A NOVEL METHOD FOR THE DETERMINATION OF DIFFUSION COEFFICIENTS

IN AMORPHOUS POLY(3-HYDROXYBUTYRATE)

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ABSTRACT

A novel approach is proposed for the determination of the diffusion coefficient of certain drugs in amorphous poly(hydroxybutyrate) (PHB), which can be a reliable alternative to the conventional permeation based measurements. The method requires the preparation of PHB films with various concentrations of the drug and if the latter absorbs in the visible wavelength range, its concentration gradient in the polymer film as well as the time dependence of the latter can be analyzed quantitatively by following changes in color. Color can be converted into concentration with the help of adequate calibration and thus the dependence of additive concentration on space (x) and time (t), i.e. the $c(x,t)$ function, can be determined relatively easily. The fitting of the numerical solution of Fick's second law onto the measured values provides directly the targeted diffusion coefficient. The comparison of diffusion coefficients obtained by the proposed approach to values published in the literature proved that the new method provides reliable results and requires reasonable time and effort at the same time.

1. INTRODUCTION

Among microbial polyesters, poly(3-hydroxybutyrate) (PHB) gained considerable attention mainly because numerous fermentation techniques offer rather simple and cost efficient ways to produce it with recombinant E. coli strains [1-4]. Using it in medical and generally *in vivo* applications [5-13] seems to be obvious, since the polymer yields only nontoxic metabolites [14-17]. One of the most important and most intensively studied area is the use of poly(3-hydroxybutyrate) as drug carrier matrix [18-23].

The physical and chemical properties of the matrix must be inevitably known in order to develop a PHB based drug carrier device with actual commercial potential. If we consider this application, the kinetics of drug release must be determined, but quantitative description requires the knowledge of the diffusion coefficient. This characteristic might be measured and/or calculated by a number of approaches already published in the literature [24-37].

The simplest way to determine the unknown diffusion coefficient of a liquid substance is to immerse the polymer into it and measure weight as a function of time [24-31]. The rate of the sorption is related to the diffusion coefficient. However, the calculation of the diffusion coefficient generally yields results strongly depending on the method applied. The mathematically exact way of evaluation requires the application of the numeric solution of Fick's second law, as proposed by Fujita in 1952 [24] and later by Crank and Park [25]. The solution yields a sum of functions, i.e.

$$\frac{m_t}{m_\infty} = 1 - \frac{8}{\pi^2} \sum_{m=0}^{m=\infty} \frac{1}{(2m_0+1)^2} \exp\left[-\frac{D(2m_0+1)^2 \pi^2}{l^2} t \right] \qquad (1)$$

where m_0 is the initial, m_∞ the final weight of the sample, m_t is the weight measured at time t, D the diffusion coefficient and l the thickness of the sample. Even though the solution is mathematically exact, its direct fitting onto the measured points is difficult either with

conventional linearization-based methods or with more advanced nonlinear iteration algorithms. In order to overcome this problem, several authors simplified the mathematically exact solution [26-31].

Iordanskii [28], for example, introduced a model derived from the solution above (see **Eq. 1**), which provides acceptable results only at longer times, in the region of $m_t/m_\infty > 0.5$

$$\frac{m_t}{m_\infty} = 1 - \frac{8}{\pi^2} \exp\left[-\frac{D}{l^2} t\right] \tag{2}$$

On the other hand, Sultana [30] considered exclusively the beginning of the process and applied an even more simplified derivative of Eq. 1

$$\frac{m_t}{m_\infty} = 2\left[-\frac{D}{\pi l^2} t\right]^{1/2} \tag{3}$$

As the right hand side is expressed as the square root of time, the function inevitably diverges as t→∞, but the authors claimed that their model provides an appropriate estimate in the proximity of t=0 [30]. Yoon [31] used an even more simplified approach and calculated the diffusion coefficient of water in PHB by the substitution of the measured data into the following model

$$D = \frac{\pi}{16} \left[\frac{d\left(\frac{m_t}{m_\infty}\right)}{d\left(\frac{\sqrt{t}}{l}\right)}\right]^2 \tag{4}$$

in which the calculation of the differential expression requires the simple measurement of consecutive points at the beginning of the sorption study [31].

Permeation studies represent another approach for the determination of the diffusion coefficient. In these measurements the diffusion of an arbitrary permeant is monitored from a donor cell to an acceptor cell through a membrane made of the investigated polymer [32-

34]. Monitoring in the acceptor cell can be done by the measurement of pressure, in the case of gases, or by the determination of the concentration of the diffusing molecule by spectroscopic methods for other substances [32]. The vast majority of publications assumes an exact linear correlation between the permeation and the diffusion coefficients [32-34], and the latter is obtained by the division of the measured permeation parameter by a linear factor (*S*), the physical meaning of which is defined rather diversely. The simple correlation between the two coefficients is the following [32-34]

$$D = \frac{P}{S} \tag{5}$$

The controversy determining the diffusion coefficient from permeation measurements lies in the interpretation of the linear factor, *S*. Bergstrand [33], for example, substituted the parameter (*S*) simply with the thickness of the film and claimed to obtain exact results. However, others question his conclusions. Iordanskii [27] interpreted *S* as a solubility related parameter, and used the Flory-Huggins interaction parameter derived from group contributions to calculate it. However, the Flory-Huggins lattice model has a number of weaknesses and the calculation of the interaction parameter from solubility parameters is a further simplification. Because of these difficulties in the calculation of the diffusion coefficient, Poley [34] omitted this step and published only the permeation coefficients themselves.

