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Release of polyphenols from starch-chitosan based films containing thyme extract

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Abstract

The release kinetics of thyme extract polyphenols (TE) from chitosan (CH), pea starch (S) and CH:S blend films in different solvents was evaluated, as well as their antioxidant activity in each release media. Pure starch films showed the fastest delivery rate and the highest delivery ratio of polyphenols, although the corresponding release media exhibited the lowest antioxidant capacity. TE provided CH based films with remarkable antioxidant activity, despite the lower polyphenol release obtained in all solvents, due to the strong polyphenols-chitosan interactions. The maximum amount of polyphenols delivered was found in the acetic acid solution, due to the high solubility of CH. The incorporation of tannic acid (TA) into CH films promoted cross-linking effect, which delays the TE release rate in water and ethanol aqueous solutions, except for CH:S:TA films. Thus, the polarity of the solvents and the polyphenols-matrix interactions markedly affected the polyphenol release and the antioxidant activity of the films.

Keywords: starch, chitosan, thyme, tannic acid, antioxidant activity, kinetics.

1. Introduction

Microbiological degradation and oxidation are the most common causes of food deterioration (Mozafari *et al.,* 2006; Quintavalla and Vicini, 2002). Current trends in new packaging technologies for food preservation are focused on the development of alternatives for replacing synthetic additives with natural antioxidant and antimicrobial compounds.

Polyphenols are one of the most numerous and universal groups of natural antioxidants from plant sources, such as herbs and spices. The antioxidant nature of polyphenols is related with their ability to chelate metals, inhibit lipoxygenase enzyme activity and act as free radical scavenger (Garrido and Borges, 2013).

Aromatic herbs, such as thyme, have been traditionally used as healthy food ingredients obtained through an aqueous extraction process (Stojanovic *et al.,* 2012). *Thyme serpyllum* L. is a rich source of polyphenols with reported antibacterial, antifungal, and antioxidant effects (Safaei-Ghomi, Ebrahimabadi, Djafari-Bidgoli and Batooli, 2009; Trifkovic *et al.,* 2014). Despite the great potential of thyme essential oil, its uses in food preservation are limited mainly due to its intense aroma, toxicity problems and possible changes in the organoleptic properties of the food (Sánchez-González, Vargas, González-Martínez, Chiralt and Cháfer, 2011). Thus, the attention has shifted towards the use of hydrophilic extracts, which contain phenolic acids (rosmarinic acid, caffeic acid and its oligomers), flavonoids, hydroquinone derivatives, terpenoids and biphenyl compounds (Fecka and Turek, 2008; Mihailovic-Stanojevic *et al.,* 2013). Thyme extract polyphenols, have been also used as crosslinkers being the main chemical pathway related with the oxidization of diphenyl moieties of phenolic acids or other polyphenols, producing quinone intermediates that react with nucleophiles (mainly amino or sulfhydryl side chains) to form covalent C-N or C-S bonds with the phenolic ring (Azeredo and Waldron, 2016).

The direct food application of polyphenols is limited by their relatively rapid actuation (Perazzo *et al.,* 2014). The combined use of natural antioxidants and packaging materials could contribute to increase their effectiveness of their food application on, thus limiting the oxidative reactions and extending their action during a longer time. Starch is one of the most abundant low-cost hydrocolloids with recognized film-forming ability. In spite of the biodegradability, flexibility and transparency of starch films, their lack of adequate mechanical and barrier properties. Improvement strategies are based on the mixture of starch with other polymers (e.g. chitosan), which allow for producing films with better properties. Chitosan is a cationic hydrocolloid with interesting film-forming properties (Elsabee and Abdou, 2013). Chitosan presents a great potential for a wide range of food applications due to its biocompatibility and non-toxicity (Krochta and Mulder-Johnston, 1997; Sánchez-González *et al.* 2011). Numerous studies have shown the antioxidant and antimicrobial activity of chitosan (Dutta, Tripathi, Mehrotra and Dutta, 2009; Kumar, 2000; Yen, Tseng, Li and Mau, 2007; Yen, Yang and Mau, 2008). The antibacterial activity of chitosan can be related with the interaction between the positive charges of chitosan and the negatively charged residues of macromolecules on the microbial cell surface (Siripatrawan and Harte, 2010). Chitosan films have good mechanical properties (Butler, Vergano, Testin, Bunn and Wiles, 1996), but their application is limited by their high water vapor permeability (Caner, Vergano and Wiles, 1998; Vargas, Albors, Chiralt and González-Martínez, 2011).

Previous works have shown that the partial replacement of starch with chitosan in starch-based films can improve the mechanical properties of starch-based films at the same time that the antimicrobial effect is promoted (Bonilla, Atarés, Vargas and Chiralt, 2013). Moreover, the physical properties of chitosan based-films can be improved by the addition of tannic acid (TA), a natural polyphenol with antioxidant and crosslinking capacity (Aelenei, Popa, Novac, Lisa and Balaita, 2009; Rivero, García and Pinotti, 2010). TA crosslinking properties as regards chitosan have been related with

electrostatic interactions (ionic complexations in acidic conditions), ester linkages and hydrogen bonds (Silva-Weiss, Bifani, Ihl, Sobral and Gómez-Guillén, 2013). In this sense, Talón *et al.* (2017) showed that tannic acid interacted with chitosan chains, acting as a crosslinking agent and enhancing the tensile behaviour of chitosan-based films.

Tannic acid shows antioxidant and antimicrobial properties due to its multiple phenolic groups (Cao, Fu and He, 2007; Rivero *et al.,* 2010). The potential antioxidant effect of the active compounds present in bioactive films are affected by the kinetics of their release into the food surface, which in turn depends on several factors such as the food product characteristics and film microstructure. In order to design efficient active films and to increase their functionality, release studies are required. However, to the best of our knowledge, the kinetics of release of active ingredients in hydrocolloid-based films has been little explored. Moreover, to define the phenomena that occur during the release by means of mathematical models will allow for simulating similar behaviours in new delivery systems (Buonocore, Del Nobile, Panizza, Corbo and Nicolais, 2003).

The aim of this work was to evaluate the kinetics of the release of thyme extract polyphenols from chitosan-pea starch films in solvents of different polarity. Moreover, the effect of the addition of tannic acid into the polymeric matrix was also studied.

2. Materials and methods

2.1 Raw materials

To prepare the films, high molecular weight chitosan (Batch MKBH57816V, Sigma-Aldrich, Madrid, Spain), tannic acid (Sigma-Aldrich, Madrid, Spain), pea starch (Batch W469V, Roquette Laisa SA, Valencia, Spain,), glacial acetic acid (Panreac Química

SLU, Barcelona, Spain) and glycerol (Panreac Química SLU, Barcelona, Spain) were used.

