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Investigation of Release Kinetics of Gallic Acid Loaded Microorganisms in Different Environments

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Abstract: *Encapsulation is widely used in food, textile and pharmaceutical fields. The use of antioxidants, as a loading material of encapsulation of this study, contributes to the treatment of some diseases by neutralizing free radicals that occur as a result of oxidation. One of the compounds with the highest antioxidant effect among more than 4000 flavonoid types is gallic acid. It is found mostly in grapes, carob, sumac and green tea. It has been stated in several studies that this compound inhibits cancerous cells without damaging healthy ones. On the other hand, because of their supportive effects on health and reducing the risks of diseases, probiotics belonging to functional foods are preferred in recent years. In this study, antioxidant release mechanisms of gallic acid loaded probiotic-membraned-spheres present in different medium were investigated with selected models. Firstly, the spheres were shrinked with different salt concentrations, and then loaded with different gallic acid concentrations. Released gallic acid amounts were determined by Folin-Ciocalteu method. It has been observed that the loaded microorganisms usually release in the stomach regardless of the parameters studied. The model that fits the release mechanism was found as the Higuchi model in the gastric media and the Korsmeyer-Peppas model in the post-gastrointestinal and intestinal medium.*

Key Words:*Lactobacillius acidophilus, Gallic acid, Release, Kinetic, Stomach, Bowel*

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I. Introduction

Chemical prevention of cancer is defined as delaying, preventing or eliminating the risk of the development of diseased cells with the help of drugs and vitamins, and drugs used in cancer treatments are expected to protect healthy tissues and cells [1]. Antioxidants taken from vegetables and fruits are among the best agents that perform these expected effects. These molecules reduce the harmful effects of free radicals on health (such as causing cancer and cardiovascular diseases) [2] by binding oxygen and metals to themselves and stopping the reactions of free radicals [3]. Gallic acid (3,4,5-trihydroxybenzoic acid; $C_7H_6O_5$), a natural antioxidant, is an organic acid (Figure 1) and is abundantly found in plants such as grapes, carob, sumac, and green tea [4, 5]. As a result of studies performed on different types of cancer, gallic acid has been found to have a protective effect on healthy cells as well as its therapeutic effect [6, 7].

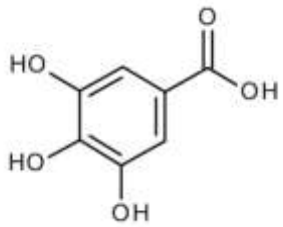


Figure 1: Molecular structure of gallic acid [5]

Bioactive components and probiotics are frequently used in alternative medicine [8, 9, 10]. Probiotics have positive effects on human health and they can remain active in the stomach and intestinal environments without being damaged due to resistancy to the highly acidic conditions [11, 12]. It is known that one of the probiotic microorganisms called *Lactobacillius acidophilus* regulates intestinal pH and reduces intestinal disturbances [13]. There are several studies in the literature considering the loading and release of antioxidants into different types/sizes of capsules [14,15,16], but as far as authors know, no study had been determined in which the cell membrane of probiotics used as building material of the spheres.

The active substance of the drug taken orally in capsule form is transferred to the cell as a result of diffusion [17]. The release of drug in controlled drug release systems, however, occurs according to various mechanisms. Various mathematical models have been developed to explain these mechanisms (Table 1). While

the zero-order kinetic model is based on the constant release of the active substance at a given dose, the first-order kinetic model states that the release rate of the active substance is directly proportional to the concentration [18, 19]. The Higuchi model introduced in 1961, which was the first mathematical model to define the drug release profile due to diffusion, was originally designed for planar systems, and adapted to different geometric and porous systems over time [20]. Another mathematical model used in defining the drug release due to diffusion is called The Korsmeyer-Peppas, and it assumes homogeneously distributed drug in the medium, constant diffusion flux and the optimum release medium are present [20]. In 1931, Hixson and Crowell revealed the release mechanism resulting from the change in the surface area of the particles, and determined that one third power of the released amount was proportional to the surface area of the particles [20]. The mathematical equations of the models were given in Table 1. In that equations, c_0 and c_t represent concentrations at time t=0 and at time t (mg), respectively; c_∞ is the amount of active ingredient (mg) released at the end of the release period examined; k_0 and k_i are the zeroth and first order kinetic constants, respectively; k_H is Higuchi dissolution coefficient; n is the exponential term; k_{KP} and k_{HC} represent constants of corresponding models.