Recently more advanced quantitative and/or qualitative measurement techniques are used to overcome the deficiencies of sorption and permeation methods [35-37]. Yang et al. [35] followed the diffusion of water molecules into a PHB-HV polymer phase by FTIR spectroscopy on films immersed into water. They used the absorption band appearing at 3400 cm^{-1} for the determination of the amount of water absorbed. They calculated the diffusion coefficient by applying a mathematical model similar to the numeric solution of

Fick's second law [25]. The authors obtained a diffusion coefficient which agreed well with the one published by Iordanskii [26,27]. The main drawback of the approach is that the permeant must be a liquid in order to be able to submerge the polymer into it. The method developed by Kosenko [37] to study molecular diffusion in polymers is based on NMR measurements [37]. He discusses diffusion by the interpretation of time dependent NMR spectra, but does not calculate the actual values of the diffusion coefficient.

The goal of our work was to propose a method which addresses the issues discussed above. In our arrangement, the drug is dispersed homogeneously in an amorphous PHB film and then the formation of a concentration gradient is initiated along the length of the polymer sample. The time dependence of the concentration gradient is followed and recorded continuously. If the diffusion of the drug obeys Fick's laws, which is generally the case, a numeric solution of Fick's second law is expected to provide a fairly accurate approximation of the measured data. The fitting of the numeric solution onto the measured points yields directly the diffusion coefficient, which would have been otherwise especially difficult to calculate.

2. EXPERIMENTAL

2.1. Materials

Poly(3-hydroxybutyrate) (Mirel M2100, ≥99.5 % purity) was obtained from Metabolix Ltd. in the form of granules with an approximate crystallinity of 60 %. Technical grade chloroform and denatured ethanol were supplied by Molar Chemicals Ltd. with the purity of 98 and a 96 %, respectively. The drug applied as model compound for the determination of diffusion coefficient was quercetin, [2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-chromen-4-one], a natural antioxidant found in several fruits and vegetables,

and it was purchased from Sigma-Aldrich with ≥95 % purity.

2.2. Film preparation

Films were cast onto a glass surface from 3 m/m% chloroform solution of poly(3-hydroxybutyrate). The solution contained quercetin at the concentration of 0.5, 1.0, 1.5, 2.0 and 2.5 mg/ml (mg quercetin/ml solution). Subsequently, the films were stored at constant temperature (25 °C) and relative humidity (50 %). The diffusion measurements were done on rectangular strips of 40 x 100 mm dimensions cut from the films.

2.3. Diffusion experiments

100 ml ethanol was poured into a 500 ml Erlenmeyer flask and then the 40 mm long edge of the film was submerged into it. The other end of the film was fastened at the top of the flask with a paper clamp to keep the film in vertical, upright position throughout the entire measurement. A photo was taken from the film just before dipping its end into the solvent and then further photos were recorded in every 60 minutes over a period of 7 hours. Each film was photographed eight times to capture the temporary state of the concentration gradient along the 10 cm length of the studied PHB film. In order to minimize the influence of systematic and stochastic errors inherent to the nature of the approach, each film was placed in front of the same white sheet in a room having constant lighting conditions.

2.4. Data processing

The photographs taken from the films were cut to size in order to ensure that each pixel of the image corresponds to a discrete point on the film. The images were then resized to 400 x 160 pixel resolution. A software was developed in MATLAB environment, which converts the hexadecimal color codes of the bitmaps to 400 x 160 x 3 multidimensional

matrices containing the RGB (red, green and blue channel) values of the picture. From the RGB values, the software calculates the HSL (hue, saturation and luminosity) values as well. The saturation parameter depends linearly on the amount of quercetin in the polymer. Since the concentration and thus the color gradient develops along the 10 cm length of the film, the pixels in the other, vertical direction are assumed to have the same saturation value.

During signal processing the measured data are inevitably loaded with a stochastic noise, but the signal to noise ratio can be further improved. This is done by the averaging of saturation values along the horizontal coordinate resulting in the minimization of the bias caused by the stochastic noise. As a consequence, the 400 x 160 matrices containing the saturation parameters are reduced to 400 x 1 arrays, in which each element contains the average of 160 values.

3. RESULTS AND DISCUSSION

The results are discussed in several sections. The determination of concentration gradients and the quantification of primary results are presented first, followed by the discussion of release kinetics. Numerical analysis leading to the targeted diffusion coefficient is described in the next section, while the advantages and the drawbacks of the approach are discussed in the last part of the paper.

3.1. Concentration gradients, quantification

The determination of the diffusion coefficient (D) of a small molecular weight compound is based on the development of a concentration gradient in a film, the quantification of the gradient and the calculation of D by applying Fick's laws. The concentration gradient is visible by the naked eye, if the compound used as probe has a distinct color and discolors the polymer. Photos taken from amorphous PHB films before

starting the experiment (**Fig. 1a**) and after 5 hour release (**Fig. 1b**) are shown in **Fig. 1.** The mere observation of the films in **Fig. 1b** shows that the right (lower) edge of the film became completely transparent, while the left retained its original color, i.e. the concentration gradient hoped for develops indeed during the release experiment. However, the determination of the unknown diffusion coefficient requires an adequate quantitative analysis of the images shown above.