Folin-Ciocalteu reagent was obtained from Sigma-Aldrich (Madrid, Spain) whereas phosphorus pentoxide (P_2O_5), sodium carbonate (Na_2CO_3) and sodium chloride (NaCl) were provided by Panreac Química SLU (Castellar el Vallés, Spain).

Thyme (*Thymus serpyllum L.*) was kindly provided by The Institute of Medicinal Plant Research ''Dr. Josif Pančić'' (Belgrade, Serbia). The aqueous extract was prepared by a conventional water-extraction method. In this way, 200 mL of boiled distilled water was poured over 10 g of dry thyme herb. The extraction process was carried out for 30 min, at room temperature, and with intermittent stirring. The obtained extract was filtered through a medical gauze (Stojanovic *et al.,* 2012; Trifkovic *et al.,* 2014). After filtration, the extract was lyophilized in a freeze drier under vacuum pressure (Alpha 1–2, Martin Christ, GmbH, Osterode am Harz, Germany). Thyme extract powder was kept stored under vacuum and refrigeration conditions.

2.2. Measurement of thyme extract polyphenols

The total content of polyphenols was determined by the Folin-Ciocalteu method (Stojanovic *et al.,* 2012). To perform this analysis, 0.5 mL of Folin-Ciocalteu reagent was mixed with 1.5 mL of Na_2CO_3, 0.1 mL of sample and distilled water to complete 10 mL. After 2 hours, the absorbance of the samples was measured at 765 nm, in triplicate, by means of a UV-vis spectrophotometer (Evolution 201, Thermo Scientific). Gallic acid was used as a standard and the results were expressed as $mg \cdot L^{-1}$ of Gallic acid equivalents (GAE).

Rosmarinic acid (RA) is the predominant phenolic compound present in thyme extract (Mihailovic-Stanojevic *et al.,* 2013). In this study, the amount of RA was obtained by means of high-performance liquid chromatography (HPLC) performed with a Waters

2695 Alliance HPLC System (Alliance, USA) equipped with a photodiode detector (Waters 2996, Alliance). The concentration of RA was determined by using a Brisa LC2 column (C18, 250 × 4.6 mm, 5µm) and a C18 pre-column. 20 µL of each sample, previously filtered through a 0.45 µm filter and injected in the HPLC system. The elution was performed with the following solvents: 0.2% (v/v) formic acid (solvent A) and pure methanol (solvent B) with a flow rate of 1 mL/min. The gradient used was: 26% solvent B (in the first minute); in the second minute, a linear gradient from 26% to 50% solvent B was used and it was kept constant until during 2 minutes; from minute 4 to minute 7 a linear gradient from 50% to 95% solvent B was used and it was kept constant until minute 19; the last minute was used to return to the initial conditions. The chromatograms were recorded at 278 nm and rosmarinic acid peak was confirmed by comparing the retention times and the UV-spectra with the HPLC standard. The integrated peak areas of the standard solutions were obtained and graphs representing concentration versus area was prepared. Lineal regression was used to calculate the corresponding concentrations of samples (R^2=0.998). All analyses were performed in triplicate.

2.3. Film preparation and characterization

Films were produced by means of casting method. Different film-forming dispersions (FFDs) based on pure chitosan (CH) or pure starch (S) or a mixture of both polymers (CH:S) were obtained. In FFDs based on chitosan, tannic acid (TA) was also added as a cross-linking agent (Rivero et al., 2010).

Chitosan (2% w/w) was dispersed in an aqueous solution of acetic acid (2% v/w) under magnetic stirring at 40°C and 150 rpm for 24 h. Glycerol was added in a polymer:glycerol ratio of 1:0.2 (w/w). To obtain the CH:TA film forming dispersion, TA was added to the CH dispersion in a CH:TA ratio of 1:0.04 (w/w).

Starch dispersions were prepared by dissolving 2% (w/w) of pea starch in distilled water, while heating at 95°C for 30 min to promote starch gelatinization, under continuous stirring. Afterwards, glycerol was added in a polymer:glycerol ratio of 1:0.2 (w/w).

Chitosan and starch dispersions were mixed in the adequate proportion to obtain a CH:S ratio of 1:4 (w/w). TE was incorporated to the FFDs at a polymer:TE ratio of 1: 0.15. As control samples, films without TE were also prepared (CH, CH:TA, S, CH:S, CH:S:TA). All FFDs were homogenized by using a rotor-stator (Ultraturrax Yellow Line DL 25 Basic, IKA Janke and Kunjel, Germany) for 4 min at 13500 rpm, and degassed by means of a vacuum pump. The FFDs were poured into levelled Petri dishes (8.7 cm in diameter) and the amount of polymer remained constant (56.62 g of polymer/m^2). After drying for 48 hours under controlled conditions (T=25°C and RH=50%) and prior to further analysis, the films were conditioned in a desiccator at 25°C with a supersaturated NaCl solution (a_w= 0.75) until they reached constant weight.

2.3.1. Water content, solubility and film thickness

Conditioned films were cut into small pieces to measure the moisture content in triplicate by means of a gravimetric method. The samples were desiccated 48 hours in a vacuum oven (Vacio TEM-T) at 60°C and then were stored in a desiccator with phosphorus pentoxide until they reached constant weight.

Solubility test was carried out with the films that were dehydrated to measure their water content. Distilled water was added in a film:water ratio of 1:25 (w/v). Samples were and kept for 24 h at 25°C. After that, water was removed and the samples were placed in a convection oven (J.P. Selecta, S.A., Barcelona, Spain) at 60°C for 24h. Afterwards, samples were stored in a desiccator with P_2O_5 for two weeks until constant weight. The solubility of the films was obtained from the difference between the initial

and final dry weight of the films (Ortega-Toro, Morey, Talens and Chiralt, 2015). The test was performed in triplicate.

For the measurement of film thickness, three samples of all formulations conditioned at 25°C and 75% RH were used. Thickness was measured in six random points of each sample by means of a digital electronic micrometer with an accuracy of 0.001 mm (Palmer model COMECTA, Barcelona).

