Enzymatic reactions are the basis of many biotechnological manufacturing and biomedical diagnostic procedures that require effective methods of monitoring over the reaction course. In the current paper, we present the results of the development of a new approach within the differential microwave dielectrometry method for the non-invasive monitoring of the course of enzymatic reactions based on the complex permittivity changes of these reactive mixture solutions in real time at a fixed frequency of 31.82 GHz. The dynamic studies of the dielectric parameters of selected enzymatic systems containing a protein substrate (immunoglobulin G, human serum albumin) and enzyme trypsin. The developed differential microwave dielectrometry setup has been performed to verify the proposed approach effectiveness for the enzymatic reaction monitoring in biomedical practice and the food industry. Our microwave dielectrometry results have been validated by the results of the UV-Vis spectrophotometry method for selected enzymatic systems. We propose a new approach to use the differential microwave dielectrometry method with high sensitivity (in average 0.5% and 3±5% for the real and imaginary parts of the complex permittivity, respectively) to estimate the course of enzymatic reactions in real time.

**Keywords**: high loss liquids, complex permittivity, enzymatic reactions, electromagnetic wave propagation, differential microwave dielectrometry, enzyme trypsin, human serum albumin, immunoglobulin G.

1. Introduction

Development of effective physical methods for the continuous monitoring of the processes in mixtures of biomolecules (e.g., protein substrate, enzyme, solvent, et al.), including enzymatic reactions mixtures, is one of the urgent problems of current biotechnological and biomedical applied researches and practice [1, 2]. Some biotechnologies related to the food and drugs production are based on the process of the enzymatic hydrolysis of proteins in a biochemical reactor. Until now, the majority of analytical chemical methods for the monitoring of the enzymatic hydrolysis of proteins are discrete [1, 2]. Such methods require the sampling from the reactive zone and the following chemical reaction with a coloring agent, which specially added to the probing sample.
Non-invasive impedance methodology for investigating the enzymatic reactions within a dielectric polymer microchip is presented in [3]. The method was shown to be highly sensitive, enabling the real-time monitoring of the enzymatic reaction between glucose oxidase and glucose with a limit of detection of 10 μm. However, some potential difficulties, when investigating the enzymatic reactions using the impedance methodology, include: validating the results with using other complementary analytical techniques to confirm the identity and concentration of the reaction products. In work [4], the feasibility of using a surface plasmon resonance (SPR) to monitor the progress of enzymatic reactions in real time was demonstrated. The authors also observed a linear correlation between the enzymatic reaction rate and the SPR signal change, which suggests that SPR can be used as a quantitative measurement tool for enzymatic reactions. Article [5] presents the results of the real-time monitoring of the enzymatic activity of the wild-type APOBEC3G enzyme using the fluorescence resonance energy transfer and circular dichroism spectroscopy. The authors faced difficulties in developing an assay system that accurately reflects the activity of APOBEC3G and in interpreting the complex interactions between the enzyme and its substrate RNA.

The spectrophotometric method is a widely used technique for determining the concentration of the end product of the enzymatic hydrolysis of proteins [6]. The method is based on the measurement of the absorbance of the colored product formed during the hydrolysis reaction, which is proportional to the concentration of the product. The main disadvantage of the spectrophotometric method is that the hydrolysis reaction was interrupted at various time points to determine the degree of hydrolysis, which may not reflect the actual extent of hydrolysis during a continuous process.

Based on the methods mentioned above, there are today no fast and continuous non-invasive methods for the monitoring of the enzymatic reactions. Moreover, the problem is also the irrelevance of the evaluation results, because the sampling and measurements (e.g., spectrophotometry measurements) take up to several tens of minutes, while, in the reaction zone, the enzymatic process is going on [1, 2]. The necessity to develop effective physical methods is due to the high probability of a contamination of the reaction zone during the sampling and the need to stop or limit the enzymatic process for the time of a sampling. That is why the search for methods of the dynamic monitoring of the enzymatic hydrolysis is of current interest.

