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► To cite this version:

Nasreddine Benbettaieb, Ruth Cox, Michelle Gilbert, Frédéric Debeaufort. How the temperature and salt content of food simulant affect the release of tyrosol or phenolic acids from bioactive films?. *Future Foods*, 2021, pp.100040. 10.1016/j.fufo.2021.100040 . hal-03211745

HAL Id: hal-03211745

<https://hal.science/hal-03211745v1>

Submitted on 13 Jun 2023

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1 **How the temperature and salt content of food simulant affect the release of**
2 **tyrosol or phenolic acids from bioactive films?**

3
4 **Nasreddine Benbettaieb^{1&2}, Ruth Cox³, Michelle Gilbert³ and Frédéric Debeaufort^{1&2}***

5 ¹ Univ. Bourgogne Franche-Comté, UMR PAM A 02.102, F-21000 Dijon, France

6 ² IUT-Dijon-Auxerre, Dpt BioEngineering, 7 blvd Docteur Petitjean, F-20178 Dijon Cedex,
7 France

8 ³ Cork Institute of Technology, Bioscience Dpt, Rossa Av., Bishopstown, Cork, Ireland

9
10
11 * Corresponding author:

12 Univ. Bourgogne Franche-Comté, UMR PAM A 02.102, F-21000 Dijon, France

13 Tel: +33 380 77 2388; fax: +33 380 77 4011.

14 E-mail address: frederic.debeaufort@u-bourgogne.fr (F. Debeaufort)

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20 **Abstract**

21 Active films made of chitosan and fish gelatin blends containing 5% (w/wt of polymers) of
22 tyrosol, ferulic acid or caffeic acid were prepared by the casting method. The kinetic of release
23 was assessed in water or in 1% (w/v) NaCl solution, at three temperatures (4, 25 and 32°C).
24 Partition ($K_{f/S}$) and the effective diffusivity (D) was modelled which allowed to characterize
25 the release mechanism. Tyrosol has a faster release at a higher concentration than the other
26 antioxidants, because of its structure and a lower level of intermolecular interactions with the
27 biopolymer matrix. There is less effect of temperature and of the salt content on the release of
28 tyrosol contrarily to the phenolic acids. The NaCl solution (food simulant) induced
29 conformational changes in the biopolymer network due to the modifications of the interactions
30 between the gelatin, chitosan and antioxidants, inducing a less strengthen network and a faster
31 release.

32 **Keywords:** edible films; gelatin-chitosan network; effective diffusivity; partition; NaCl

33

34 **1. Introduction**

35 Nowadays, the shelf life of food could be prolonged by using antioxidant and barrier packaging
36 materials without a negative effect on consumers (Zhu *et al.*, 2012). This is considered as one
37 of the promising innovative technology for food preservation able to reduce food loss (Zhu *et*
38 *al.*, 2012). Natural antioxidants are incorporated into biopolymeric matrices intended to be in
39 contact with the food product with a very low restriction to contact ability according safety and
40 regulation reasons (Otero-Pazos *et al.*, 2016). Phenolic acids and tyrosol are recognized
41 effective natural antioxidants for food and pharmaceutical applications and GRAS classified,
42 mainly extracted from plants (coffee, olive oil and leaves) (Banerjee *et al.*, 2017) and very
43 often cited for the development of sustainable active bio-based packaging films (Benbettaïeb
44 *et al.*, 2019).

45 Natural biopolymers (proteins or polysaccharides) are manufactured for packaging materials.
46 Indeed, this trend tends to satisfy the consumer demand for safe materials but also satisfy to
47 environmental issues by using renewable and biodegradables matters coming from agri-food
48 by-products and wastes (Benbettaïeb *et al.*, 2018). Chitosan is a polysaccharide with
49 biodegradable, biofunctional, and intrinsic antimicrobial properties which is able to entrapped
50 bioactive compounds (Moradi *et al.*, 2012). Gelatin, is obtained from the hydrolysis of collagen
51 collected in animal slaughterhouses and fish processing plants where collagen is considered as
52 a waste. Gelatin has excellent film-forming abilities and is able to interact with chitosan (Hoque
53 *et al.*, 2011, Benbettaïeb *et al.*, 2014). At appropriate pH, the gelatin and chitosan have opposite
54 charges which allow to settle ionic interactions able to strengthen and stabilize the gelatin-
55 chitosan network. Chemical interaction between chitosan and gelatin was previously
56 demonstrated using FTIR in a previous work (Benbettaïeb *et al.*, 2014).