2.3.2. Antioxidant activity of films

The antioxidant capacity of the films with thyme extract was expressed as Trolox equivalent antioxidant capacity (TEAC), which was estimated by the ABTS radical cation decolorization assay. This method is based on the scavenging of stable blue–green ABTS radical cation ($ABTS^{\bullet+}$), which is formed by chemical oxidation of ABTS (Re *et al.,* 1999). The amount of ABTS radical cation scavenged by antioxidants was measured by monitoring the decrease in absorbance of ABTS radical cation. Briefly, in order to oxidize ABTS to $ABTS^{\bullet+}$, 5 mL of ABTS water stock solution (7 mM) was mixed with 88 µL of potassium persulfate (140 mM) and incubated in the dark for 12-16 h, at the room temperature. Prior to analysis, the ABTS•+solution was diluted with ethanol to absorbance value of 0.70 (± 0.02), measured at 734 nm. Films antioxidant activity was measured in solvents of different lipophilic-hydrophilic nature: water, 3% (w/v) acetic acid solution, 10% (v/v) ethanol solution and 20% (v/v) ethanol solution. Film specimens (59.4 cm^2) were cut in small bits and placed into 50 mL vials containing 25 mL of each of the four different solvents. After 24 hours, an aliquot of 10 µL of sample was withdrawn and added to 2.0 mL of the diluted ABTS•+solution. The absorbance of the obtained sample was measured after exactly 6 min. Absorbance values were compared to the blank sample, which was prepared by adding the 10 µL of ethanol to 2

mL of the diluted ABTS•+solution. The analysis were performed in triplicate and the results, were expressed as Trolox equivalents.

2.4. Kinetics of the polyphenols release

Four different solvents were used to perform the release studies: water, 3% (v/v) acetic acid solution (AA3%), 10% (v/v) aqueous ethanol solution (E10%) and 20% (v/v) aqueous ethanol solution (E20%). A piece of film of 59.4 cm^2 was cut in small bits and placed into 50 mL vials containing 25 mL of each of the four different solvents. Release studies were carried out during 48 h at 25°C. Successively, aliquots of 100 μl of sample were taken at different film-solvent contact times and the total phenolic content was determined in triplicate as previously described in Section 2.2. The results were expressed as amount of gallic acid equivalents per gram of polymer (mg GAE/g polymer).

2.4.1. Mathematical modelling of polyphenols release

Two empirical models were applied to determinate the release profiles of experimental data.

The generalized expression (equation 1) of the Korsmeyer-Peppas model (Siepmann and Peppas, 2012) was used to investigate the possible coupling of the relaxation of the polymer in contact with the solvent with the diffusion of the active compound through the polymer matrix.

$$\frac{M_t}{M_\infty} = kt^n \qquad \text{(Equation 1)}$$

where M_t/M_∞ represents the fraction of active compounds releases at time t, k is the rate constant of the film, related to the diffusion process and n is the diffusional exponent that provides information about the mechanisms involved in the release process. Thus, a n value of 0.5 means that the release takes place through Fickian diffusion, whereas if the n value is higher than 0.5, known as anomalous transport, the diffusion and the polymer relaxation rates are coupled. If the n value is lower than 0.5, a quasi-Fickian diffusion for the active release can be considered (Siepmann and Peppas, 2012) describing the transport mechanism.

Peleg's model (Peleg, 1988), described by equation 2, was applied to experimental data in order to predict the release kinetics.

$$M_t = \frac{t}{k_1 + k_2 t} \qquad \text{(Equation 2)}$$

where M_t is total phenolic content at time t, k_1 is the kinetic constant of the model that is inversely related to the mass transfer rate at the beginning of the process, and k_2 is a constant of the model that is related to the asymptotic value, which can be related to the equilibrium value ($1/M_\infty$ where M_∞ is the amount of active compound released at equilibrium).

2.5. Statistical analysis

Results were submitted to analysis of variance (ANOVA) using Statgraphics Centurion XVI software (Manugistics Corp., Rockville, Md.). Fisher′s least significant difference (LSD) procedure was used at the 95% confidence level.

3. Results and discussion

3.1. Water content, solubility, thickness and antioxidant activity of the films

The equilibrium moisture content of the films stored at 75% RH - 25°C is shown in Table 1, together with the thickness, solubility and antioxidant activity.

In order to understand the possible interactions of polyphenols from thyme extract with the polymeric matrix, pure polymer films, without polyphenols (TE), were also evaluated. In these films, the highest equilibrium moisture content were obtained for pure CH and CH:TA films (0.242 and 0.22 g water/g dry solids, respectively). This was explained by the higher hydrophilic character of chitosan film as compared to starch-based films (Bonilla *et al.,* 2013), and it can be associated with the higher water binding capacity of chitosan molecules. Chitosan-starch films showed a lower water content (0.142 g water/g dry solids) and similar solubility values to that of pure chitosan or pure starch films (6.8 g film/L). The incorporation of tannic acid into the polymeric matrix reduced the average water content of chitosan-starch films (0.131 g water/g dry solids), without affecting its solubility. In a similar way, the addition of thyme extract polyphenols to the polymeric matrix, containing or not tannic acid, promoted a slight decrease in the water content of the films, which can be explained by the interactions between these hydrocolloids with polyphenols (Chung, Wong, Wei, Huang, Lin, 1998). A similar effect was observed by Wang, Dong, Men, Tong and Zhou (2013) when incorporating tea polyphenols into chitosan-based films at the same equilibrium conditions.

As shown in Table 1, significant differences ($p < 0.05$) were detected in terms of film thickness depending on the formulation. Film thickness depends on the organization of the polymer chains, on the interactions between the polymer chains and on the polyphenol and water content of the film matrix. Pure chitosan films showed the highest

thickness values (91 ± 11 μm), and presented a more open structure as previously observed by Talón *et al.,* 2017, which is also consistent with their greater moisture content, as commented on above. Starch-based films showed lower thickness values (55 ± 11 μm), and a more compact organization of the polymer chains (Talón *et al.,* 2017). The mixture of the two polymers yielded intermediate thickness values (58 ± 2 μm). In general, two opposite trends were observed in terms of the thickness of films containing polyphenols; in pure chitosan films the incorporation of polyphenols led to a significant decrease in the thickness of the films whereas in CH:S films the addition of polyphenols yielded thicker films. Polyphenols (TE and TA) and chitosan have opposite charge in acidic media (Gibis, Ruedt, and Weiss 2016) and therefore their mixture may cause interactions that lead to a compaction of the polymer chains and hence a reduced thickness. This was not observed in CH:S mixtures, possibly due to the screening effect of CH surface charge.