Since the systems in which enzymatic reactions take place are water solutions of organic substances, they are characterized by the strong absorption in the microwave range. The real and imaginary parts of the complex permittivity (CP) of water change rapidly in this microwave frequency range [7]. As a result of the interaction of biomolecules with the water solvent, a layer of bound water is formed on the surface of the dissolved biomolecules. The bound water layer is characterized by a relaxation time (time of establishing the thermodynamic equilibrium) much longer than that of ordinary water. Reducing the mobility of the near-surface water molecules leads to a decrease of the CP of the solution as a whole [8, 9]. The open-ended waveguide method is used to study of the CP of liquids, because it provides a non-destructive and non-invasive way of measuring [10]. For example, the electrical properties of contaminated soil were discussed in [8] with a focus on the response of bound water in the soil samples with the use of open-ended coaxial probe in the frequency range from 0.2 to 6 GHz. The accuracy of the CP measurement is about 5% from the actual values for commercially kits from Keysight Technologies [11]. The open-ended waveguide technique for CP measurements of samples with various biological cells was effective in examining a number of biological processes in the cells, including the evaluation of the cytoplasmic membrane permeability, glucose concentration monitoring, etc. [12]. However, in the measurement of the CP of liquid samples with open-ended coaxial probes, the inaccuracies can be caused by the location of the coaxial probe in a liquid and a variation in the liquid volume in the measured system. By using the open-ended waveguide technique, the CP measurement uncertainty can be reduced by using the calibration standards with known permittivity values and by minimizing the effects of parasitic elements [10].

Standard liquids such as water are used as reference liquids when applying the dielectrometry and open-ended coaxial measurement of the CP [8, 13–15], because they have well-known dielectric properties. Additionally, water is often used as a reference point for the comparison with other liquids, allowing
Table 1. Comparison of the differential microwave dielectrometry method with some other microwave methods

<table>
<thead>
<tr>
<th>Microwave methods</th>
<th>Sample type</th>
<th>Tested sample volume</th>
<th>Accuracy of the real (\varepsilon^') and imaginary (\varepsilon^'') CP parts determination</th>
<th>Operating frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Differential microwave dielectrometry (this work)</td>
<td>Water-protein and trypsin solution</td>
<td>7 ml</td>
<td>0.5 and 3(\pm)5%</td>
<td>31.82 GHz</td>
</tr>
<tr>
<td>Whispering-gallery-mode resonator technique [22]</td>
<td>Albumin-water solutions</td>
<td>Sub-microliter/ nanoliter</td>
<td>1.4 and 0.7%</td>
<td>30–40 GHz</td>
</tr>
<tr>
<td>Dielectrometry [20]</td>
<td>Water, NaCl-, albumin-water solutions</td>
<td>A few (\mu)l</td>
<td>6%</td>
<td>10 MHz(\pm)10 GHz</td>
</tr>
<tr>
<td>Dielectric spectroscopy [12]</td>
<td>Biological cells samples</td>
<td>A few (\mu)l</td>
<td>4%</td>
<td>0(\pm)40 GHz</td>
</tr>
<tr>
<td>Open-ended coaxial cable [8]</td>
<td>Soils</td>
<td>–</td>
<td>5–20%</td>
<td>0.2(\pm)6 GHz</td>
</tr>
<tr>
<td>Open-ended cut-off waveguide reflection method [13]</td>
<td>Water, methanol, ethanol, isopropanol</td>
<td>0.06 ml</td>
<td>0.08(\pm)12.32 and 0.02(\pm)11.9%</td>
<td>0.5(\pm)3 GHz</td>
</tr>
<tr>
<td>Microstrip split-ring resonator [21]</td>
<td>Water, hexane, methanol, chloroform</td>
<td>A few (\mu)l</td>
<td>0.05(\pm)2.3%</td>
<td>3 GHz</td>
</tr>
<tr>
<td>Open-ended coaxial probe from Keysight [11]</td>
<td>Liquids and conformable solids</td>
<td>From milliliters to microliters</td>
<td>0.05(\pm)0.1</td>
<td>0.01(\pm)50 GHz</td>
</tr>
</tbody>
</table>

Table 1 provides a comparison of some parameters for the proposed microwave dielectrometer with some other microwave methods.

for relative comparisons between different materials [16–19]. When using the resonator techniques, in particular, the microfluidic sensor [20, 21], the sensitivity of the CP determination is limited for a liquid with high CP. But, for a low CP, we have highly sensitive results for small changes in the CP of liquids.