57 When antioxidants are incorporated into bio-based films, they could migrate into the food or
58 in liquid simulant. The release of these natural antioxidant in the media permits to reduce the
59 amount of chemical additives loaded in foods. Reduction of synthetic additives in food, as well
60 as in packaging material is sought by both the consumers and regulations. Therefore, the
61 substitution of synthetic antioxidants by natural ones (Ma *et al.*, 2017, Piñeros-Hernandez *et*
62 *al.*, 2017), such as plant extracts (Qin *et al.*, 2015), essential oils (Moradi *et al.*, 2016) and
63 phenolic compounds (flavonoids, tannin and phenolic acid) is of great interest (Benbettaïeb *et*
64 *al.*, 2018). The release consists of the transfer by diffusion of small molecules from the
65 packaging material into the food or into food simulants. Diffusivity is a molecular mechanism
66 described by the Fick's laws, considering both an effective diffusion coefficient (D) and a
67 partition coefficient ($K_{f/S}$). D reveals how fast the antioxidant (=migrant) is transferred whereas
68 the $K_{f/S}$, equal to the ratio of antioxidant amount between the packaging and food (or simulant)
69 when diffusion reaches the equilibrium. Some parameters related to the food or to the
70 antioxidant itself (polarity, solubility, hydrophobicity, pH, ionic strength, water activity..), or
71 related to the surroundings (temperature, pressure, relative humidity...) may affect the behavior
72 of the antioxidant release. The polymer matrix characteristics are also strongly responsible of
73 the release mechanism such as the network mesh, its polarity, its density or crystallinity (Tapia-
74 Blácido *et al.*, 2018, Gutiérrez and Álvarez 2017). Also, chemical interactions between the
75 polymer chains and the antioxidant could affect the release process. Indeed, salt could induce
76 “salting-in” or “salting-out” effects with proteins, which improved the release (lower retention
77 and faster diffusion) or reduced the release respectively (Endo *et al.*, 2011). The aim of the
78 present work was to investigate the effect of the salt content and of the temperature of the
79 medium on the release kinetics of three natural antioxidants incorporated in bio-based
80 chitosan-gelatine films. A mathematical modelling was used in order to model the kinetics of
81 release. In this way, we chose three temperatures: 4°C as refrigeration temperature of many

82 foods, 25°C is the consumption temperature and 32°C is the ambient temperature in sub-arid
83 regions. The NaCl content of food simulant was chosen at 1% (w/v) corresponding to the
84 maximum recommended concentration of salt in foods according the European Commission
85 (2016).

86 **Hypothesis:** temperature and NaCl presence in food simulant affect differently the release of
87 tyrosol or phenolic acids from a gelatine-chitosan film.

88

89 **2. Materials and methods**

90 **1.2. Preparation of the active film**

91 The bioactive films were prepared according to Benbettaïeb *et al.*, (2018): 2 grams of chitosan
92 powder were dispersed in 100 mL of water-acetic acid solution (1% v/v) under a 6000 rpm
93 agitation (Ultra Turrax RW16 basic- IKA-WERKE) at 25°C. In the same time, another solution
94 of 6% (w/v) of fish gelatin powder was dispersed in water at 70°C. The two solutions were
95 mixed at a ratio 1:1 (w/w) under stirring at 50°C. The pH was adjusted at 5.5 in order to favor
96 electrostatic interactions between the two biopolymers and to prevent phase separation. At this
97 pH, the gelatin is negatively charged (the iso-electric point of gelatin is about 4.5) while
98 chitosan is positively charged (pKa of the chitosan amino group is about 6.5). This favors the
99 ionic interactions between amino groups ($-\text{NH}^{3+}$) of chitosan and carboxylate ($-\text{COO}^-$) groups
100 of gelatin which allows to strengthen the network and thus improves the film-forming
101 properties.