Table 1 shows the antioxidant activity of the released active compounds after 24 h of contact with solvents of different polarity. The antioxidant activity was expressed as Trolox equivalent antioxidant capacity (mM TEAC). The higher the values, the lower the antioxidant activity. Both factors, type of solvent and film had a significant effect on the antioxidant activity of the film matrix, being the interaction between these factors also significant ($p<0.05$). The effect of TA addition on the film antioxidant activity depended on the type of solvent and on the polymer matrix. For CH:S:TE films and in solvents of high polarity (water and AA3%) the addition of TA significantly increased the antioxidant activity of the films ($p<0.05$), whereas the opposite effect was observed in both 10% and 20% ethanol. In CH:TE films, and all types of solvent, the addition of TA did not have significant effect on the film antioxidant activity. The lowest antioxidant activity regardless the solvent polarity was obtained for S:TE films, but CH:S blend films containing TE (without TA) led to a significant increase in film antioxidant activity. Differences in the polyphenol release from the different films into the simulants and

potential oxidation of the compounds during the film processing can explain the different antioxidant activity observed in each case.

Table 1. Moisture content, water solubility, thickness and antioxidant activity in different solvents (H2O, AA3%: 3% (w/v) acetic acid aqueous solution, E10%: 10% (v/v) ethanol aqueous solution, E20%: 20% (v/v) ethanol aqueous solution). Mean values and standard deviation, in brackets.

Film	MC % (g water/100 g dry solids)	Water solubility (g solubilized film/100 g film)	Thickness (mm)	Antioxidant activity (mM TEAC)			
				H_2O	AA3%	E10%	E20%
CH:TE	21.61 (0.09)^{c}	21.1 (1.1)^{ab}	87 (4)^{c}	0.5 (0.2)^{ab,1}	1.3 (0.2)^{b,2}	0.69 (0.09)^{ab,1}	0.69 (0.12)^{ab,1}
CH:TA:TE	20.9 (0.4)^{c}	22.7 (1.0)^{bc}	84 (3)^{c}	0.76 (0.05)^{b,12}	0.64 (0.07)^{a,1}	0.68 (0.07)^{ab,1}	0.95 (0.07)^{b,2}
S:TE	12.4 (0.3)^{a}	23.7 (1.3)^{c}	50 (3)^{a}	1.70 (0.19)^{c,12}	1.45 (0.07)^{b,1}	2.1 (0.4)^{c,3}	2.26 (0.04)^{d,3}
CH:S:TE	14.0 (0.6)^{b}	19.3 (1.4)^{a}	71 (5)^{b}	0.69 (0.09)^{b,1}	0.65 (0.10)^{a,1}	0.53 (0.07)^{a,1}	0.51 (0.04)^{a,1}
CH:S:TA:TE	14.6 (0.7)^{b}	20.2 (0.3)^{a}	70.3 (1.4)^{b}	0.41 (0.09)^{a,1}	0.58 (0.05)^{a,1}	0.90 (0.14)^{b,2}	1.3 (0.3)^{c,3}

^{abcd} Different letters in the same column indicate significant difference among formulations ($p<0.05$)
^{123} Different numbers in the same row indicate significant difference among solvents ($p<0.05$)

3.2. Polyphenols release kinetics

The total polyphenolic content (TPC) of pure thyme extract incorporated to the films was 44.845 ± 3.696 mg GAE/g polymer. Tannic acid (TA), which was incorporated to the polymer matrix to act as a crosslinking agent (Rivero *et al.,* 2010), is also a polyphenolic compound. However, in the release study, the absorbance values obtained in the release studies performed with films containing TA (without TE) were subtracted from the absorbance obtained in the release studies carried out with the corresponding films containing both TA and TE. Therefore, the reported results (Table 2) show the polyphenols released from thyme extract (without considering the release of polyphenols from TA).

The release of active compounds from a polymeric matrix can be influenced by several phenomena that occur in successive steps: polymeric swelling, which allows the water diffusion, macromolecular matrix relaxation, and active compounds diffusion through

the polymeric matrix into the outer solution until thermodynamic equilibrium is achieved (Buonocore *et al.,* 2003; Sánchez-González *et al.,* 2011). In the present study, the release of polyphenols was affected by different factors, such as solvent migration to starch, chitosan or chitosan-starch matrices, the polymer solubility and the diffusion of the polyphenols to the food simulant.

Table 2 shows the total polyphenol (TP) from TE released to the solvent after 24 hours, when equilibrium was assumed on the basis of kinetic curves. Two trends, which were not affected by the type of solvent were observed; the highest amount of polyphenols was delivered from pure starch films. In this matrix, the values of total polyphenols released ranged from 22.3 to 23.8 mg GAE/g polymer, which represented 49.7 – 53.0% of the total polyphenols incorporated in the films after 180 min of release. This can be explained by the high solubility of starch matrix, without crosslinking effect with the phenolic compounds, which leads to the rapid release of polyphenolic compounds.

Table 2. Total polyphenols (TP) released (mg GAE/g of polymer), total rosmarinic acid (RA) released (mg RA/g of polymer), percentage RA of released, total polyphenols retained (TPR) in the matrix (mg GAE/g of polymer) and percentage of polyphenols retained in the matrix, as a function of different solvents (H_2O, AA3%: Acetic acid aqueous solution (3%, w/v), E10%: Ethanol aqueous solution (10%, v/v), E20%: Ethanol aqueous solution (20%, v/v).

Solvent	Film	TP (mg GAE released /g polymer)	RA (mg RA / g polymer*)	% RA released from film	TPR (mg GAE/ g polymer)	% TP retained in the matrix**
H_2O	**CH:TE**	4.68 (0.17)^{b,2}	-	-	40.16	89.55
	CH:TA:TE	2.4 (0.5)^{a,2}	-	-	42.47	94.71
	S:TE	20.3 (0.8)^{d,1}	3.8 (0.3)^{b,1}	49.10	24.56	54.77
	CH:S:TE	7.7 (0.5)^{c,}2	0.05 (0.00)^{a,1}	0.66	37.18	82.90
	CH:S:TA:TE	4.8 (0.3)^{b,1}	-	-	40.09	89.39
AA3%	**CH:TE**	7.46 (0.16)^{b,3}	-	-	37.32	83.21
	CH:TA:TE	7.4 (0.5)^{b,3}	-	-	37.39	83.37
	S:TE	20.0 (0.3)^{d,1}	-	-	24.86	55.43
	CH:S:TE	11.7 (0.5)^{c,3}	-	-	33.12	73.86
	CH:S:TA:TE	4.6 (0.4)^{a,1}	0.57 (0.00)^{a,1}	7.39	40.24	89.72
E10%	**CH:TE**	4.35 (0.10)^{b,1}	-	-	40.50	90.30
	CH:TA:TE	1.1 (0.5)^{a,1}	-	-	43.78	97.62
	S:TE	20.5 (0.7)^{e,1}	3.70 (0.13)^{b,1}	47.97	24.37	54.35
	CH:S:TE	6.9 (0.2)^{d,12}	0.70 (0.04)^{a,2}	9.11	37.92	84.56
	CH:S:TA:TE	5.1 (0.2)^{c,1}	0.43 (0.00)^{a,1}	5.53	39.74	88.61
E20%	**CH:TE**	5.1 (0.3)^{a,12}	0.13 (0.00)^{a,1}	1.64	39.72	88.57
	CH:TA:TE	-	0.06 (0.02)^{a,1}	0.80	44.85	100.00
	S:TE	21.1 (0.5)^{c,2}	4.8 (0.8)^{d,2}	61.67	23.72	52.88
	CH:S:TE	6.6 (0.2)^{b,1}	2.23 (0.02)^{c,3}	28.90	38.25	85.30
	CH:S:TA:TE	6.5 (0.3)^{b,2}	0.98 (0.16)^{b,1}	12.74	38.37	85.55