Color gradient was converted into concentration gradient by calibration. Films were prepared with various, known concentrations of quercetin and their color was determined to obtain a calibration curve. In order to improve the signal to noise ratio, the concentration of quercetin was set to rather large values to obtain films with an intense yellow color (**Fig. 1**). However, this approach has its drawbacks as well; at large concentrations of the absorbing molecules, the concentration vs. absorbance correlation is not linear (**Fig. 2**). Due to the nonlinear correlation between quercetin concentration and the calculated saturation values, the calibration curve was approximated with a third order (cubic) polynomial. Saturation values were converted into actual concentrations by using the parameters of the regression curve (1.872, 1.802, -0.02433, 1.092E-4 for the constant, linear, second and third order term, respectively).

Release experiments of different duration were carried out in order to obtain the time dependence of diffusion. Using the calibration correlation discussed above, all of them were converted into concentration gradients, which are presented in **Fig. 3**. The gradients clearly correspond to the expectation, the concentration value at the upper end of the film indicates the initial value of quercetin added to the film, while it is zero at the other end, at the spatial coordinate of 100 mm. Fick's second law is applied to calculate the diffusion coefficient of quercetin in the amorphous PHB film from the time dependence of the concentration gradients shown in **Fig. 3**.

3.2. Release kinetics

Quercetin molecules move continuously from the upper end of the film towards the ethanol phase as long as any drug remains in the PHB film. The amount of drug entrapped in the polymer phase at a given moment can be determined from the volume integral of concentration as a function of the spatial coordinates in the following way

$$m_q = \int_{x_0}^{x_1} \int_{y_0}^{y_1} \int_{z_0}^{z_1} c_q(x, y, z)\, \mathrm{d}x\mathrm{d}y\mathrm{d}z \tag{6}$$

where m_q is the total amount of quercetin in the film, while c_q its concentration at the given location. However, in our case the cross section of the film has an approximately constant geometry along the x axis, which provides the possibility to simplify the volume integral shown above considerably, i.e.

$$m_q = A \int_{x_0}^{x_1} c_q(x)\, \mathrm{d}x \tag{7}$$

The solution of **Eq. 7** yields the amount of quercetin molecules present in the polymer at a given moment and its time dependence describes release kinetics quantitatively. Quercetin concentration calculated in the way presented above is plotted against time in **Fig. 4** showing the kinetics of the diffusion of the additive in PHB.

The rate of drug release depends on the amount of drug still present in the polymer, therefore, it is faster at the beginning of the experiment and slows down with time. Diffusion is a first order process and it can be approximated by an exponential function (**Eq. 8**)

$$c_q = C_0 \left[1 - \exp\left(-\frac{t}{\tau} \right) \right] \tag{8}$$

where C_0 is the initial concertation of quercetin in the polymer phase, while τ is the time constant of diffusion. Such a function was fitted to the experimental points and is shown by the continuous line in **Fig. 4**. Although **Eq. 8** provides an acceptable approximation of the

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measured data, it is not an exact solution, but only highlights the global characteristics of the time dependent release of the drug. For a detailed discussion of the exact representation of dissolution, please refer to section 3.3.

The release kinetics of quercetin from PHB is influenced by a number of factors some depending on the physical and chemical properties of the carrier matrix, while others on the drug itself. The properties of the polymer were kept constant in this study, but we investigated the effect of the initial concentration of quercetin on the kinetics of its release from the polymer film.

The time constant of diffusion (τ) is plotted against the initial concentration of quercetin in **Fig. 5**. As shown by the figure, release rate depends strongly on the initial concentration of the drug, at larger initial quercetin concentration less time is required for the release of a given amount. This correlation can be attributed to and explained by the first law of Fick stating that the molar flux of a diffusing matter is determined by the concentration difference between adjacent spatial coordinates, larger difference leads to faster diffusion. However, the first law of Fick does not provide any information about the time dependence of the process, the diffusion coefficient can be determined by the fitting of Fick's second law to the quantitative results presented in **Fig. 3**.

3.3. Diffusion coefficient

Fick's second law is usually expressed in the form of a partial second order linear differential equation

$$\frac{\partial}{\partial t}[c(x,t)] = D \frac{\partial^2}{\partial x^2}[c(x,t)] \qquad (9)$$

which, at the initial and boundary conditions of our experiments, i.e. constant initial concentration profile and permanently zero concentration at one particular end of the

investigated film, does not have an analytical solution. On the other hand, **Eq. 9** can be solved numerically. In order to find an appropriate numerical solution for **Eq. 9**, a software was written in the Origin C development environment.

Since the numerical solution of Fick's second law yields a function with one dependent (concentration) and two independent (spatial coordinate, x and time, t) variables, one might present it as a surface of concentration values over the x and t (position and time) plane as shown in **Fig 6**. The $c(x,t)$ surface presented in the figure contains all the information of a release study. On the other hand, it is rather difficult to interpret and/or understand such a three dimensional plot. As a consequence, a number of discrete concentration profiles developing at various times has been selected and they are plotted against the spatial coordinate in **Fig. 7**. The correlations plotted in the figure are very similar to those presented earlier in **Fig. 3**.