Based on the above-mentioned results of the CP measurements in biological samples (related to the changes of the state of water molecules in the samples), we assume that a change in the number of bound water molecules associated with the course of enzymatic biochemical reactions also can lead to a change in the CP of the reactive system solutions. In particular, various kinetic processes in the reaction system related to the interaction of the dissolved molecules with the solvent and the participation in the reaction should directly affect the absorption of the electromagnetic wave by the tested liquid. Thus, dielectrometry CP measurements in the microwave frequency range can allow us to obtain information about the state of water molecules in the biochemical systems with enzymatic processes and to estimate the course of biochemical reactions in these systems. In addition, using the dielectrometry method in biochemical reactors to trace the course of enzymatic reactions, one can significantly reduce production costs and eliminate the contamination of the solution in the bioreactor during a sampling.

Understanding the urgency of the development of the dynamic and non-invasive methods of monitoring of enzymatic processes, we will propose, for the first time, to use the microwave differential microwave dielectrometry method for the monitoring of the enzymatic hydrolysis of proteins without the interrupting of the reaction course. For measurements, we used the microwave dielectrometer earlier developed by our scientific team and tested by numerous comparisons with other results described in our papers [16–19]. The biologically significant reactions of hydrolysis of the human immunoglobulin G (IgG) and human serum albumin (HSA) proteins by enzyme trypsin were selected as the reactions to be examined in the current study. Table 1 provides a comparison of some parameters for the proposed microwave dielectrometer with some other microwave methods.
2. Materials and Methods

2.1. Sample preparation

In our dielectrometry measurements, we used the proteolytic enzyme, which catalyzes the hydrolysis of proteins to amino acids and oligopeptides, such as trypsin from bovine pancreas ("Merck", EU) with a concentration of 0.1%. To prepare the enzymatic reaction mixture for differential microwave dielectrometry measurements, we used the following protein solutions: 1) HSA water solution 2.5% ("Biopharma", Kyiv, Ukraine) with trypsin 0.1%; 2) HSA water solution 10% with trypsin 0.1%; 3) IgG water solution 10% ("Biopharma", Kyiv, Ukraine) with trypsin 0.1%. The process of enzymatic reactions was monitored at room temperature of \(22 \pm 1\) °C.

For UV–VIS spectrophotometry measurements, we used the water solution of IgG with a concentration of 10% ("Biopharma", Kyiv, Ukraine) which was diluted to a final protein’s concentration of 0.1% in distilled water. Separately, we prepared trypsin water solutions (1:1) (Merck, EU).

2.2. The waveguide differential microwave dielectrometry method

We used a differential microwave dielectrometer (Fig. 1, a) for the study of the enzymatic reaction course that was previously used for the measuring of the high-loss liquids like water solutions [16–19]. In the dielectrometer manufacturing, we used the generator in the microwave frequency range, where the frequency dispersion of the real and imaginary CP parts of water is observed. The cuvette consists of two identical cells (Fig. 1, b). The one cell contains a tested liquid and another one contains a reference liquid. As a reference liquid in this investigation, we used distilled water. The liquid volume in each measuring cell is 7 ml. The main component of each cell is the cavity body (1, Fig. 1, b) which is made in the form of a copper cup of the radius \(b = 10\) mm and inner quartz rod (2, Fig. 1, b) with the radius \(a = 2.5\) mm.

We used a generator at an operating frequency of 31.82 GHz [17]. The local oscillator at 31.82 GHz is a phase-locked loop transistor VCO at the frequency 7955 MHz with a reference quartz frequency standard, further multiplication by four and power amplification. The amplitude modulation of a microwave carrier with a frequency of 100 kHz and a synchronous detection were used in order to increase a signal-to-noise ratio. A Schottky diode and a low-noise amplifier were used as a microwave detector with a conversion factor of 25 mV/microW. The microwave power output from the generator is a rectangular waveguide whose cross-section is \(7.2 \times 3.4\) mm. The same waveguides are used in the microwave unit of the dielectrometer, except for the dielectrometer measuring cuvette.

The method of dielectrometry used in our experiments belongs to the methods of the differential type, and this is provided by the functional design of our dielectrometer. Specifically, the power of the microwave generator is divided by means of T-joint into two arms of the bridge, each of which contains a measuring cell with a liquid. To balance the bridge, it has an electrically controlled attenuator and a phase shifter located in the arms. After passing through the cells, the signals of two arms of the bridge are summed up by means of T-joint and fed to the detector.
At the first measurement step after the filling of both cells with the reference liquid, the temperature balance in differential cavity cells is achieved. Then, according to the microcontroller program, the minimum of the detector receiver signal is automatically found by tuning an attenuator and a phase shifter alternately. We register the readings of measurement amplitude $A_r$ (left graph on the computer monitor, Fig. 1, a) and the measurement phase $\varphi_r$ shifter (right graph on the computer monitor, Fig. 1, a) corresponding to the selected minimum. Then we empty one of the cells and fill it by the tested liquid which has the dielectric constant and loss tangent different from the reference liquid. As a result, the bridge balance is destroyed. This balance is recovered according to the procedure described above, and the measurement attenuator $A_t$ and the measurement phase shifter $\varphi_t$ readings correspond to a new minimum position.