Table 1: Some mathematical models used in drug release kinetics

Model	Mathematical equation
Zero order	$c_0 - c_t = -k_0 * t$
First order	$\ln c - \ln c_0 = -k_i * t$
Higuchi	$c_t - c_0 = k_H * t^{1/2}$
Korsmeyer-Peppas	$c_t/c_\infty = k_{KP} * t^n$
Hixson-Crowell	$c_0^{1/3} - c_t^{1/3} = k_{HC} * t$

In this study, the cell membrane of the probiotic microorganism was used for a frame of the capsule and loaded with gallic acid having anticarcinogenic effect. By examining the released amounts of the capsules in three different simulation environments, the kinetic model expressing the physical nature of the release of each were determined. The study findings aim to contribute to the literature not only the usability of probiotic membranes as capsulation material and the effect of parameters on loading process, but also the location and mechanism of release of gallic acid. They may open the door to studies in which this new capsulation frame used for other drug raw materials.

II. Material And Methods

II.1. Materials

Lactobacillius acidophilus (ATCC 43121) and other chemicals at analytical grade (sodium tartrate, casein peptone, meat and yeast extract, D-glucose, dipotassium hydrogen phosphate, Folin-Ciocalteu reagent, Tween-80, diammonium hydrogen citrate, sodium acetate trihydrate, magnesium sulfate, manganese sulphate, calcium carbonate, sodium chloride, acetic acid, sodium acetate, disodium hydrogen phosphate dihydrate, citric acid, lecithin, maleic acid, sodium hydroxide and sodium carbonate) were purchased from InterLab and Yıldız Chemicals.

II.2. Growth of the microorganism

The MRS broth containing 10g of casein peptone, 8g of meat extract, 4g of yeast extract, 5g of D-glucose, 2g of dipotassium hydrogen phosphate, 1mL of Tween-80, 2g of diammonium hydrogen citrate, 8.25g of sodium acetate trihydrate, 0.04g of magnesium sulfate, 0.008g of manganese sulfate was prepared and autoclaved at 120ºC for 15 minutes (Hirayama HV-50). Then it was cooled up to 37^0C (the appropriate temperature for inıoculation of probiotic) in the incubator (Ildam). After that, lyophilized microorganism was inoculated in 10mL of cooled medium in sterilized ESCO Laminar Flow Cabinet, and it was kept there to acclimate to the environment during 2 hours. This period was reported as the required time period of maximal growth [21]. At the end of this period 200µL of inoculated microorganism solution was inserted into 250mL of MRS medium. The microorganisms were left to grow for 48 hours in the incubator at 37ºC. Finally, they were filtered and washed with distilled water (120x120 FilterLab) and used in the further stages of the study.

II.3. Shrinking and loading of the microorganism

Shrinking of microorganisms was achieved by osmosis, and reverse osmosis method was used for loading the capsules with gallic acid. Microorganisms were added into salt solutions prepared at three different concentrations (0.1-0.2 and 0.3M) and kept for an hour while stirring at 50rpm [22, 23]. In the reverse osmosis,

at the same conditions used in osmosis, shrunken microorganisms were added into gallic acid solutions prepared at different concentrations (0.5 and 1g / 100mL). At the end of the period, the microorganisms separated from the environment by filtration were washed with distilled water and used in release experiments.

II.4. Investigation of release of gallic acid from capsules

At the body temperature (37ºC), the release profiles of all produced capsules were examined at three medium simulation solutions namely stomach, the intestine and the intestine after the stomach. For this purpose, the stomach (237.02mM sodium chloride, 17.12mM acetic acid, 29.75mM sodium acetate, 1: 1 milk / pH = 5 buffer) and the intestinal simulation solutions (3mM sodium tartrate, 0.2mM lecithin, 19.12mM maleic acid, 34.8mM sodium hydroxide, 68.62mM sodium chloride) were prepared [24] and the determined amount of capsules were added to each mixture at a stirring speed of 120rpm. The amount of gallic acid released was generally followed every 10 minutes until the gallic acid concentration stabilized, whereas in the case of rapid release the samples were taken more frequently (2,4,6,8 min).