102 Glycerol (plasticizer) was added at 10% (w/w of dry matter). Each antioxidant compound was
103 loaded in separate film-forming solution under stirring at concentration 5% (w/wt of dry
104 mater). 30 mL of the mixed solution was poured into plastic Petri dishes and dried at 45% RH
105 at 25°C for 1 day in a climatic chamber (KBF 240 Binder, ODIL, France). The films were then

106 peeled off from the Petri dishes after drying and stored in controlled conditions (25°C - 50 %
107 RH) prior analysis.

108 **2.2. Methodology of the release kinetic measurement**

109 The release kinetics of the antioxidants from the films was carried out by determining the rate
110 of migration from the biopolymer film when immersed in water or in 1% (w/v) NaCl solution
111 (food stimulant) adapted from (Benbettaïeb *et al.*, 2018).. The release process was followed by
112 the determination of the concentration of antioxidant in the food simulant at 4, 25 and 32°C.
113 To do that, 60mg of each film sample were immersed in 100 mL of the simulant in a glass vial
114 and kept under magnetic agitation at 120 rpm. The ratio film/simulant corresponds as close as
115 possible to the regulation for migration tests described in the Directive EC/89/109 (European
116 Commission 1989). Then, 2 mL of the release medium were periodically sampled and the OD
117 was read until equilibrium. The 2mL sampled were returned into the simulant in order to keep
118 constant the volume until equilibrium. The antioxidant concentration of the samples was
119 determined by UV-vis spectrophotometry (Biochrom WPA Lightwave II UV/Visible
120 spectrophotometer) at 222 nm for tyrosol, 325 nm for ferulic and caffeic acid. A series of
121 standard solutions for each antioxidant was used for calibration, according to the Beer-
122 Lambert's law. The release of each antioxidant was carried out at in triplicate. The solubility
123 of the antioxidant in water was not a limitation as the concentrations used are very low
124 compared to their limit of solubility. Indeed, the tyrosol has a water solubility of 124 g/L,
125 ferulic acid of 15 g/L and caffeic acid of 1 g/L. If all the antioxidant loaded in films are released,
126 according to the ratio film/simulant used, the maximum concentration potentially reached in
127 the simulant would be 0.03 g/L.

128

129

130 **2.3. Determination of the effective diffusion (D) and partition (K_{f/s}) coefficients of the**
 131 **antioxidants**

132 The effective diffusion coefficient (D, m².s⁻¹) was determined using a relationship derived from
 133 the Fick's second law equation (transient state) for a unidirectional diffusion in one-dimension
 134 (Crank 1975). This model considered a limited volume of film in a limited volume of solution
 135 (simulant or water) for a plane sheet (film). The model describing this phenomenon (Equation
 136 1) considers 1) the initial maximum concentration of the antioxidant in the film is uniformly
 137 distributed at t=0; 2) the initial concentration in the simulant equals to zero at initial time; there
 138 is no boundary effect (because the medium is perfectly stirred); and the diffusivity is supposed
 139 non-concentration-dependent nor time-dependent.

$$140 \quad \frac{C_t}{C_\infty} = 1 - \sum_{n=1}^{\infty} \frac{2\alpha(1+\alpha)}{1+\alpha+\alpha^2q_n^2} \exp\left(-\frac{Dq_n^2t}{L^2}\right) \quad \text{Equation 1}$$