Then we calculate the phase difference $\Delta \varphi = \varphi_t - \varphi_r$ and the amplitude difference $\Delta A = A_t - A_r$ of the $HE_{11}$ wave propagates in measuring cavity cells along the quartz rod with the surrounding high-loss liquid (Fig. 1, b). The instrumental measurement errors of the phase and amplitude determination are equal to $\delta \varphi_{\text{meas}} = \pm 0.025 \text{deg}$ and $\delta A_{\text{meas}} = \pm 0.0005 \text{dB}$, respectively. The instrumental measurement errors depend on the metrological characteristics of such measuring elements as the phase shifter and the attenuator. The longitudinal movement of the piston of the phase shifter for one step of engine rotation corresponds to the phase change by the value $\delta \varphi_{\text{meas}}$, and the piston positioning error does not exceed one step. The amplitude measurement error is determined by the low bit of the digital analog convertor, which controls the attenuation of the p-i-n attenuator and does not exceed the value of $\delta A_{\text{meas}}$. By measuring the phase and amplitude differences of the $HE_{11}$ wave in the case where the liquid in one cell is different from the reference one, we obtain the real $\Delta \varepsilon' = \varepsilon'_t - \varepsilon'_r$ and imaginary $\Delta \varepsilon'' = \varepsilon''_t - \varepsilon''_r$ CP parts differences non-zero values according to the procedure given below.

It is necessary to note that we calibrated our device before each measurement series by measuring different water-ethanol solutions, since their dielectric characteristics are known and are presented in many literature references [13, 14, 19].

2.3. Procedure of the CP determination of the tested liquid

For the structure of our measuring cavity cells with central dielectric rod surrounded by a liquid layer, it is possible to obtain an analytic solution of the characteristic equation (1) based on the solution of the Maxwell equations [19]. The characteristic implicit transcendence complex equation in the simplified form can be written as a function of the following variables:

$$F = F(\varepsilon'_r, \varepsilon'_t, \varepsilon''_t, h'_r, h''_t, h'_t, h''_t),$$  

where $\varepsilon'_r, \varepsilon'_t, \varepsilon''_t$ are the real and imaginary CP parts of the reference and tested liquids; $h'_r, h''_t, h'_t, h''_t$ are the real and imaginary parts of complex propagation coefficients of the $HE_{11}$ wave passing via the measurement cells that are filled by the reference and tested liquids; the symbols $r$ and $t$ are marked for the reference and tested liquids, respectively. The solution of Eq. (1) for a two-layered waveguide structure involves finding the roots of the equation. This can be done numerically using computational methods such as the shooting method. The real and imaginary CP parts of the tested liquid are determined by changing the characteristics of the propagating wave in the layered waveguide structure with the tested liquid. In our case, such characteristics as the attenuation $h''_t$ and the phase $h'_t$ coefficients values which are used to determine the CP of the tested liquid. The determination of the attenuation $h''_t$ and the phase $h'_t$ coefficients of the tested liquid is as follows. The wave attenuation $\Delta h''_t = \Delta A/l$ and the phase $\Delta h'_t = \Delta \varphi/l$ coefficients differences of the $HE_{11}$ wave propagates in the measuring cells with liquids are calculated, where $l = 2b$ is the quartz rod lengths. The attenuation coefficient $h''_t$ and the phase coefficient $h'_t$ of the wave in the cell with the tested liquid are calculated using rhe formulas: $h''_t = h''_r + \Delta h''_t$ and $h'_t = h'_r + \Delta h'_t$, where $h''_r$ and $h'_r$ received from solving the direct problem of the characteristic equation (1) using the known CP values of the reference liquid.

By solving the inverse problem for the measuring cell which is the characteristic implicit transcendence complex equation (1) by using the obtained value of $h''_t$ and $h'_t$, we performed the computer calculation of the real and imaginary CP parts of the tested liquid, $\varepsilon_t = \varepsilon'_t + i \varepsilon''_t$. To extract the real and imaginary CP parts of the tested liquid, we designed a

specific software program application which implemented in C++ Borland Builder 6.0. More detailed description of our differential microwave dielectrometry method and the procedure of CP determination are described in [19].