The amount of gallic acid in the medium was determined by Folin-Ciocalteu analysis, which is an easy and reproducible spectrophotometric method. This method is an oxidation-reduction reaction based on the conversion of gallic acid in basic medium to its oxidized form by reducing the Folin-Ciocalteu reagent [25]. The Folin-Ciocalteu reagent here acts as an oxidizing compound and the absorbance of the blue color formed by the reaction of folin with gallic acid is measured. 300 µl of each sample taken from the simulation solutions was added into an anlyzing solution containing 1.5mL of Na_2CO_3 solution (20%, (w/w)), 5.1 mL of distilled water, 100 µl of gallic acid, 0.5mL of folin and left in a dark environment at room temperature for 2 hours to complete the reaction. At the end of the period, the absorbance values of the samples were measured by UV spectrophotometer (UV-vis Carry 60) at 765 nm. With the help of the calibration curve equation given below (Equation 2.1), [26] these values were used to calculate the release amount as gallic acid equivalent (GAE). Each experimental study was carried out with at least three parallel and repeated until the standard deviation value was 0.0046. The average of the values providing this standard deviation were used in the calculations of methematical models.

$$Absorbance = 0.01532 * concentration \text{ (µg/mL)} \; R^2 = 0.9989 \textit{(2.1)}$$

The the most releasing conditions of each of the medium were investigated in detail for determination of release kinetics of the capsules in the stomach, post-gastrointestinal and intestinal environments. Zero-order, first-order, Higuchi, Korsmeyer-Peppas and Hixson-Crowell mathematical models were used in this period. Linearization technique was used to determine the model constants of each. By comparing the regression values of the lines obtained, the model closest to a value of "1" was chosen as the model that best expresses the release mechanism. In order to visually express the fit of the model and experiment, the obtained model parameters were inserted into the model equation and the amount of gallic acid expected from a corresponding model equation to be released at the specified time was calculated (c_{calc}) and these values were compared with the amount of released gallic acid determined in experiments (c).

III. Results

The released amounts from each of the capsules that were shrunk by different salt concentrations and loaded with different amounts of gallic acid in stomach, post-gastrointestinal and intestinal simulation environments were summarized in Table 2. Comparing all of the findings obtained via all of the parameters, the capsules that shrunk by 0.3M NaCl solution and loaded by 0.5g GA/100mL achieved maximum release (~ 0.08 mg GA) in the post-gastrointestinal environment, whereas those that shrunk by 0.1M NaCl solution and loaded by 0.5g GA/100mL caused the lowest release (~ 0.005mg GA) in the same medium. It has been determined that shrinking of capsules by low or moderate salt content produced release of GA in the stomach and intestine if they were loaded at high concentrations, and in the stomach if they were loaded with a low concentration of GA. The capsules tend to release in the post-gastrointestinal environment, if high salt content and low GA content were used in the production of them. In addition, it was determined that the capsules that were shrunk by 0.3M salt could be loaded with approximately 0.1mg of gallic acid, and approximately 76% of this amount was released in the post-gastrointestinal environment.

The highest releases were obtained by using capsules that were shrunk by 0.2M NaCl and loaded by 1g GA/100mL in the stomach simulation, using those that were produced by 0.3M NaCl and 0.5g GA /100mL in the post-gastrointestinal simulation, and by using those that were produced by 0.2M NaCl and 1g GA/100mL in the intestinal simulation medium. If high concentration of gallic acid loading was used, the amount of salt in the shrinking process did not have any significant effect, whereas if low gallic acid concentrations were used, the amount of salt caused serious differences. Doubling the salt concentration doubled the released amount of GA in

the stomach and intestine medium, and tripling of it resulted a prevention of releasing off the capsule up to the the intestine, i.e. after passing through the stomach.

At the same salt concentrations, when the effect of GA concentration on the release was examined, it was observed that the spheres loaded by 1g GA/100mL and shrunk by 0.1M NaCl released the highest GA in all medium. For 0.2M and 0.3M NaCl usage, high amount of GA loading produced the best release in the stomach and intestine, whereas loading with low amount of GA caused better release in the post-gastrointestinal media.