141 C_t (mg/L), the antioxidant concentration over time in the release medium; C_∞ (mg/L), the
 142 antioxidant concentration at equilibrium in the release medium; L, the half thickness of the film
 143 (m); q_n, the non-zero positive roots of tan(q_n) = -αq_n (n values ranging from 1 and 6); α
 144 determined from $\alpha = \frac{V_s}{(K_{f,s} \times V_f)}$; V_s, the release medium volume (m³); V_f, the film volume (m³)
 145 and K_{f,s} = $\frac{C_{f,\infty}}{C_{s,\infty}}$ the partition coefficient of the antioxidant between film and simulant (solution)
 146 at equilibrium; and C_{f,∞} and C_{s,∞}, the equilibrium concentrations of the antioxidant (mg/L) in
 147 the film and in the food simulant, respectively.

148 Equation 1 fit to the experimental data by minimising the sum of the square of the differences
 149 between measured and predicted values, using the Levenberg–Marquardt algorithm, and
 150 considering D as adjustable parameter. Modelling was performed using MATLAB/Simulink

151 software environment (MATLAB 8.5-2015). The means value of effective diffusion of each
152 formulation was calculated from the triplicate analyses.

153 The coefficient of determination (R^2) and the Root Mean Square Error (RMSE, mg/L) were
154 determined from each measure:

$$155 \quad \text{RMSE} = \sqrt{\frac{(\hat{y} - y)^2}{N}} \quad \text{Equation 2}$$

156 where, \hat{y} and y were respectively predicted and experimental residual values, and N , is the
157 number of experiment point.

158 There was an acceptable fitting between the theoretical values and the experimental data for
159 the all antioxidants ($R^2 > 0.94$ and $\text{RMSE} < 0.1$ mg/L) for the three temperatures and for the
160 salt content.

161

162 **2.4. Statistical analyses**

163 All the data were at least triplicated and the statistical analysis was done using an independent
164 sample t-test with the statistical software SPSS 13.0 (SPSS Inc., Chicago, IL). A standard
165 deviation (p -value < 0.05) at the 95% confidence level was used to compare the data according
166 the NaCl content and the temperature.

167

168 **3. Results and Discussion**

169 At pH 5.5, gelatin is negatively charged (the iso-electric point of gelatin is about 4.5) while
170 chitosan is positively charged (pK_a of the chitosan amino group is about 6.5). This favors the
171 ionic interactions between amino groups ($-\text{NH}_3^+$) of chitosan and carboxylate ($-\text{COO}^-$) groups
172 of gelatin. At this pH, both ferulic acid ($pK_a=4.58$) and caffeic acid ($pK_a 4.62$) exhibit negative

173 charges whereas tyrosol (pKa=10.17] has positive charges. This implies the antioxidants could
174 modify differently the electrostatic interactions between gelatin and chitosan, modifying the
175 network structure and thus changing the retention/release behaviors.

176

177 Phenolic acids are slower released and in a less amount than that of tyrosol (**Figure 1**). Less
178 than 10 min was necessary to achieve the release of tyrosol, whereas the release of others
179 antioxidants required at least 30 or 40 min whatever the temperature or the nature of the liquid
180 medium (water or 1% NaCl solution). Moreover, tyrosol was less retained in the film,
181 corresponding to a retention of 30-40%. The higher retentions of ferulic acid or caffeic acid
182 (up to 65%) could be attributed to the charge-charge interactions occurring between the
183 polymer network and the acids. Indeed, this is related to the negative groups (carboxylate
184 group) of the phenolic acids and to the positive groups of gelatine or chitosan (ammonium
185 groups). The charge-charge interactions between phenolic acid and protein (and or chitosan)
186 could explain the stronger impact of caffeic and ferulic acid on the properties of the chitosan-
187 gelatin film. In contrast, tyrosol (without carboxylate ($-\text{COO}^-$) groups), is not able to make a
188 charge-charge interaction with the protein or chitosan (Benbettaieb *et al.*, 2018). The effect of
189 phenolic acids on film structure and release was displayed by FTIR: the shifts on amide I and
190 amide III band are more related to the changes in the structural organization of the network
191 which induced the changes in diffusivity and kinetic. Caffeic and ferulic acids are thus able to
192 cross-link the biopolymers as previously displayed for the same films. Consequently, the
193 effective diffusion coefficient (**Table 1**) of the phenolic acids in water is from 5 to 18 times
194 smaller than that of the tyrosol that is not involved in the network strengthening (no
195 crosslinking). Also, the chemical structure of tyrosol without double bond in the lateral chain
196 makes it more flexible and thus could enhance its diffusion in polymers compared to caffeic or
197 ferulic acids.