The quantitative analysis and the fitting of a function to the correlations of **Fig. 7** requires the knowledge of the unknown diffusion coefficient. Since D is not known, an iteration procedure must be applied to determine its value. Starting with an arbitrarily selected value of the diffusion coefficient, the iteration is carried out until the fitted function corresponds to the one calculated from **Eq. 9**. The result of the fitting procedure is demonstrated in **Fig. 8** comparing the measured and calculated concentration gradient for a selected polymer film. As the figure shows, the agreement between the calculated and measured profile is excellent.

The fitting procedure was carried out for polymer films containing the model drug in various concentrations and the diffusion coefficient of the compound was determined in each case. The values obtained are presented as a function of the initial quercetin content of the films in **Fig. 9**. Under ideal conditions the iteration should have resulted in five equal diffusion coefficients, since according to the principles of the diffusion theory it is expected

to be independent of both the dependent (concentration) and the independent (spatial coordinate, time) variables. However, the calculated diffusion coefficients depend slightly on concentration, diffusion increases with increasing amount of quercetin in the films. The deviation from the expected behavior needs further considerations and explanation.

3.4. Considerations, discussion

One of the possible explanations might be related to the physical ageing of the polymer. PHB, like the majority of the family of poly(hydroxyalkanoates) and most of the aliphatic polyesters, undergoes relatively fast and extensive physical ageing. During physical ageing a number of physical and chemical parameters of the polymer change considerably with time. One of these parameters is free volume which plays an important role in diffusion. The direct determination of free volume is difficult. Positron annihilation spectroscopy is the most frequently used technique these days [38], but both the theory behind and the measurement itself are rather complicated. On the other hand, observations related to the physical characteristics of the polymer film used for the study might give information about the effect of additive concentration on free volume and the change in the value of diffusion coefficient with increasing concentration.

In our case, physical ageing could be followed relatively simply by the measurement of the thickness of the film as a function of time and quercetin concentration. The initial ~50 μm thickness of the film decreases below 40-45 μm in several hours, if the film does not contain any quercetin. On the other hand, the thickness of films containing the drug changes only slightly or not at all even after longer times. During physical ageing the conformation of the macromolecules approaches to equilibrium resulting in a reduction of free volume. It has been shown earlier that the physical ageing process of poly(lactic acid) was considerably modified by basically all additives introduced into it including, fillers, wood flour, glycerol,

other polymers, etc. [39]. The addition of these second components led to faster cold crystallization and a decrease in the glass transition temperature of the polymer. Obviously, the additives modified the mobility of the molecules resulting in a structure closer to equilibrium. Apparently quercetin has a similar effect on the structure of PHB and physical ageing as well as free volume depends on the amount of the drug added. Increased mobility of the polymer chains results in faster diffusion and larger diffusion coefficients and the small, but clear increase in its value as a function of quercetin content. Further study is needed to prove the tentative explanation offered above and account for the slight dependence of the diffusion coefficient on physical ageing, i.e. time.

Another issue that must be considered is the reliability of the method proposed and that of the diffusion coefficient determined. Since the diffusion of quercetin in PHB has not been studied yet, we must compare our results to those published in the literature for other compounds. In this case, however, we must keep in mind that sample preparation and measurement conditions as well as the calculation methods were different and all influence the obtained values. The diffusion coefficient determined by us is compared to published data in **Table 1**. Since the rate of diffusion is determined by the size of the diffusing molecule and its physical-chemical properties, an approximate size and the dipole momentum of the studied materials are also included in the table.

One of the molecules the diffusion of which is quite widely investigated in PHB is water. Water is much smaller than quercetin, but it is capable of forming strong hydrogen bonds with the ester groups of the polymer. Iordanskii [26,27], Miguel [29], Sultana [30], Yoon [31] and Yang [35], all determined and published diffusion coefficients. The values are scattering in the wide range of $1.1 \cdot 10^{-11}$ cm^2/s – $7.0 \cdot 10^{-8}$ cm^2/s, but most of the values are found in between $2.3 \cdot 10^{-9}$ cm^2/s and $7.0 \cdot 10^{-8}$ cm^2/s. Taking into consideration the differences in the measurement techniques and the applied mathematical methods, the

agreement among the results reported is fairly acceptable.

Since one of the possible uses of PHB is as a carrier material in controlled release devices, the diffusion of various active molecules was also determined in this polymer. Bonartsev [28], for example, studied the diffusion of two frequently used drugs (indomethacin and dipyridamole) in PHB. Based on dissolution data, the author calculated the apparent diffusion coefficients with a method which considers only the final part of the dissolution correlation (see **Eq. 2**). The value of the diffusion coefficient showed considerable concentration dependence, but depended also on the average molar mass of the polymer and on the thickness of the film. The reported values of $1.7 \cdot 10^{-7}$ cm^2/s - $2.6 \cdot 10^{-6}$ cm^2/s [28], are much larger than those obtained for water [26, 27, 29-31, 35]. Sanchez-Garcia investigated the diffusion of a model drug (limonene) in PHB nanocomposites [36], but determined the diffusion coefficient also in a polymer not containing any reinforcement. The actual value reported by Sanchez-Garcia is $2.0 \pm 0.2 \cdot 10^{-8}$ cm^2/s [36], which is rather close to the values determined by us in spite of procedural differences. The similarity of our results to published data confirms that the approach proposed in this paper yields reasonable diffusion coefficients in a simple way, which is much less tedious than the sorption, permeation or other spectroscopy based experiments often used in practice.