Table 2. Gallic acid (mg) release of capsules in different environments

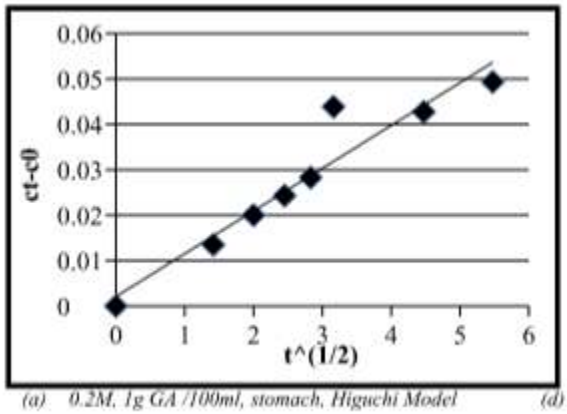
In stomach simulation solution						
	1g GA /100ml			0.5g GA /100ml		
t	0.1M NaCl	0.2M NaCl	0.3M NaCl	0.1M NaCl	0.2M NaCl	0.3M NaCl
0	0.00001	0.00001	0.00001	0.00001	0.00001	0.00001
2	0.0002	0.0135	0.0059	0.0012	0.0006	0.0007
4	0.0008	0.0200	0.0087	0.0045	0.0008	0.0012
6	0.0037	0.0243	0.0196	0.0069	0.0043	0.0051
8	0.0076	0.2830	0.0357	0.0097	0.0091	0.0097
10	0.0096	0.0438	0.0431	0.0243	0.0412	0.0235
20	0.0298	0.0427	0.0452	0.0152	0.0247	0.0240
30	0.0359	0.0493	0.0426	0.0211	0.0383	0.0209
40	0.0353	0.0434	0.0451	0.0202	0.0358	0.0235
50	0.0392	0.0468	0.0452	0.0147	0.0451	0.0198
60	0.0386	0.0465	0.0437	0.0180	0.0383	0.0246
70	0.0398	0.0512	0.0425	0.0178	0.0422	0.0240
80	0.0271	0.0458	0.0440	0.0152	0.0414	0.0207
90	0.0292	0.0484	0.0432	0.0176	0.0441	0.0241
100	0.0337	0.0530	0.0387	0.0166	0.0475	0.0242
110	0.0352	0.0524	0.0457	0.0173	0.0432	0.0334
120	0.0406	0.0484	0.0453	0.0203	0.0418	0.0244
In post gastrointestinal simulation solution						
	1g/100ml GA			0.5g/100ml GA		
t	0.1M NaCl	0.2M NaCl	0.3M NaCl	0.1M NaCl	0.2M NaCl	0.3M NaCl
0	0.00001	0.00001	0.00001	0.00001	0.00001	0.00001
10	0.0073	0.0064	0.0056	0.0027	0.0101	0.0698
20	0.0055	0.0059	0.0060	0.0050	0.0111	0.0783
30	0.0056	0.0079	0.0083	0.0035	0.0119	0.0666
40	0.0066	0.0053	0.0069	0.0045	0.0106	0.0843
50	0.0039	0.0142	0.0061	0.0068	0.0091	0.0691
60	0.0074	0.0058	0.0070	0.0047	0.0105	0.0680
70	0.0060	0.0046	0.0082	0.0069	0.0102	0.0753
80	0.0064	0.0089	0.0064	0.0048	0.0099	0.0689
90	0.0070	0.0076	0.0086	0.0051	0.0106	0.0839
100	0.0044	0.0058	0.0057	0.0057	0.0104	0.0717
110	0.0049	0.0059	0.0071	0.0039	0.0130	0.0729
120	0.0054	0.0065	0.0068	0.0046	0.0094	0.0774
In intestinal simulation solution						
	1g/100ml GA			0.5g/100ml GA		
t	0.1M NaCl	0.2M NaCl	0.3M NaCl	0.1M NaCl	0.2M NaCl	0.3M NaCl
0	0.00001	0.00001	0.00001	0.00001	0.00001	0.00001
10	0.0358	0.0405	0.0321	0.0072	0.0096	0.0069
20	0.0371	0.0425	0.0407	0.0064	0.0116	0.0094
30	0.0367	0.0434	0.0384	0.0066	0.0119	0.0075
40	0.0368	0.0399	0.0389	0.0064	0.0111	0.0072
50	0.0367	0.0412	0.0427	0.0066	0.0112	0.0080
60	0.0349	0.0409	0.0393	0.0076	0.0089	0.0075
70	0.0381	0.0403	0.0415	0.0072	0.0107	0.0076
80	0.0402	0.0418	0.0420	0.0064	0.0113	0.0089
90	0.0397	0.0418	0.0413	0.0063	0.0087	0.0085
100	0.0413	0.0401	0.0392	0.0061	0.0110	0.0072
110	0.0413	0.0426	0.0421	0.0057	0.0117	0.0064
120	0.0394	0.0414	0.0422	0.0062	0.0129	0.0107

Kinetic studies have been carried out by using selected model equations in accordance with the results obtained in simulation solutions in which the highest release produced; i.e. for stomach 0.2M NaCl, 1g GA/100ml, for post-gastrointestinal 0.3M NaCl, 0.5g GA/100ml and for intestine 0.2M NaCl, 1g GA/100ml of salt and GA concentrations, respectively. The analysis was realized up to the equilibrium and the results were given in Table 3. Choosing of the best model expressing release kinetics was applied by two approaches considered simultaneously; having the regression coefficient closest to one and fitness of the concentration values calculated from the model to the experimental data. The best models were determined as the Higuchi model for the stomach, the Korsmeyer-Peppas model for the post-intestinal and intestinal medium (Figure 2).

