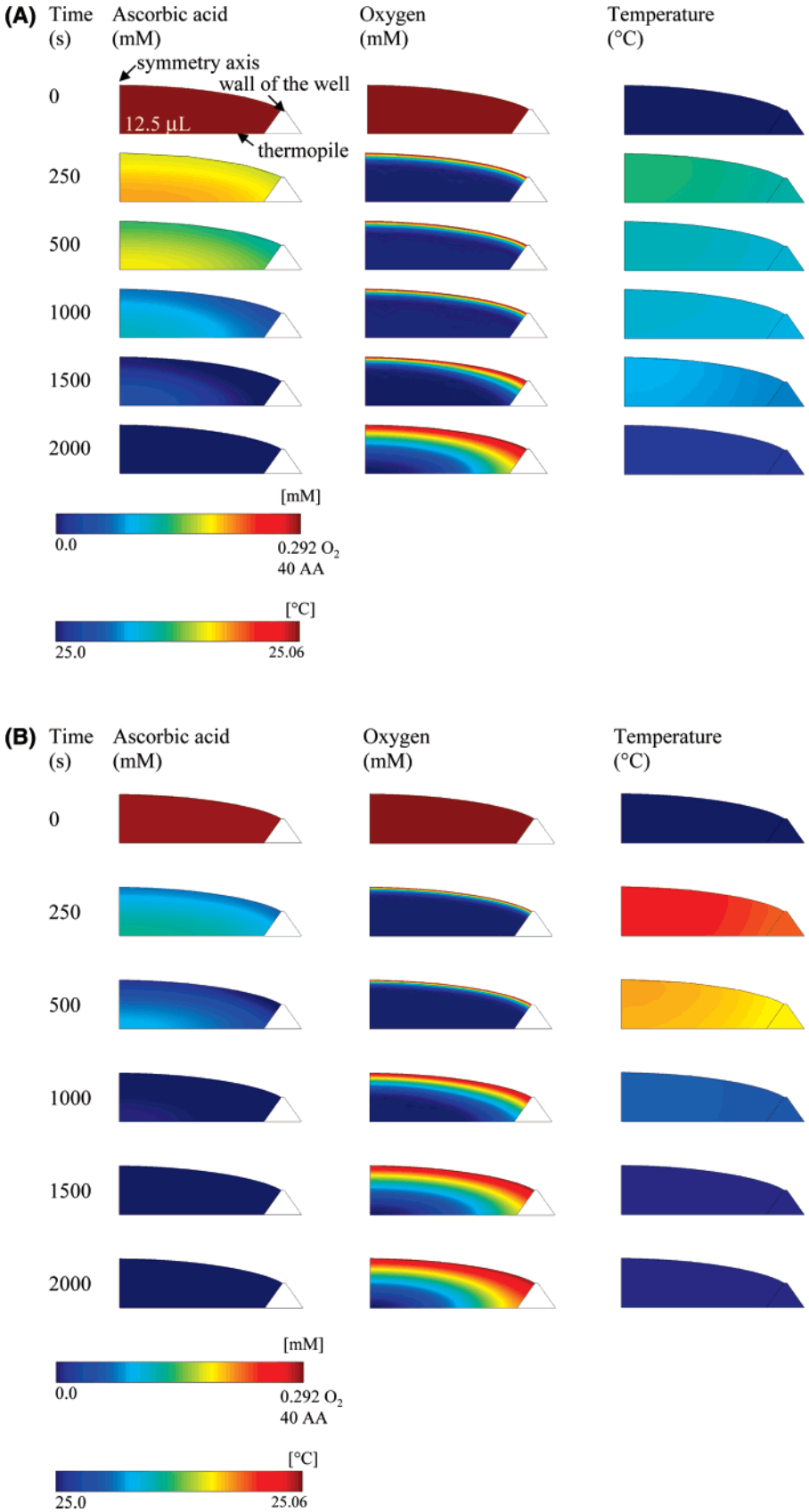

Figure 7. Model simulations of the oxygen concentration, ascorbic acid concentration, and temperature profile in the well of the microplate differential calorimeter during the analysis of an ascorbic acid concentration of 40 mM. The profiles are given on six different time steps. Set A is for 0.5 U of ascorbate oxidase, set B for 2.5 U of ascorbate oxidase.

Table 3. Ascorbic Acid Content of the Analyzed Food Supplements, Pain Reliever, and Fruit Juices[a]

sample	theoretical (mM)	HPLC method (mM)	MiDiCal method ± SD (mM)	<LOQ
Redoxon (1)	113.6	120.6 ± 0.5	113.6 ± 4.4	no
Redoxon (2)	113.6	118.3 ± 0.7	118.6 ± 11.8	no
UPSA-C (1)	113.6	118.6 ± 2.3	116.0 ± 5.0	no
UPSA-C (2)	113.6	118.8 ± 1.6	118.5 ± 10.4	no
aspirin C (1)	27.3	31.6 ± 0.8	33.8 ± 0.8	no
aspirin C (2)	27.3	28.7 ± 0.4	35 ± 1.8	no
orange (1)		3.3 ± 0.1	1.8 ± 0.3	no
orange (2)		4.7 ± 0.2	4.2 ± 0.8	no
orange (3)		3.7 ± 0.1	3.2 ± 0.4	no
orange (4)		2.4 ± 0.1	1.5 ± 0.2	yes
orange (5)		3.2 ± 0.1	2.0 ± 0.2	no
lemon (1)		3.0 ± 0.1	1.6 ± 0.5	no
lemon (2)		3.2 ± 0.1	2.8 ± 0.5	no
lemon (3)		2.3 ± 0.1	1.5 ± 0.2	yes
lemon (4)		1.8 ± 0.1	1.4 ± 0.3	yes
grapefruit (1)		2.7 ± 0.1	2.3 ± 0.3	no
grapefruit (2)		1.8 ± 0.1	1.5 ± 0.3	yes
grapefruit (3)		1.8 ± 0.1	1.0 ± 0.2	yes
grapefruit (4)		1.7 ± 0.1	1.5 ± 0.2	yes
lime (1)		2.6 ± 0.1	1.9 ± 0.2	no
lime (2)		1.6 ± 0.1	1.5 ± 0.4	yes
lime (3)		2.0 ± 0.1	1.1 ± 0.1	yes