4. CONCLUSIONS

In this work, an approach is proposed for the determination of the diffusion coefficient of certain drugs in amorphous PHB which can be a reliable alternative to the conventional permeation based measurements. If the drug absorbs in the visible wavelength range, its concentration gradient in the polymer film as well as its time dependence can be analyzed quantitatively by following changes in the color of the film. Color can be converted into concentration with the help of adequate calibration and thus the dependence of additive

concentration on space and time, i.e. the $c(x,t)$ function can be determined relatively easily. The fitting of the numerical solution of Fick's second law onto the measured values provides directly the targeted diffusion coefficient. The comparison of diffusion coefficients obtained by the proposed novel approach to values published in the literature proved that this new method provides reliable results in a relatively simple and easy way.

5. ACKNOWLEDGEMENTS

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Table 1   Comparison of the diffusion coefficients of different compounds. Size and

dipole moment are included for reference.

Permeant	Size[a] (Å)	Dipole moment (D)	Diffusion coefficient (cm^2/s)	Ref.
Water	1.63	1.78	$2.3 \cdot 10^{-9}$-$7.0 \cdot 10^{-8}$	26, 27, 29-31, 35
Indomethacin	13.7	1.83	$2.0 \cdot 10^{-7}$-$2.5 \cdot 10^{-6}$	28
Dipyridamole	14.5	2.55	$1.7 \cdot 10^{-7}$-$2.6 \cdot 10^{-6}$	28
Limonene	8.32	0.44	$2.0 \pm 0.2 \cdot 10^{-8}$	36
Quercetin	11.8	1.43	$3.1 \cdot 10^{-8}$-$3.7 \cdot 10^{-8}$	this work

a) Longest edge of the smallest cuboid capable of containing each atom in the molecule
   calculated with MOPac (Molecular Orbital Package) version 17.0.012e.

CAPTIONS

Fig. 1   Photographs of amorphous PHB films containing 2.0 mg/g quercetin a) before the release test, b) after 5 hours.

Fig. 2   Calibration curve for the determination of the quercetin concentration of PHB films from their color.

Fig. 3   Concentration gradients of quercetin in PHB films recorded with a time interval of one hour throughout a period of 7 hours. Initial quercetin content: 22.4 mg/g.

Fig. 4   Total amount of quercetin dissolved in the matrix polymer plotted against release time. Initial quercetin content: 1 mg/g. An exponential function was fitted to the experimental data to describe kinetics quantitatively (continuous line).

Fig. 5   Effect of initial quercetin concentration on its rate of release (1/h) from PHB into ethanol.

Fig. 6   Numerical solution of Fick's second law calculated by using the initial and boundary conditions corresponding to the release experiments used.

Fig. 7   Two dimensional representation of the calculated $c(x,t)$ surface.

Fig. 8   Fitting of the calculated concentration gradient (dashed line) onto the measured one (solid line). Initial quercetin concentration: 22.4 mg/g, release time: 5 hours.