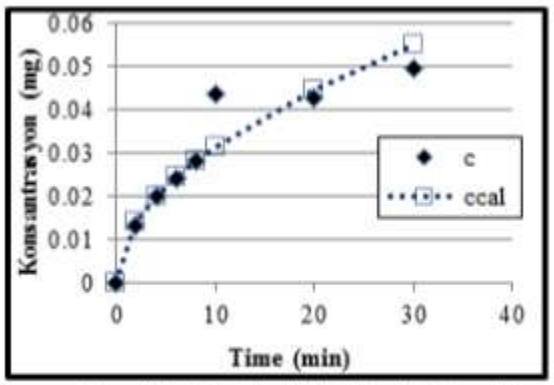
The consistency between the experimental data and the concentration values calculated from the respective model equation was shown in Figure 2; (d), (e), and (f). The equilibrium concentration was reached nearly the same time durations (approximately 30 min) in all of the medium invrestigated as shown in Figure 2-d in detail, as an example. Due to the rapid release in the first minutes, there were some scattering values between the model and the experimental data, and also huge differences were observed especially longer time durations, which means that the models were unable to determine the equilibrium concentration. According to the Higuchi model, the initial drug concentration is assumed to be higher than the solubility of the cell membrane of the microorganism, ideal ambient conditions are assumed as maintained up to the saturation concentration, and the diffusion coefficient is assumed to be constant. If the released drug concentration reaches 10% of the saturation concentration, the amount of the released drug causes the ambient conditions non-ideal and the highly neutralized medium does not change with the diffusion of the drug [27]. According to the results, those assumptions were valid up to 30 minutes. When the highest release in the intestinal medium data were examined, it was determined that the model that best explains the release was found as Korsmeyer-Peppas (Figure 2 (c)), and the agreement between the concentration values suggested by the model and the experimental concentration values were shown Figure 1 (f), for all time durations studied. From the slope of the graph, the value of the diffusion exponent was found as 2.6271 (n> 0.89), so it was determined that the release of the drug from the system was explained by super case II transport [27]. The model that best explains the release kinetics of the capsules passing through the stomach to the intestine (post-intestinal condition) was found as Korsmeyer-Peppas (Figure 2 (b)), and the concentration values calculated with the model were consistent (Figure 2 (e)). Since the slope in the release graph is n> 0.89, it was determined that the diffusion in this media could be explained by super case II transport, as in the case of intestine. Super case II transport, which is a non-Fickian diffusion process, occurs when the solvent activity is high and the diffusion rate is higher than the dissolution rate of the cell membrane [28]. In the study, the diffusion was supported with the holes in the cell membrane and with the activity of the simulation solutions to dissolve the cell membrane of the microorganism. In the literature, polymer-solvent systems described with super case II diffusion are frequently encountered. In the study conducted by Weisenberger and Koenig in 1990, it was revealed that methanol diffusion into pMMA was explained by super case II transport, and acetone diffusion to poly (vinyl chloride) was found similar [29].

Table 3: The results of release kinetics in simulation solutions studied

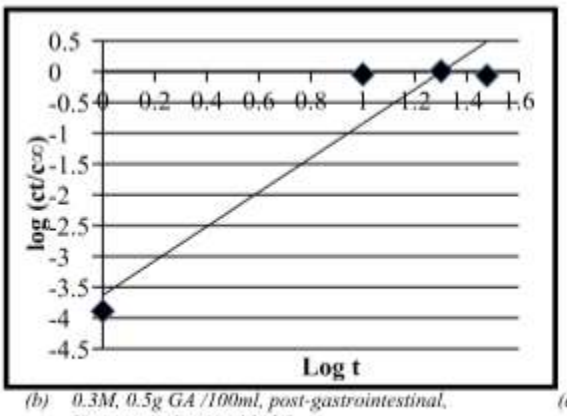
	Stomach			**Intestine**			**Post-gastrointestinal**		
Model	Intersection	Slope	R^2	Intersection	Slope	R^2	Intersection	Slope	R^2
Zero order	0;0	0.0009	0.0893	0;0	0.0018	0.5060	0;0	0.003	0.3706
First order	0;0	0.5992	0.3219	0;0	0.3582	0.4380	0;0	0.3800	0.4224
Higuchi	0;0	0.010	0.9081	0;0	0.0093	0.8648	0;0	0.0147	0.7920
Korsmeyer -Peppas	0;-0.3431	0.1871	0.6939	0;-3.3662	2.6271	0.9148	0;-3.6294	2.7854	0.9095
Hixson-Crowell	0;0	0.0007	0.4003	0;0	0.0140	0.4565	0;0	0.0168	0.4091