* Total amount of rosmarinic acid in the thyme extract is 7.7 ± 0.8 mg RA/ g of polymer. ** Total amount of polyphenols in the thyme extract is 45 ± 3 mg of GAE/ g of polymer.

[abcd] Different letters in the same column indicate significant difference among formulations ($p<0.05$)

[123] Different numbers in the same row indicate significant difference among solvents ($p<0.05$)

Figure 1 shows the percentage of TP polyphenols released as a function of time in the 4 evaluated solvents. In S:TE films, the polyphenols release decreased after 180 minutes in all solvents, which can be related with polyphenols oxidation and it is in agreement with the lower antioxidant activity observed in these films, as commented on above. A similar effect was observed by Perazzo *et al.,* (2014) when incorporating a high content of green tea polyphenols into cassava starch films. This suggests that polyphenols should be incorporated in starch-based matrices at limited concentrations in order to preserve their effectiveness. As regards the release of polyphenols from pure CH films, no significant differences in the amount of released polyphenols were noticed; and lower amounts of polyphenols were released in all solvents, as compared to starch-based films. This can be explained by hydrogen bonding and ionic interactions between the amine groups of chitosan and phenolic groups (Mayachiew and Devahastin, 2010; Kanatt, Chander and Sharma, 2008; Aelenei *et al.,* 2009; Trifkovic *et al.,* 2015), which led to the retention of high amounts of polyphenols into the CH matrix. The highest amount of polyphenols released was obtained in the 3% acetic acid solution (7.46 mg GAE/g polymer), due to the higher solubility of chitosan polymer in acid media. The addition of TA into chitosan and chitosan-starch films led to a reduction in the amount of polyphenols released probably due to the previously reported crosslinking effect of TA (Talón *et al.*, 2017). A similar effect when incorporating TA in chitosan films where TA acted as a cross-linker and led to a more rigid closed and compact matrix was shown by Rivero *et al.* (2010). These results were consistent with the lower water content of the films containing tannic acid as compared to pure CH films (Table 1).

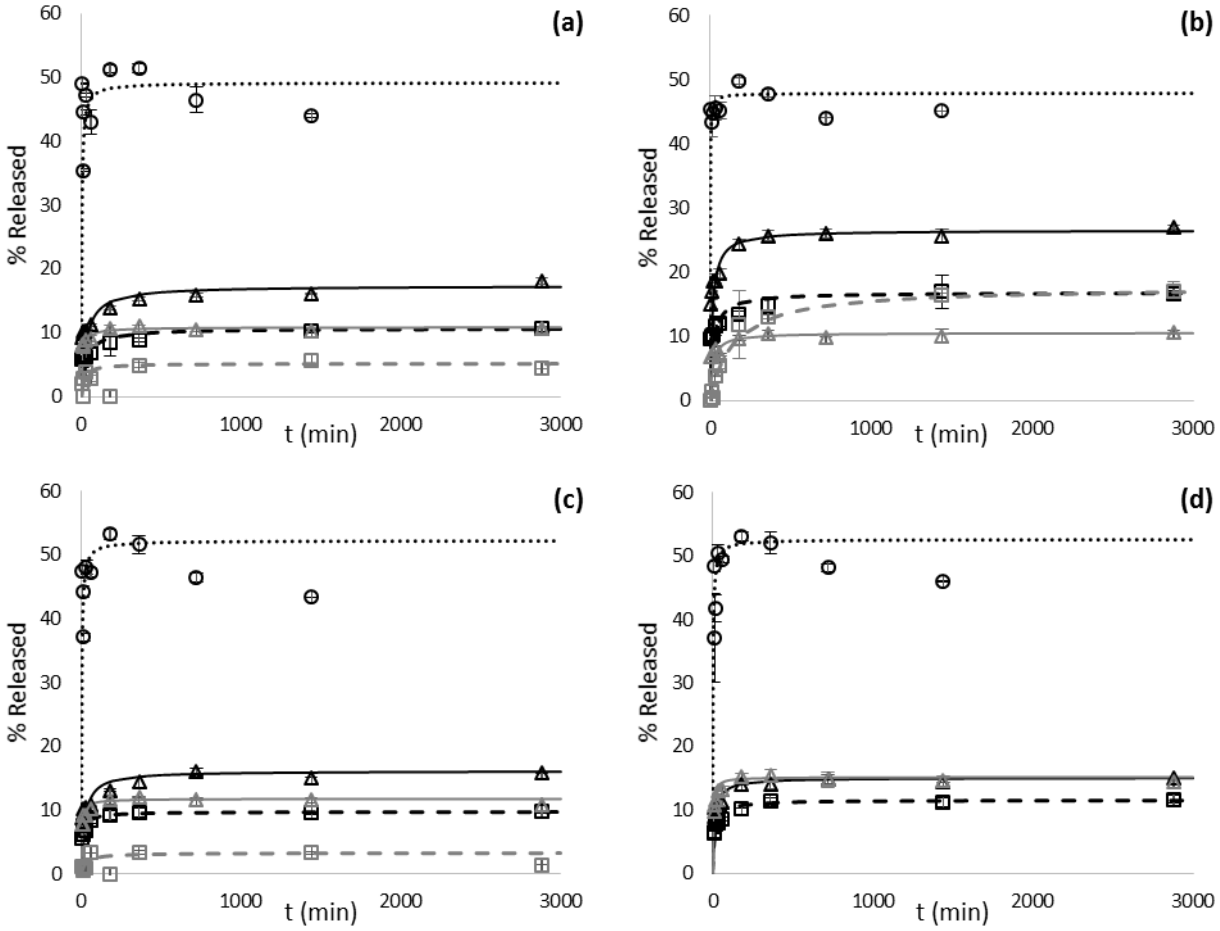

Figure 1. Percentage of total polyphenol released at 25ºC as a function of time in different solvents (a: H_2O; b: Acetic acid aqueous solution (3% w/v); c: Ethanol aqueous solution (10% v/v); d: Ethanol aqueous solution (20% v/v); experimental data (□ CH:TE; ○ S:TE; △ CH:S:TE; □ CH:TA:TE; △ CH:S:TA:TE) and fitted Peleg´s model (- - - CH:TE, — CH:S:TE, ⋯ S:TE, - - - CH:TA:TE, — CH:S:TA:TE).