### 2.4. Dynamic conditions of the dielectrometry measurements

The determination of the real and imaginary CP parts under static conditions was performed at room temperature, and the temperature sensors (7) determine the temperature of liquids in both measuring cavity cells to be 22 °C. But the enzymatic reaction course monitoring was performed under dynamic conditions, and we observed the temperature deviation not more than 1 °C for the liquids in the measuring cavity cells during 40 min of the enzymatic reaction. The growths of the temperature of liquids in both cells during the enzymatic reaction were equivalent. This temperature deviation appeared as a result of the heating of cells associated with the influence of the microwave power. We find the differences of the real \( \Delta \varepsilon' \) and imaginary \( \Delta \varepsilon'' \) CP parts from dynamic measurements of the tested liquid in one cell relative to the reference liquid in another cell at the same temperature in both cells (see Fig. 3). The observed CP differences \( \Delta \varepsilon' \) and \( \Delta \varepsilon'' \) occur as a result of the increase in the amount of free water molecules in this reaction mixture associated with the enzymatic biochemical reactions.

We have excluded the temperature deviation from the microwave heating which occurs under dynamic measurement conditions to eliminate this influence on the CP values of the tested liquid. For this purpose, we use developed a special technique that is as follows. We measure the phase \( \Delta \varphi \) and amplitude \( \Delta A \) differences of the \( HE_{11} \) wave passing through each measurement cavity cell at current room temperature value. Knowing the CP of the reference liquid, \( \varepsilon_r = \varepsilon'_r + i\varepsilon''_r \), at the current temperature value and solving the characteristic equation (1) as the direct problem we find the wave phase \( h'_r \) and attenuation \( h''_r \) coefficients of the tested liquid at the current temperature measuring. The next step is solving the inverse problem and obtains the CP of the tested liquid \( \varepsilon_t = \varepsilon'_t + i\varepsilon''_t \) from the characteristic equation (1) at the same temperature using measured data. All measurements results were presented in this paper are obtained according to the procedure of the excluded the temperature deviation described above.

### 2.5. UV-VIS spectrophotometry method

We compare the dielectrometry measurement results with the UV-VIS spectrophotometry method results using GeneQuant 1300 spectrophotometer (USA, General Electric). The proteins (HSA and IgG) hydrolysis time depending process was monitored by falling the peptide bond absorption decreasing at 210 nm and interval in 30 sec by the use of spectrophotometer’s program “Kinetics”.

### 3. Results and Discussions

#### 3.1. Enzymatic reaction course monitoring by the differential microwave dielectrometry method

Our approach is based on the determination of physical parameters such as CP of the reaction mixture during the hydrolysis reaction and can be carried out non-invasively and continuously without any sampling from the reaction mixture. When the required properties of the reaction mixture are achieved (namely, obtaining the required ratio of hydrolysis products such as amino acids and oligopeptides), we can stop the hydrolysis reaction. By making the measuring cells as flow-through, thanks to the available drain holes (Fig. 1, 6), it is possible to continuously determine CP of the reaction mixtures without sampling, whereas the state-of-art methods mentioned in the introduction require a continuous sampling of the reaction mixture to monitor the course of the reaction.

Figure 2 presents the dependence of the differences for the real \( \Delta h' = h'_t - h'_r \) and imaginary \( \Delta h'' = h''_t - h''_r \) parts of the complex wave propagation coefficient for distilled water (1), and for the tested water solutions of protein IgG with the concentration equal to 2.5%, HSA with the concentration equal to 2.5 and 10% (points 2–4) and for water-protein enzymatic reaction mixture (lines 5–7), respectively. The error bars shown in the plots below include the average measurement error values based on the five-measurement series \( \delta h' = \pm 0.001 \text{ cm}^{-1} \) and \( \delta h'' = \pm 0.015 \text{ dB/cm} \) and the instrumental measurement errors \( (\delta \varphi = \pm 0.053 \text{ deg/cm} \) and \( \delta A = \pm 0.004 \text{ dB/cm} \). With an increase of the enzymatic reaction time, we observe a decrease of the real and
Fig. 2. The dependence of the differences for the real \( \Delta \tilde{h} \) (a) and imaginary parts \( \Delta \tilde{h}'' \) (b) of the complex wave propagation coefficient on IgG/HSA trypsinolysis reaction time. Numbers (1) denotes the base zero point with respect to water), points IgG water solution (2.5% concentration in water solution) (2), HSA water solution (2.5% concentration in water solution) (3), HSA water solution (10% concentration in water solution) (4), IgG (2.5%) with trypsin (0.1%) (5), HSA (2.5%) with trypsin (0.1%) (6), HSA (10%) with trypsin (0.1%) (7).