198 The temperature influences significantly the release in the water of the two phenolic acids,
199 reducing the time to reach equilibrium and increasing the amount of antioxidant released,
200 whereas it seems having only a very weak effect on the tyrosol release behaviour (**figure 1**).
201 However, and as expected, the release mechanism is temperature dependent. The increase of
202 temperature leads to a greater biopolymer chain mobility that makes the effective diffusion
203 higher for all the compounds and whatever the medium (**Table 1**). Indeed, D increases for all
204 the antioxidants from 2 to 4 times when the temperature rises from 4 to 32°C, but this seems
205 also dependent on the medium. As the charge-charge and ionic interactions are temperature
206 dependent (Howell 2005), the partition coefficient of the phenolic acids is significantly
207 affected, reduced up to 80 times in water, but much less when NaCl solution was used. The
208 $K_{f/S}$ of tyrosol is much lower than that of phenolic acids because tyrosol is very hydrophilic
209 ($\log P = 0.62$) and water soluble (water solubility $ws = 124 \text{ g.L}^{-1}$) compared to the caffeic acid
210 ($\log P = 1.42$, $ws < 1 \text{ g.L}^{-1}$) or ferulic acid ($\log P = 1.64$, $ws = 15 \text{ g.L}^{-1}$). It is thus logical that
211 tyrosol is more and faster released in water with the temperature increase.

212 The NaCl presence in the liquid medium induced conformational change within the biopolymer
213 matrix because it diffuses from the solution into the film. This modifies the interactions
214 between the gelatin and chitosan, inducing a less strengthen network and thus a faster release
215 particularly for the phenolic acids. Indeed, the enhanced ionic strength of the food simulant
216 increased the activity of the phenolic compounds, thus improving the driving force for
217 migration into the simulant. The diffusion coefficient of all the compounds is from 2 to 7 times
218 higher when NaCl was added (**Table1**). In the case of tyrosol, there is no significant effect of
219 NaCl on the released amount and rate because of the retention of tyrosol in film is not related
220 to ionic or charge-charge interactions. Moreover, as demonstrated by Sow and Yang (2015), a
221 salting-out effect of NaCl on the gelatin could be supposed and thus may explain the release
222 behaviour observed. Indeed, these authors displayed that salt induce a conformational change

223 of fish gelatin from helices toward aggregates that are denser and tends to excludes small
224 molecules entrapped in. Yoshida *et al.*, (2008) and Tasaki *et al.*, (2009) demonstrated that salt
225 presence allows to faster the release of small molecules (drugs) from polyelectrolyte gels and
226 films.

227 From this work, the efficacy of active films containing natural phenolic was dependent of both
228 the media composition and the storage temperature. Understanding these factors effect on
229 diffusivity and partition could help us to tune the controlled release and adapt the concept of
230 this kind of active packaging film to food application.

231

232 **4. Conclusion**

233 The release into water or 1% (w/v) NaCl solution of the active compounds entrapped in films
234 based on gelatin-chitosan blends suits a Fickian behaviour. The lowest effective diffusivity and
235 released quantity were observed for ferulic acid or caffeic acid compared to tyrosol. Indeed,
236 tyrosol weakly interacted with the biopolymer chains and did not involve electrostatic
237 interactions, explaining that tyrosol release was less sensitive to NaCl. As expect, the
238 temperature increases the diffusivity and is predominant on the solubility. Indeed, even if the
239 partition coefficient of phenolic acids decreased with temperature, the rate of transfer increased
240 with temperature. The presence of NaCl allow the less strengthening of chitosan-gelatin
241 network which excludes the entrapped antioxidants and thus faster the release of phenolic
242 acids. The efficacy of active films maybe dependent of the food composition and temperature
243 for natural phenolic acid whereas it is not influenced for non-charged compounds such as
244 tyrosol.