Polyphenols release from chitosan-starch films was affected by the polarity and pH of the solvent. The amount of polyphenols in the acetic acid solution showed a maximum, due to the high solubility of CH in presence of acetic acid, and a more opened structure, which governs polyphenols release. This is in agreement with the highest antioxidant activity observed in these films in this solvent, as commented on above. The opposite trend was observed in CH:S:TA:TE films, where the TP amount showed a minimum value in the acetic acid solution. In spite of this reduction in TP content, the films preserve their antioxidant activity (Table 1).

Table 2 shows the amount of rosmarinic acid released (mg/g of polymer) from the different film formulations after 24 hours in the four different solvents. The amount of rosmarinic acid (RA) becoming from the pure thyme extract was 7.7 ± 0.8 mg/g polymer. The total release of RA depended on both film composition and the solvent. The highest amount of RA was released from pure starch films, which is in agreement with the above-mentioned higher release of polyphenols from these films. On the other hand, the release of RA was inhibited in pure CH films, while S:CH films showed intermediate results. The increase in RA release with the increase in the ethanol content of the food simulant is in agreement with the decrease in solvent polarity and the subsequent increase of the chemical affinity and solubility of RA with the solvent (Zibetti, Aydi, Claumann, Eladeb and Adberraba, 2016).

Table 2 shows total polyphenols retained (TPR) considering the theoretical content in the different matrices at the end of the release process. Two different trends were observed: the lowest retention of polyphenolic compounds in pure starch films and the enhancement effect of tannic acid on the TPR values of chitosan-based films. Polyphenols retention in pure starch based films after the release process was very similar in the four solvents and represent about 50% of the incorporated amount. The high solubility of the starch matrix (Table 1), provided a greater exposure of polyphenols and promoted a rapid diffusion of active compounds into food simulants. The theoretical 50% retention could be affected by the potential losses occurred during the film formation process, given the great sensitivity of these compounds to oxidation process, as discussed in the previous section, thus affecting the antioxidant activity of these films.

In order to evaluate the tannic acid release from films containing this compound, CH:TA and CH:S:TA films were submitted to a release study for 48 h. TA was quantified as the total polyphenol content by using the same procedure by the Folin-Ciocalteu method. The TP content of pure tannic acid was 0.889 mg GAE/mg TA. This

value agrees with that reported by Sahiner, Sagbas and Aktas (2015) and Sahiner, Sagbas, Aktas and Silan (2016), which found 0.820 mg GAE/ mg of TA. The total percentage of TA released after 24 hours (when the steady state was reached) from the CH:TA films was 10.7%, 37.5%, 16.8%, 26.5% in water, 3% acetic acid solution, 10%, and 20% ethanol aqueous solutions, respectively.

A remarkable increase in the amount of TA released with the increase in ethanol concentration was observed, but the maximum amount released was obtained for the acetic acid medium due to the high solubility of the chitosan matrix in this solvent. For CH:S:TA films, the amount of TA released was 13.2%, 27,1%, 19.0%, 24.2% in water, 3% acetic acid solution, 10% and 20% ethanol aqueous solutions, respectively. No significant differences in the TA retention in the films were observed for CH:S and CH films, except in acetic acid, where CH solubilisation implied a greater release of TA.

The addition of TA in CH:TE films promoted the retention of the polyphenols in the films matrix in the ethanol solutions, ranging from 94.7 to 100% of polyphenols retained. Although this effect was mitigated in the acetic acid medium (83.4 % of retention) due to the CH dissolution (Table 2). In addition, polyphenols retention ranged between 85.6% and 89.7% when adding TA to CH:S:TE films. In this case, no significant TA retention effect of polyphenols was observed in the 20% ethanol aqueous solution, which can be attributed to the greater solubility of phenols, which greatly affects partition coefficient of the compounds between the matrix and the solvent.

The analysis of results indicates that partition of phenols between the polymer matrix and solvent was greatly affected by the presence of CH and TA incorporation. CH bonded polyphenols to a greater extent than starch (Talón *et al.,* 2017), but TA crosslinking of CH chains also contribute to enhance the polyphenol retention in the film, thus limiting their release. Nevertheless, in acid media, both TA and TE phenols are easily delivered to the solvent from CH containing films, according to the water

solubility promotion and the partial matrix disruption. Nevertheless, even in acid media the total polyphenol delivery was lower than that obtained in CH free starch matrices. The experimental data obtained in the release studies were fitted to different models. Generally, the process of diffusion of compounds from swelling equilibrium systems can be fitted to Fick's law. However, phenomena of swelling process may produce a non-Fickian migration mechanism of compounds. To assess the pertinence of the Ficks' law fitting, the data were fitted to Korsmeyer-Peppas model for the sort time range of the process (driving force, $1-M_t/M_\infty$, lower than 5). For a thin film, according to this model, a value of n equal to 0.5 means that the release is related to a Fickian diffusion mechanism of first order; on the contrary, when n=1, polymer relaxation or degradation occurs, leading to zero-order release. An anomalous Fickian diffusion occurs when n values are between 0.5 and 1 (Mandal, Mann and Kundu, 2009; Huang, Yu and Xiao, 2007). As shown in Table 3, the values of coefficient n were far from the Fickian behaviour except for the release of polyphenols from CH:TA:TE films in acetic acid, where the behaviour was Fickian (n = 0.506). The low values of n can be attributed to the partial films solubilisation in the solvents which contribute to the compound delivery in a non-Fickian process.

Table 3. Kinetic constants of Korsmeyer-Peppas model (rate constant, k, h^{-1} and difussional exponent) at 25ºC in different simulants (H2O, AA3%: Acetic acid aqueous solution (3%, w/v), E10%: Ethanol aqueous solution (10%, v/v), E20%: Ethanol aqueous solution (20%, v/v). Mean values and standard deviation, in brackets.