Fig. 3. The dependence of the differences of the real \( \Delta \varepsilon' = \varepsilon'_r - \varepsilon'_t \) (a) and imaginary \( \Delta \varepsilon'' = \varepsilon''_r - \varepsilon''_t \) (b) CP parts on the IgG/HSA trypsinolysis reaction time. The data correspond to those in Fig. 2, as well as points and curve numbers.

imaginary parts for differences of the complex wave propagation coefficient. Thus, we can recognize small differences in the tested liquid physical parameters changing in comparison with the reference liquid.

Figure 3 shows the dependence of the differences of the real \( \Delta \varepsilon' \) and imaginary \( \Delta \varepsilon'' \) parts of CP for the data presented in Fig. 2. The behavior of CP values depending on the enzymatic reaction time can be explained by the increase of the amount of free water in the IgG/HSA water solution with trypsin due to the breaking down of IgG or HSA protein molecules into smaller peptides by trypsin. These graphs can be used for the dynamic monitoring of the enzymatic reactions. Figure 3 shows that the lower the concentration of HSA, the greater the dielectric losses for the water-protein solution, and values \( \Delta \varepsilon' \) and \( \Delta \varepsilon'' \) tend to values of pure water. The rate of the enzymatic reaction is higher for solutions with a lower HSA concentration (2.5%), the slope of the curve equals \( \Delta \varepsilon'/\Delta t = 0.02, \Delta \varepsilon''/\Delta t = 0.024 \), respectively. For solutions with a higher HSA concentration (10%), we have \( \Delta \varepsilon'/\Delta t = 0.017, \Delta \varepsilon''/\Delta t = 0.008 \), respectively. Thus, the dielectrometry method is able to demonstrate the enzymatic reaction course by the determination of the CP of water-protein solutions with trypsin during the reaction time.

It was shown the change of the CP values at the given HSA/IgG concentration in water solution (the data are marked as points 2–4, Fig. 3) in comparison with the CP values of water. The CP values tended to decrease with increasing of the proteins concentration in water solution. For example, with the increase of the HSA concentration (from 2.5% to 10%) we observe the decrease of the real and imaginary CP parts of these water-protein solutions (the data are marked as points 3 and 4, Fig. 3) and these results are in a good agreement with the CP values for the same water-protein solutions obtained in references [20–22].

Thus, at the first-time the results of our study demonstrate the possibility of the enzymatic reaction (such as trypsinolysis) monitoring in real time by determine of the real and imaginary CP parts of this reactive mixture. We suppose that the breaking down of protein molecules into smaller peptides by trypsin is the result of the increase of the amount of free water molecules in solution and this leads to increase of the real and the imaginary CP parts with the going on the reaction time. Real time reaction monitoring by described dielectrometry measurements allows
us to propose our dielectrometry approach and our dielectrometry device for dynamic monitoring of the enzymatic reactions course.

3.2. Errors parsing of the CP determination

The CP values determined by solving an inverse problem solution (1), using the computer program in which we use the highly oscillating cylindrical Bessel functions. The numerical calculation error of the real and imaginary CP parts determination are equal to $\delta \varepsilon'_{\text{num}} = \varepsilon'_{\text{direct}} - \varepsilon'_{\text{inverse}} = \pm 0.34\%$ and $\delta \varepsilon''_{\text{num}} = \varepsilon''_{\text{direct}} - \varepsilon''_{\text{inverse}} = \pm 0.62\%$, respectively, which are origin from associated with the use of highly oscillating Bessel functions.