Fig. 9   Effect of initial concentration quercetin on its diffusion coefficient in the PHB films used in the experiments.
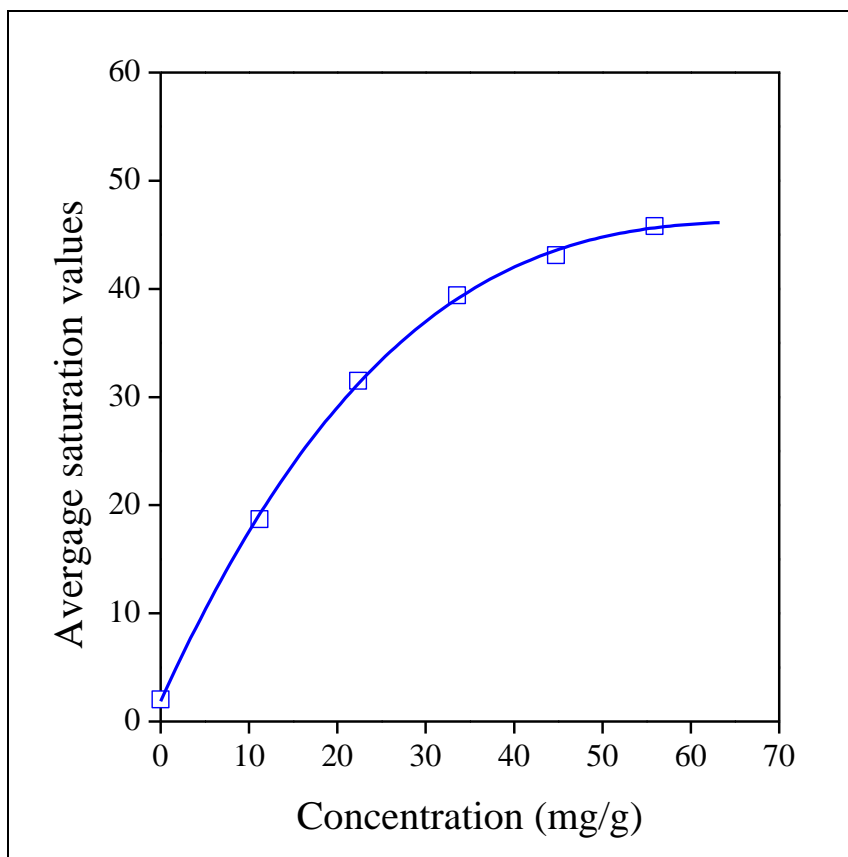