Film		H_2O	AA3%	Et10%	Et20%
CH:TE	**k**	0.45 (0.03)^{b,1}	0.483 (0.010) ^{b,12}	0.55 (0.07)^{c,2}	0.48 (0.07) ^{a,12}
	n	0.101 (0.013)^{b,1}	0.097 (0.008)^{b,1}	0.09 (0.02)^{ab,1}	0.101 (0.017)^{b,1}
CH:TA:TE	**k**	0.35 (0.13)^{a,2}	0.03 (0.02)^{a,1}	0.31 (0.05)^{a,2}	-
	n	0.16 (0.05)^{c,1}	0.51 (0.10)^{c,2}	0.16 (0.03)^{c,1}	-
CH:S:TE	**k**	0.43 (0.04)^{b,1}	0.52 (0.03)^{b,2}	0.45 (0.02)^{b,12}	0.53 (0.03)^{a,2}
	n	0.111 (0.002)^{b,1}	0.093 (0.003)^{b,1}	0.112 (0.013)^{b,1}	0.094 (0.013)^{ab,1}
CH:S:TA:TE	**k**	0.68 (0.03)^{c,2}	0.56 (0.04)^{b,1}	0.64 (0.05)^{d,12}	0.69 (0.03)^{b,2}
	n	0.055 (0.003)^{ab,1}	0.075 (0.012)^{b,1}	0.072 (0.007)^{ab,1}	0.062 (0.004)^{ab,1}
S:TE	**k**	0.84 (0.07)^{d,2}	0.87 (0.07)^{c,2}	0.74 (0.02)^{e,1}	0.72 (0.05)^{b,1}
	n	0.24 (0.014)^{a,1}	0.023 (0.009)^{a,1}	0.050 (0.003)^{a,1}	0.049 (0.004)^{a,1}

^{abcde} Different letters in the same column indicate significant difference among formulations ($p<0.05$)
^{123} Different numbers in the same row indicate significant difference among solvents ($p<0.05$)

Experimental data in terms of total released polyphenols as a function of time were also fitted to Peleg's model, and the obtained parameters are shown in Table 4. Unlike Korsmeyer- Peppas model, which is only valid for the first 60% of compound released, Peleg model can predict the value at equilibrium when time tends to infinity, thus, this model can estimate long range of values from experimental data obtained in tests of relatively short duration (Botelho, Corrêa, Martins, Botelho and Oliveira, 2013; Hines and Kaplan, 2011; Peleg, 1988). In the present study, high correlation coefficients were obtained for Peleg's model fit, as seen in Figure 2. However, in S:TE films, the model was fitted only in the first section of the release due to rapid oxidation of the polyphenols, as observed in Figure 1, where the released amount of polyphenols tends to decrease after the equilibrium was reached.

Table 4. Parameters of Peleg´s model at 25ºC in different simulants (H_2O, AA3%: Acetic acid aqueous solution (3% w/v), E10%: Ethanol aqueous solution (10% v/v), E20%: Ethanol aqueous solution (20% v/v). Constant k_1 (min·mg^{-1} GAE·g^{-1} polymer) is related to the release rate at the beginning of the process. k_2 (g polymer·mg^{-1} GAE) relates to the asymptotic value which can be related to the equilibrium value. M_∞ is the inverse of k_2 and is the amount of polyphenols released at equilibrium (mg GAE·g^{-1} polymer). Mean values and standard deviation, in brackets.

		H_2O	AA3%	E10%	E20%
CH:TE	k_1	6.2 (1.6)^{ab,2}	2.8 (1.6)^{a,1}	2.1 (1.2)^{a,1}	2.8 (1.7)^{c,1}
	k_2	0.210 (0.008)^{c,3}	0.123 (0.006)^{c,1}	0.227 (0.003)^{a,4}	0.192 (0.010)^{c,2}
	M_∞	4.76 (0.19)^{b,1}	7.5 (0.3)^{b,3}	4.40 (0.06)^{b,1}	5.2 (0.3)^{a,2}
CH:TA:TE	k_1	11 (5)^{b,12}	25 (14)^{b,2}	20 (12)^{b,2}	-
	k_2	0.43 (0.08)^{d,2}	0.123 (0.013)^{c,1}	0.67 (0.05)^{b,3}	-
	M_∞	2.4 (0.5)^{a,1}	8.2 (0.8)^{b,2}	1.503 (0.112)^{a,1}	-
S:TE	k_1	0,08 (0.08)^{a,1}	0.05 (0.03)^{a,1}	0.12 (0.03)^{a,1}	0.10 (0.05)^{a,1}
	k_2	0.046 (0.004)^{a,12}	0.047 (0.002)^{a,2}	0.0427 (0.0012)^{a,1}	0.042 (0.001)^{a,1}
	M_∞	21.7 (1.6)^{d,12}	21.1 (0.9)^{d,1}	23.4 (0.6)^{e,2}	23.6 (0.7)^{c,2}
CH:S:TE	k_1	4.0 (1.8)^{ab,2}	1.2 (0.6)^{a,1}	2.9 (0.6)^{a,12}	1.9 (0.3)^{bc,1}
	k_2	0.128 (0.008)^{b,2}	0.084 (0.002)^{b,1}	0.138 (0.003)^{a,2}	0.149 (0.006)^{b,3}
	M_∞	7.8 (0.5)^{c,2}	11.9 (0.3)^{c,3}	7.24 (0.15)^{d,12}	6.7 (0.3)^{b,1}
CH:S:TA:TE	k_1	1.7 (1.5)^{ab,12}	3.59 (1.18)^{a,2}	1.1 (0.5)^{a,1}	0.5 (0.3)^{ab,1}
	k_2	0.205 (0.007)^{c,3}	0.212 (0.009)^{d,3}	0.1876 (0.0009)^{a,2}	0.147 (0.007)^{b,1}
	M_∞	4.87 (0.17)^{b,1}	4.7 (0.2)^{a,1}	5.33 (0.02)^{c,2}	6.8 (0.3)^{b,3}

^{abc} Different letters in the same column indicate significant difference among formulations ($p<0.05$)
^{123} Different numbers in the same row indicate significant difference among solvents ($p<0.05$)