We estimated the sensitivity of determining the CP, associated with the absolute errors of the real and imaginary CP parts determination, as follows:

$$
\delta \varepsilon'_{\text{abs.}} = \sqrt{\left( \frac{\Delta \varepsilon'}{\Delta \varphi} \delta \varphi_{\text{meas.}} \right)^2 + \left( \frac{\Delta \varepsilon'}{\Delta A} \delta A_{\text{meas.}} \right)^2},
$$

$$
\delta \varepsilon''_{\text{abs.}} = \sqrt{\left( \frac{\Delta \varepsilon''}{\Delta \varphi} \delta \varphi_{\text{meas.}} \right)^2 + \left( \frac{\Delta \varepsilon''}{\Delta A} \delta A_{\text{meas.}} \right)^2}. 
$$

The absolute error values of the real and imaginary CP parts determination, for example for water-IgG with trypsin reaction mixture are equal in average to $\delta \varepsilon'_{\text{abs.}} = 0.5\%$ and $\delta \varepsilon''_{\text{abs.}} = 3\%$, respectively. This means that using our dielectrometer and approach, we can distinguish small differences in the change in protein concentration in solution during the enzymatic reaction course, associated with a change in the real and imaginary CP parts of 0.5% and 3%, respectively. The obtained error values of the real and imaginary CP parts are comparable with the corresponding errors obtained by other microwave techniques for water-protein solutions [20, 22].

3.3. Validation the differential microwave dielectrometry method with the spectrophotometric method

To validate our dielectrometry method applicability to the monitoring of enzymatic reactions, we evaluated the correlation of the differential microwave dielectrometry measurement results involving the proteins IgG/HSA in trypsin-water solutions with the results measured by the UV-Vis spectrophotometry method. For this purpose, we use the obtained imaginary part of the wave propagation coefficient $h''$ (attenuation of the $HE_{11}$ wave) at a wavelength of 9.4 nm (differential microwave dielectrometry method) and the wave absorbance $A$ at a wavelength of 210 nm (UV-Vis spectrophotometry method). As is known, Fisher’s correlation coefficient [23] is commonly used as the correlation method in the biometrical studies and can be written as

$$
F = \frac{\sum_{i=1}^{n} (x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum_{i=1}^{n} (x_i - \bar{x})^2 \sum_{i=1}^{n} (y_i - \bar{y})^2}},
$$

where $(x_i - \bar{x})$ and $(y_i - \bar{y})$ represent deviations from their respective means. We take the wave attenuation coefficient $h''$ as $x_i$ and the absorbance $A$ as $y_i$. The absorbance values obtained by two experimental methods are correlated, if the concentration ratio of 'protein:enzyme' is 1:100 (i.e., 1 mg/ml trypsin and 100 mg/ml protein). Fisher’s correlation coefficients for water solutions of IgG/HSA with trypsin are presented in Tables 2 and 3.

In Fig. 4, we compare the time dependences of the absorbance measurement results for two methods.

Table 2. Fisher’s correlation coefficient for water solutions of IgG with the trypsin concentration presented in Fig. 4. Measurement type: microwave dielectrometer (1), UV/Vis spectrophotometer (2)

<table>
<thead>
<tr>
<th>Measurement type</th>
<th>IgG concentration, %</th>
<th>Trypsin concentration, %</th>
<th>Solute ratio (trypsin : IgG)</th>
<th>$F$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.5</td>
<td>0.1</td>
<td>1:25</td>
<td>0.87</td>
</tr>
<tr>
<td>2</td>
<td>0.1</td>
<td>0.001</td>
<td>1:100</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Fisher’s correlation coefficients for water solutions of HSA with trypsin concentrations presented in Fig. 4. Measurement type as in Table 2

<table>
<thead>
<tr>
<th>Measurement type</th>
<th>HSA concentration, %</th>
<th>Trypsin concentration, %</th>
<th>Solute ratio (trypsin : HSA)</th>
<th>$F$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>0.1</td>
<td>1:100</td>
<td>0.93</td>
</tr>
<tr>
<td>2</td>
<td>0.002</td>
<td>0.00002</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2.5</td>
<td>0.1</td>
<td>1:25</td>
<td>0.64</td>
</tr>
<tr>
<td>2</td>
<td>0.002</td>
<td>0.00002</td>
<td>1:100</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 4. The time dependence of the absorbance intensity of the peptide bonds in the protein dissolved in water with trypsin in the UV/Vis spectrophotometer cavity at the wavelength 210 nm for two types of proteins: (1) IgG with the concentration 0.1% and trypsin with the concentration 0.001%; (2) HSA with the concentration 0.002% and trypsin with the concentration 0.00002% (a). Dependences of the imaginary parts of the complex wave propagation coefficient in the dielectrometer cell of the tested liquid on the reaction time for water solutions of: (1) IgG, 2.5% with trypsin 0.1%; (2) HSA, 2.5% with trypsin 0.1%; (3) HSA, 10% with trypsin 0.1% (b).