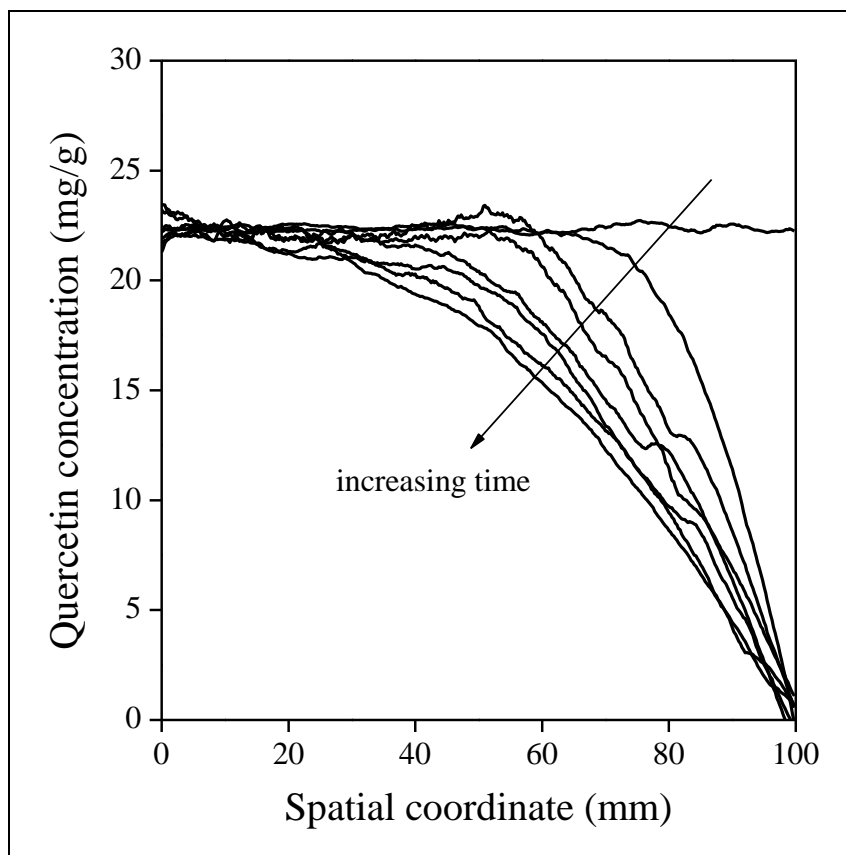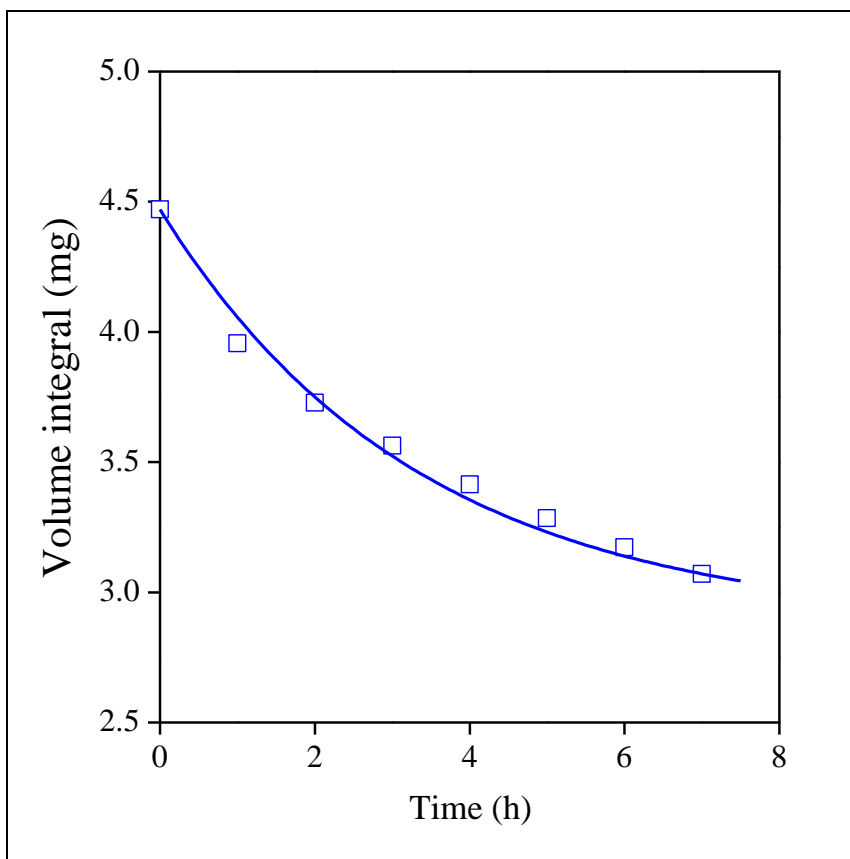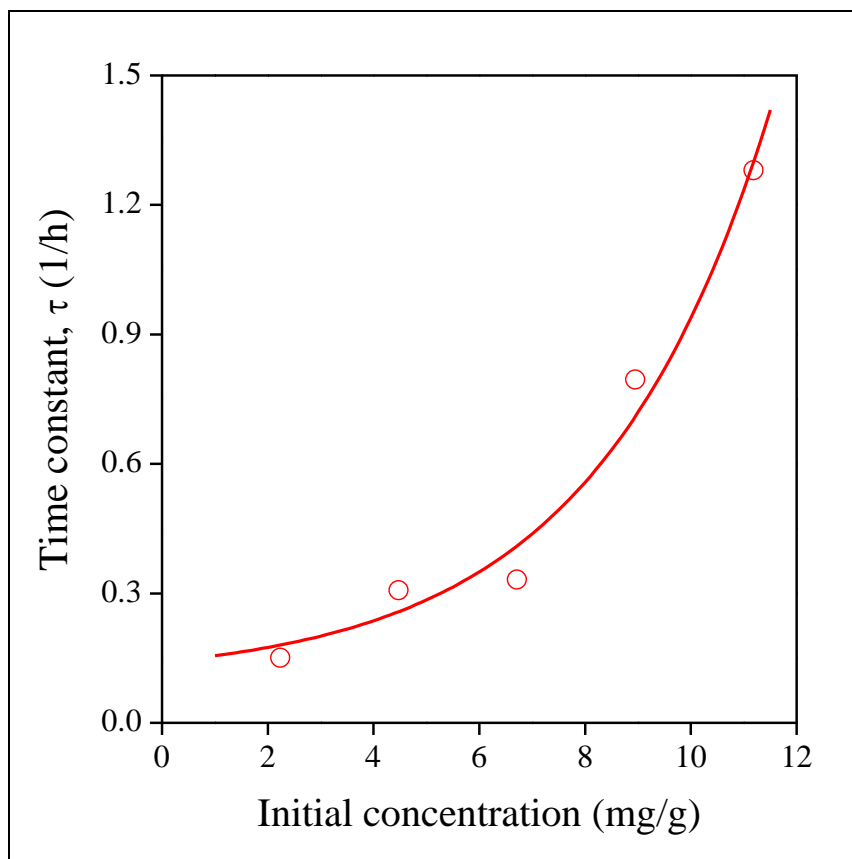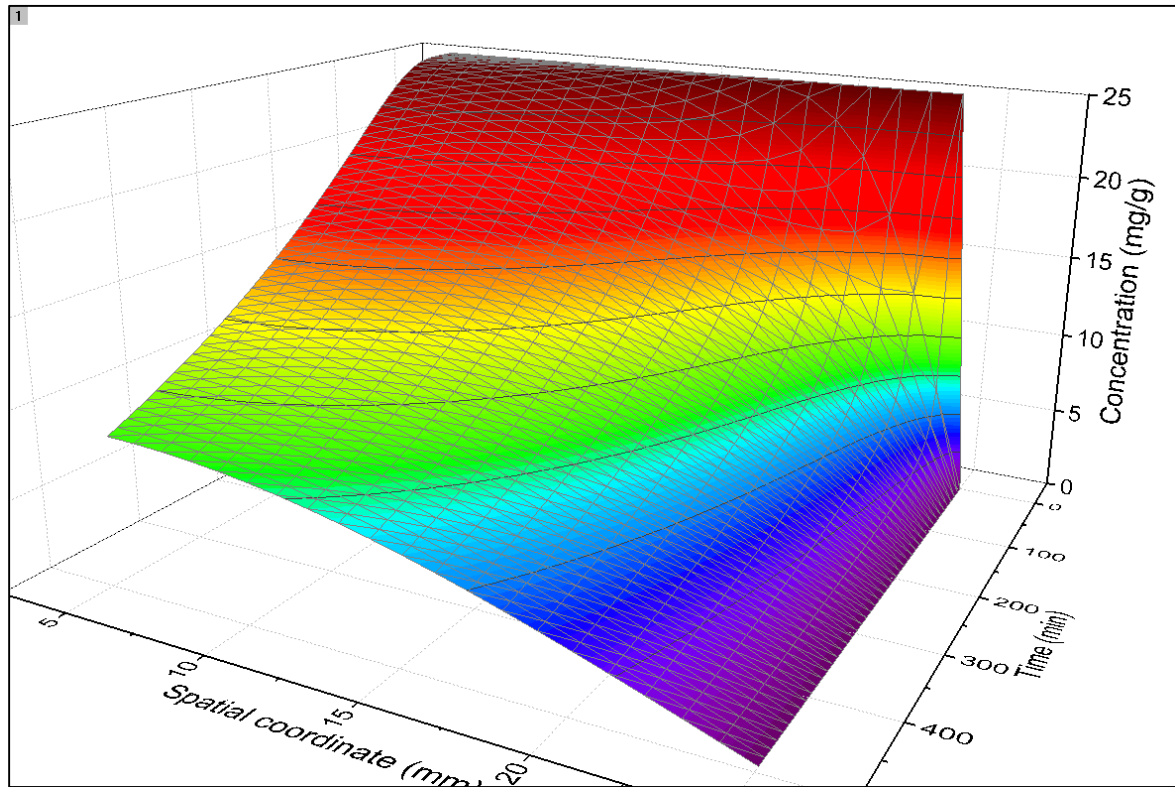