[a] Comparison was made between the content, measured by the HPLC method and the microplate differential calorimeter ($n = 4$). In addition, for the food supplements and the pain reliever the theoretical value was added in the table (SD = standard deviation, LOQ = limit of quantification).

defined[30] as respectively 3 and 10 times the standard deviation of blank value/divided by the slope of the calibration curve. This LOD corresponds to a total amount of 0.8×10^{-9} mol ascorbic acid in 1 μL of sample. Other methods described in literature obtain lower limits of detection, but considering the concentration of ascorbic acid in fresh fruits and in pharmaceutical products, this LOD should be theoretically satisfactory for the quantification of ascorbic acid in those products. A higher dynamic range was observed for the analysis with the microplate differential calorimeter (0.8−350 mM) compared to the applied HPLC method (0.031−0.51 mM). The sensitivity[31] as the relation of the output response to the initial ascorbic acid concentration is 1.4 V·s/M.

For concentrations lower than 3 mM CV values up to 25% ($n = 16$) were found. For higher ascorbic acid concentrations CV values gradually decrease from 15% (3 mM, $n = 16$)) to 5% (350 mM, $n = 4$). The interday repeatability was evaluated by the analysis of the calibration curve of 0−10 mM on three different measurement days whereby per concentration per day four different repetitions were included. An average CV value of 7.7% was obtained, indicating that the interday repeatability over several days has the same order of magnitude as the repeatability on the same day.

The longest total reaction time, including the dispensing of the initial enzyme and buffer solution, with the microplate differential calorimeter was found to be 2500 s which corresponds to the analysis of an ascorbic acid concentration of 350 mM. Since theoretically 48 samples can be analyzed simultaneously, a real maximal measurement time of ~60 s per sample is obtained which is much faster than the most methods found in literature.

4. Validation. The results of the validation analysis between the microplate differential calorimeter and the HPLC analysis are shown in Table 3. Seeing the huge difference in ascorbic acid content between the fruit samples and the pharmaceutical products containing vitamin C, difference is made between the two subsets of samples for the validation of the microplate differential calorimeter.

4.1. Pharmaceutical Products Containing Vitamin C. Three different pharmaceutical products containing vitamin C (two vitamin supplements and one pain reliever) were analyzed in duplicate. From Table 3 it can be concluded that the ascorbic acid concentration determined with the microplate differential calorimeter is very similar to the theoretical concentration and the concentration obtained with the HPLC method. CV values based on the different repetitions ($n = 4$) per duplicate indicate that the repeatability of the microplate differential calorimeter is adequate with CV values ranging from 2.3% to 9.9%. The CV values for the HPLC measurements were between 0.4% and 2.6% which indicates that the repeatability of the HPLC reference method is slightly better than that of the microplate differential calorimeter.

4.2. Fruit Juices. Citrus fruits are known as an important source of ascorbic acid. Therefore, juice from different fruits such as oranges, lemons, grapefruit, and limes were analyzed with the microplate differential calorimeter and compared to the HPLC method. Four repetitions per sample were analyzed with the microplate differential calorimeter, whereas the samples were analyzed in duplicate with the HPLC method. CV values of the analysis with the microplate differential calorimeter of the samples were about 20%, whereas the CV values for the HPLC analysis ranged from 0.4% to 3.8%. The results of the HPLC analysis indicate that the ascorbic acid concentration in a lot of samples is below the LOQ of the microplate differential calorimeter. Therefore, optimization of the device is necessary for a more accurate quantification of the ascorbic acid concentration in fruit juices. In addition, the correlation between the two methods for the remaining samples shows that higher concentrations of ascorbic

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acid were found with the HPLC method than with the microplate differential calorimeter. This is explained by the limited stability of ascorbic acid in fruit samples, whereby the presence of polyphenolic components causes the nonspecific oxidation of ascorbic acid which results in a decrease of the ascorbic acid content.[32]

CONCLUSION

In this work we studied the potential of the microplate differential calorimeter for the quantification of ascorbic acid in food and pharmaceutical products. Considering the low analysis cost per assay and the possibility to integrate the system in fully automated high-throughput applications, the device has a large potential to determine a whole range of active compounds in food and pharmaceuticals. The system was optimized for the optimal enzyme concentration and the filling order of the wells.

A mathematical model, based on reaction−diffusion equations for oxygen, ascorbic acid, and ascorbic acid oxidase, was developed to get insight in the mechanisms taking place in the wells of the calorimeter. It was found that the rate of the enzymatic reaction was slowed down due to the limited diffusion of oxygen from the surrounding air toward the center of wells.

The biosensor was calibrated and validated successfully on food supplements and a pain reliever containing vitamin C. However, further adjustments to the device are necessary to measure more accurately the ascorbic acid concentration in fruit juices. In comparison to the method described in literature the amount of enzyme needed per assay was lowered with a factor 40, which lowers the cost of analysis significantly. Furthermore, the use of an array induces additional time and cost savings resulting in an increased throughput.

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