Table 4 shows the parameters of Peleg's model, where k_1 is related to the inverse of the initial velocity of the release process and k_2 is the inverse of the total concentration released at equilibrium (M_∞), which reflects the total amount released at equilibrium in mg GAE/ g polymer. The M_∞ values coincide with the experimental determination at 24 h of film-solvent contact time, reported in Table 2. In pure CH films the greater release occurred in acid media, with similar rate as in the ethanol solutions, whereas slower release occurred in pure water, where the final release was similar as in the ethanol solutions. When CH films contained TA, the release rate was greatly reduced in all solvents showing similar values in all cases, but the final release was enhanced in acid media. This is consistent with the crosslinking effect of TA in CH films, which delays the release rate according to the higher tortuosity factor for mass transfer in the films, although the enhancement of CH solubility in acid medium let to a greater delivered amount of actives. In pure starch films the fastest delivery rate and highest delivery ratio were found, regardless the solvent, according to the weaker interactions of polyphenols with the matrix. For starch films, a decrease in the phenol content released was observed after the maximum delivered at 24 h, which can be explained by compound oxidation. This tendency to polyphenol degradation in the presence of starch could explain the lowest antioxidant capacity observed in starch films (Table 1). In CH:S blend films, release rate slightly increased with respect to CH films, as well as the total released amounts, showing similar effects of the solvents as observed for CH films. This behaviour suggests that the greatest part of the active compounds interact mainly with the CH fraction, despite the highest proportion of starch in the films. Finally, the incorporation of TA to CH:S films, provoked some similar effects to those observed in pure CH films, but the acid medium did not promote an increase in the final total amount released, which was similar to that occurred in water. However, the amount of polyphenols release in these films was higher in ethanol solutions, where the release

rate was promoted, with respect to neutral and acid aqueous solutions. Therefore, as expected, TA mainly interact with CH and the crosslinking effect occur to lesser extent in CH:S matrix, being less limiting for the polyphenol release.

The obtained results for CH and films containing TA are in agreement with that reported by Trifković *et al.* (2015), who shown an incomplete release of polyphenols from chitosan beads with a crosslinker agent (glutaraldehyde) in acid medium (pH=2.20). Likewise, polyphenol also strongly interact with the TA free CH matrix, which limit their release to the aqueous media, as compared with the starch matrix.

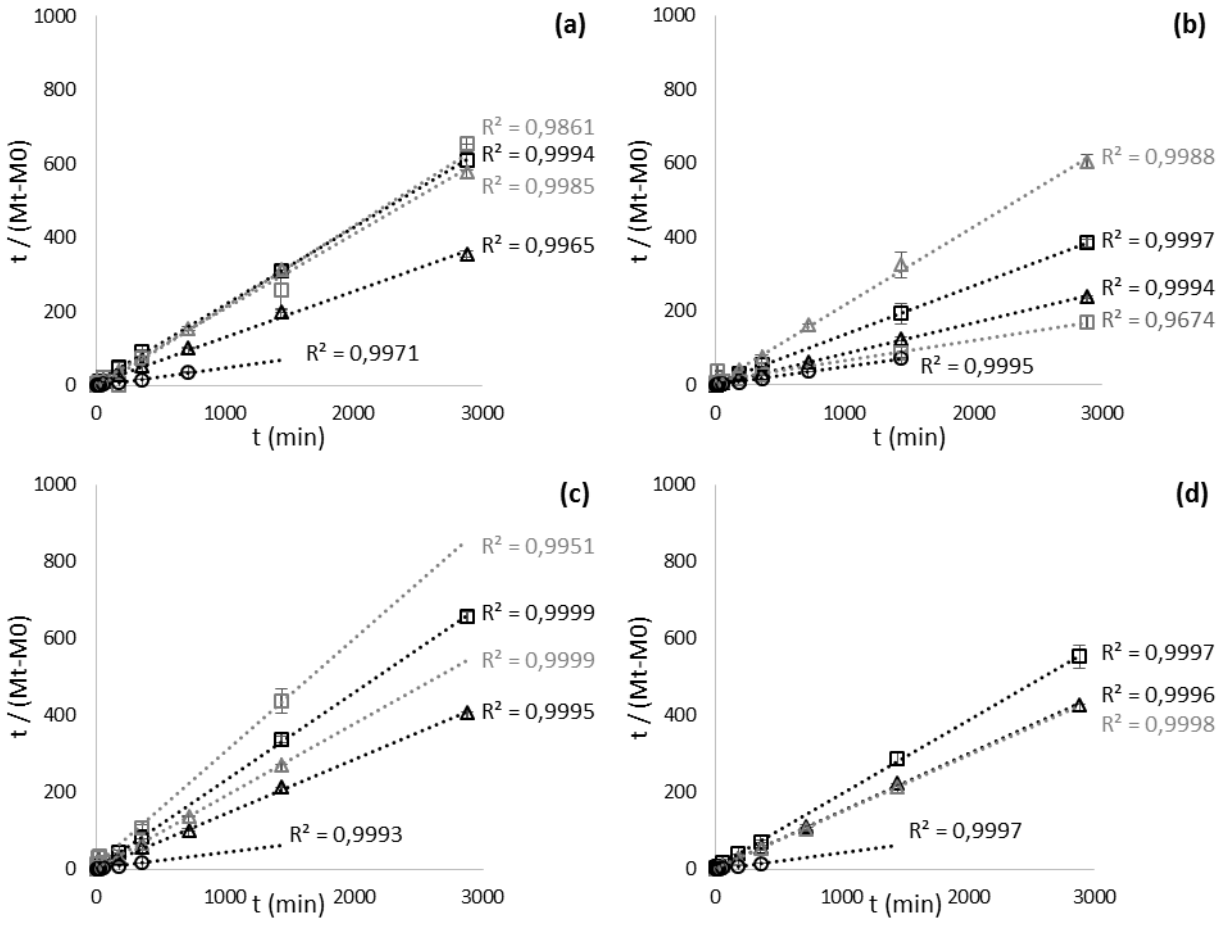

Figure 2. Application of Peleg equation to experimental data of all the films (□ CH:TE; ○ S:TE; △ CH:S:TE; □ CH:TA:TE; △ CH:S:TA:TE) in different solvents (a: water; b: 3% acetic acid; b: 10% ethanol and d: 20% ethanol).

4. Conclusions

Polyphenols from thyme extract strongly interact with chitosan chains in both pure chitosan matrix and chitosan-starch blend films. These interactions led to a reduction of polyphenols release rate in aqueous media, even at low pH where, despite the increase in the total delivered amount, this was much lower than that occurred in starch films. Incorporation of tannic acid to the chitosan films provoked the matrix crosslinking, to a greater extent in pure chitosan films, which greatly reduced the ratio and rate of polyphenol release, although in the acid medium, the increase in the chitosan solubility enhanced the total polyphenol delivery. However, in chitosan:starch blends, the cross-linking effect of tannic acid inhibited the release promotion of the acid medium, while both release rate and ratio were enhanced in ethanol solutions, thus indicating the role of chitosan-starch interaction in the blend matrix rearrangement. In fact, the total delivery of polyphenols in CH:S blend films was nearer to that occurred in chitosan films rather than to the delivery in starch films, in spite of the greater starch ratio in the blend.

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