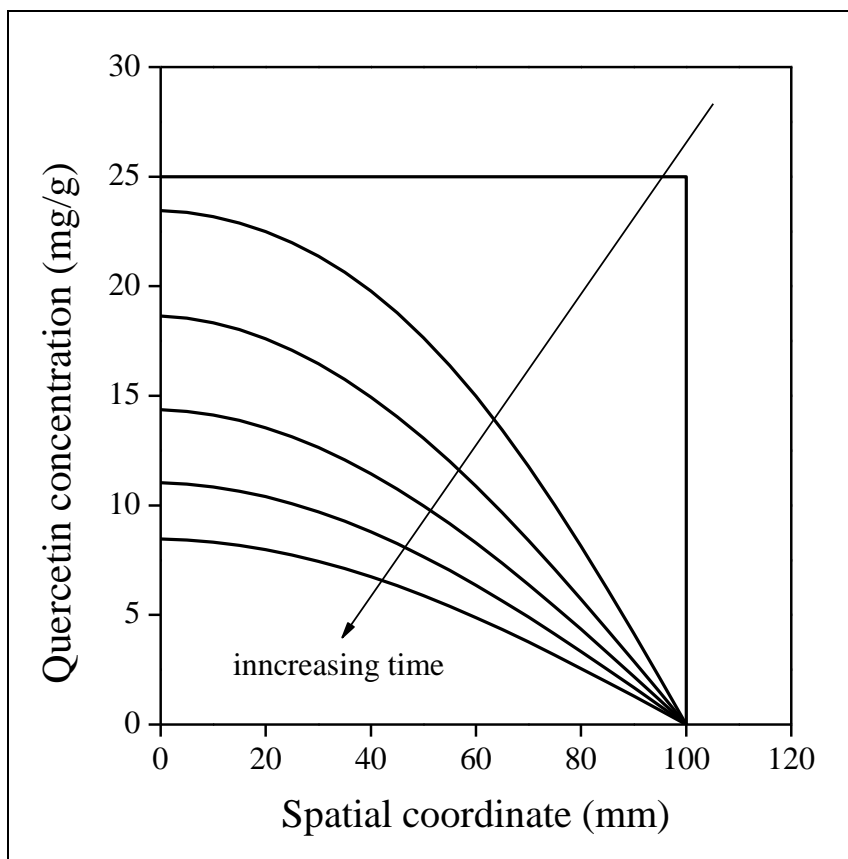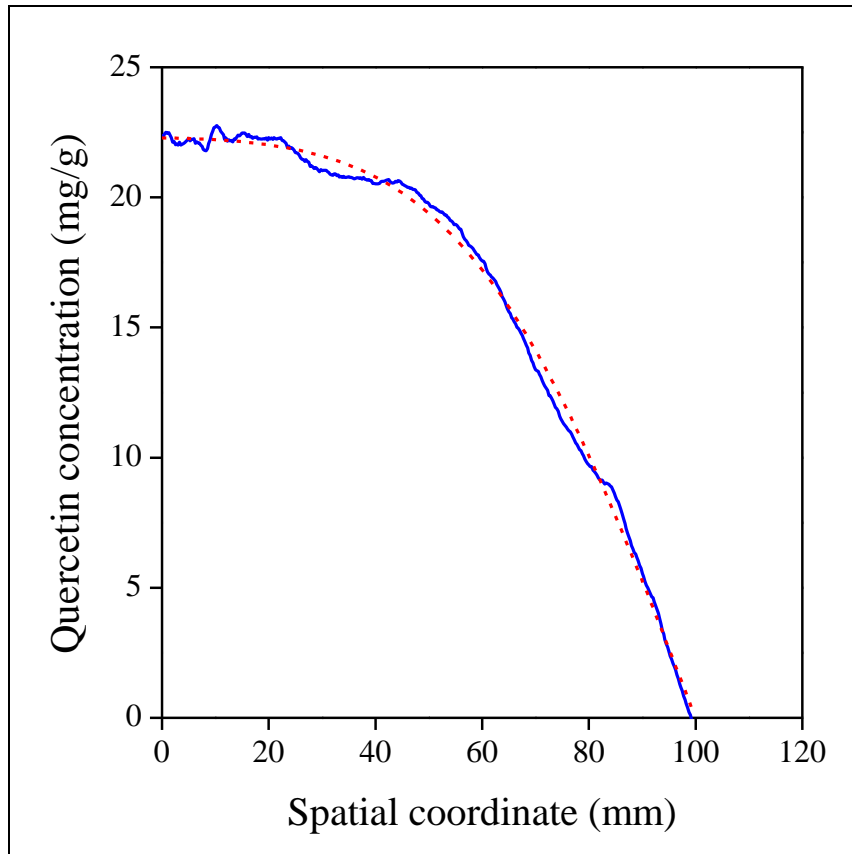
Polyák, Fig. 1

a)

b)

Polyák, Fig. 2

Polyák, Fig. 4

Polyák, Fig. 6

Polyák, Fig. 9