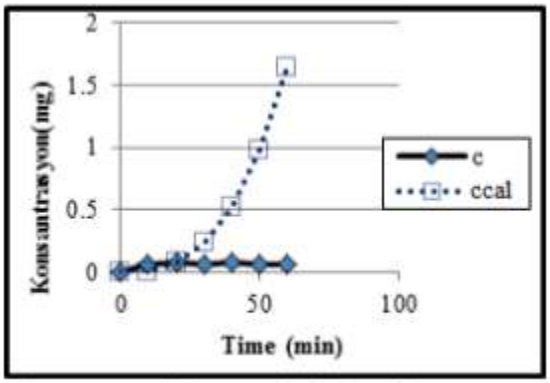
(a) 0.2M, 1g GA /100ml, stomach, Higuchi Model



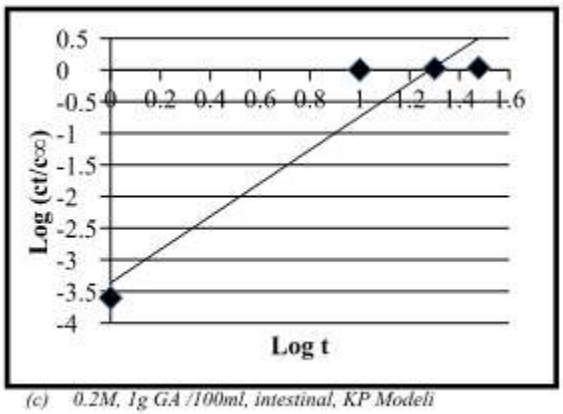
(d) The comparison of Experimental and Higuchi model (stomach)



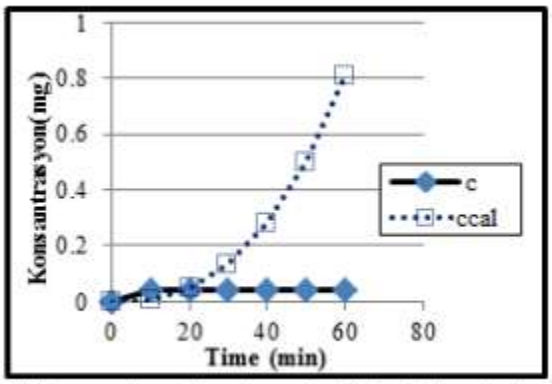
(b) 0.3M, 0.5g GA /100ml, post-gastrointestinal, Korsmeyer-Peppas Model



(e) The comparison of Experimental and KP model(post-gastrointestinal)



(c) 0.2M, 1g GA /100ml, intestinal, KP Modeli



(f) The comparison of Experimental and KP model(intestinal)

Figure 2: Release kinetics of capsules obtained at maximum release conditions of the medium

IV. Discussion

Since the best release of GA was obtained in the stomach, it has been determined that the probiotic microorganism membranes can be used in the production of GA-loaded capsules especially if the release in the stomach is needed. In addition, the maximum release would be achieved with using a loading process containing 0.2M NaCl as shrinking solution and 1g GA/100mL as loading solution.

Due to the fact that the loaded microorganisms can release both in the stomach and in the post-stomach intestine, it was concluded that the capsules produced capable of withstand the acidic environment in the stomach. However, it has also been determined that the amount of release in the post-gastrointestinal media generally decreases due to both the release of some of the loaded gallic acid in the stomach and the deformation due to acidity while passing through the stomach. Thus, the authors have been suggested that the microorganism should be covered with some extra material if the drug release in the intestine is desired. As a result of the study, it was concluded that industrial production of these capsules can be performed by using models that best explain the release profile in simulated medium.

V. Conclusion

This study approves the usabilitiy of probiotic membranes as a loading material of gallic acid. In addition, the results showed that, the parameters of shrinking and loading will significantly affect both the releasing place of the drug and release kinetics. The use of different kind of materials to cover the membranes for release in the intestine should be examined in further studies.

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