245

246 **Author statements - Conflict of interest**

247 The authors have no conflict of interest with any company or colleague.

248 The data that support the findings of this study are available from the corresponding author
249 upon request.

250

251 **Acknowledgements and funding sources**

252 This work was mainly supported by the own funds of the UMR PAM laboratory (Axe
253 transversal 3) and by the Regional Council of Bourgogne Franche-Comté (ANER project
254 ActiBioPack - ATP grant n°2020-Y-12730) and the "Fonds Européens de Développement
255 Régional (FEDER)" who invested in lab equipments. The authors wish to thanks Anne Marie
256 Seuvre and Bernadette Rollin for their help in equipment facilities.

257

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326

Figure 1

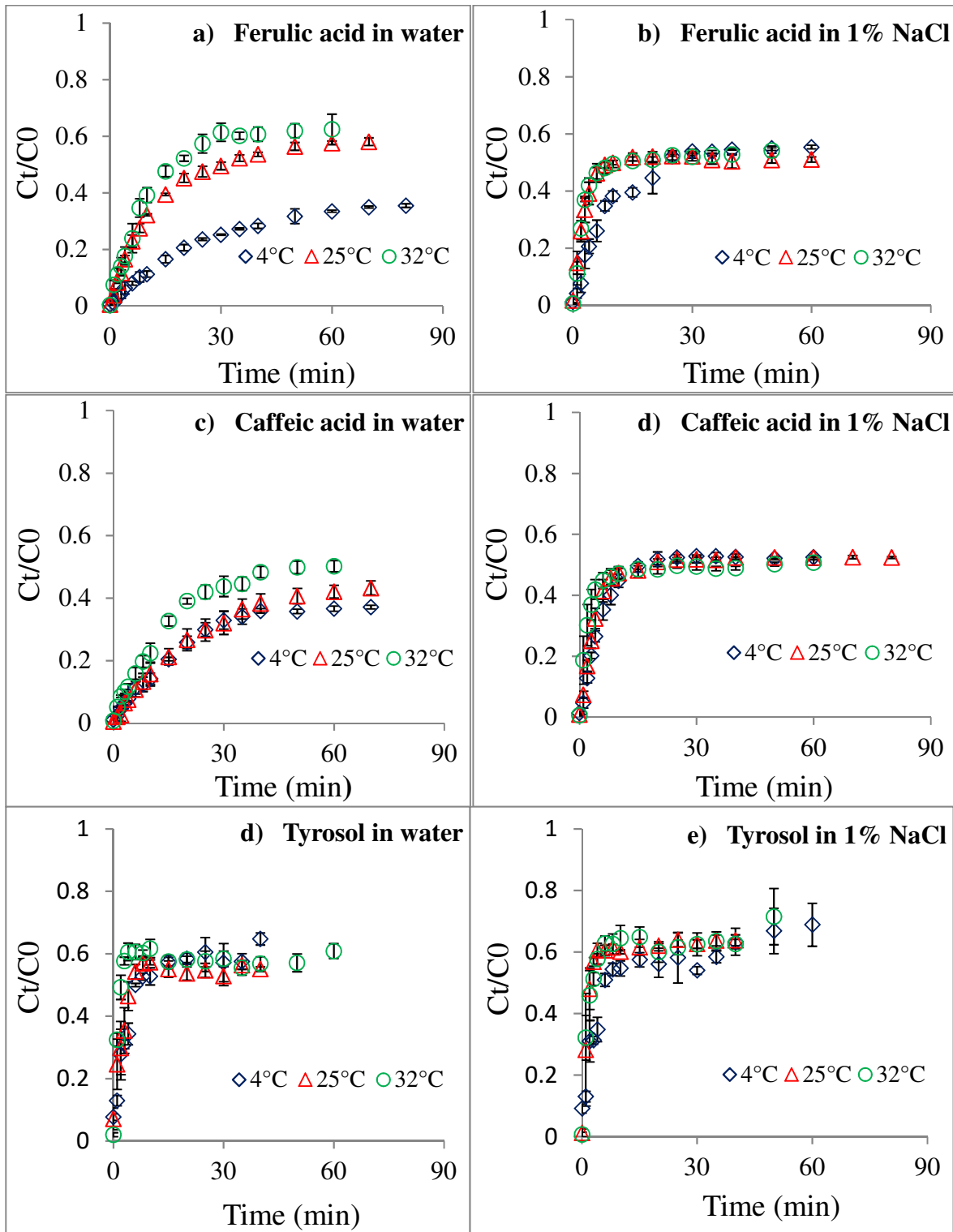
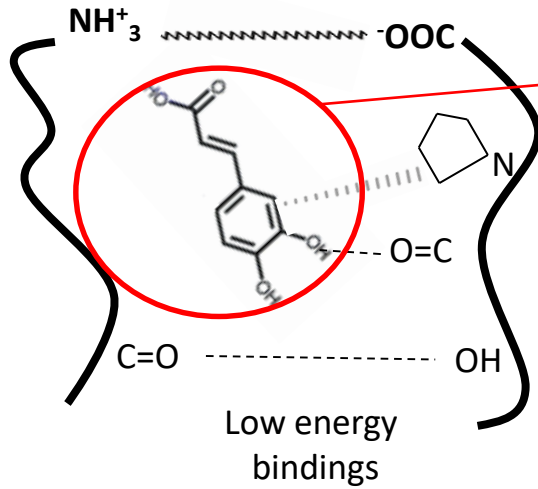