Methods for water solutions of IgG/HSA with trypsin in enzymatic reaction mixtures using Fisher’s correlation coefficient. Figure 4, a shows the linear dependences of the absorbance at the wavelength 210 nm of IgG/HSA dissolved in water with trypsin on the time. According to this dependence, it is possible to trace the enzymatic reaction course in the time, by determining the amount of IgG/HSA protein not destroyed by trypsin. We get a similar result for the time dependence of the imaginary part of the complex wave propagation coefficient of the enzymatic reaction course, which is shown in Fig. 4, b. The linearity of the time dependence of the absorbance for protein solutions with trypsin is related to a change in the concentration of proteins in the solution due to the reaction with trypsin (Fig. 4 a and b). The results are in a good agreement with those obtained in [6, 24].

We have found a good correlation for the time dependence of the absorbance of the tested solution within two methods. In this case, the Fisher correlation coefficient is equal to 0.87 for the IgG water solution with trypsin (Fig. 4, a and b, curve 1-IgG) and to 0.93 for HSA at the concentration 0.002% with trypsin at the concentration 0.00002% and HSA at the concentration 10% with trypsin at the concentration 0.1% (Fig. 4, a and b, curve 2-HSA). When we compare the absorbance values for the solution with the ratio of the concentrations of trypsin and HSA/IgG equal to 1:100, the Fisher correlation coefficient is close to unity. But if we take different ratios of concentrations in the experiments executed by both methods, the Fisher correlation coefficient will significantly deviate from 1 (e.g., we have got its value as low as 0.647). For the same ratio of concentrations in the compared methods, we have Fisher correlation coefficient close to unity, and we can say that our microwave dielectrometer method has been validated by the known UV-Vis one.

We can see from Fig. 4 that the decreasing of the time dependence of the absorbance correlates with the decreasing of the number of peptide bonds –CO–NH– owing to the hydrolysis of a protein (IgG or HSA) by trypsin. Fisher’s correlation coefficient takes values that are close to unity, which testifies to an excellent correlation and to the possibility of using a dielectrometer for the dynamic monitoring of the enzymatic protein hydrolysis. Although various concentrations of the enzymatic substrate (like IgG or HSA) were taken for research, the ratio of the 'protein : enzyme' must be remained the same (in our case, it is 1 : 100) to have the opportunity to compare and verify our considered methods.

Thus, in this investigation, we have confirmed the repeatability of our differential microwave dielectrometry method and approach for the monitoring of the enzymatic hydrolysis of proteins, and the verification of the proposed method with the spectrophotometric method has been successfully performed.

4. Conclusion

We have demonstrated, for the first time, the effectiveness of the usage of the differential microwave dielectrometry as a non-destructive and express method for the monitoring of the enzymatic reaction course in real time. The approach proposed and tested in this study is based on the results of the CP determination in the reactive mixtures (containing selected proteins such as IgG and HSA, enzyme trypsin, and water as a solvent) during the hydrolysis of proteins. It
is shown experimentally that the real and imaginary parts of the CP are increasing with the enzymatic reaction progress in time. The obtained results can be explained by changing the balance of bound and free water molecules in the reaction systems with the trypsinolysis reaction course. We suppose that it relates to decreasing the amount of water molecules bound to molecules of polypeptides (products of the hydrolysis of HSA or IgG proteins) in the course of the reaction in comparison with the amount of bound water molecules at the beginning of the enzymatic process. The applied microwave differential microwave dielectrometry method of CP determination is characterized by the high sensitivity (for real and imaginary CP parts in average as 0.5% and 3±5%, respectively).

The dielectrometry measurement results of the enzymatic reaction course are confirmed by the results of the UV-Vis spectrophotometry for solutions of IgG/HSA and trypsin, as evidenced by the calculated Fisher’s correlation coefficients close to unity. So, we can say that our differential microwave dielectrometry method has been validated by the widely known UV-Vis one.

Thus, the proposed microwave differential microwave dielectrometry approach and the setup are prospective to be applied for monitoring the dynamic protein hydrolysis process in the biotechnological practice. We believe that, by making our measuring cells flow-through, we will get the possibility to apply our dielectrometry approach on the industrial scale for the monitoring of enzymatic reactions.


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