Table 1

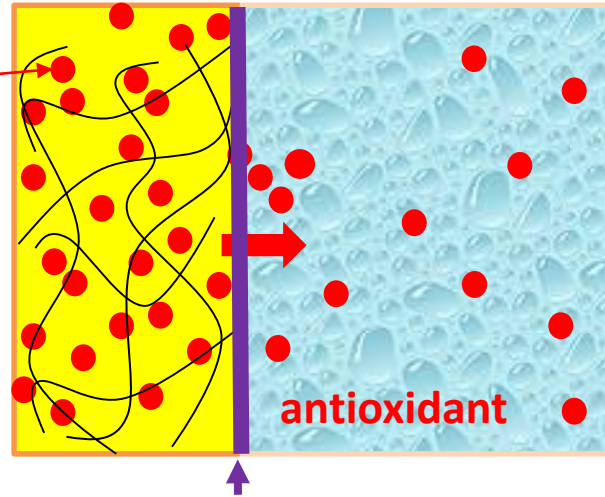
	NaCl content (% w/v)	Temperature (°C)	Tyrosol	Ferulic acid	Caffeic acid
Diffusion coefficient (D, 10⁻¹² m²/s)	0	4	2.21±0.20 ^{A,a}	0.45±0.03 ^{B,a}	0.48±0.07 ^{B,a}
		25	5.58±1.75 ^{A,b}	0.59±0.03 ^{B,b}	0.33±0.05 ^{C,b}
		32	9.86±1.33 ^{A,c}	1.00±0.09 ^{B,c}	0.55±0.06 ^{C,a}
	1	4	2.00±0.95 ^{A,a}	1.09±0.17 ^{A,B,c}	1.42±0.12 ^{A,B,c}
		25	8.50±2.29 ^{A,b}	3.24±0.69 ^{B,d}	1.61±0.28 ^{C,c}
		32	8.21±0.80 ^{A,b,c}	3.67±0.73 ^{B,d}	3.87±1.57 ^{B,d}
Partition coefficient (K_{T/S})	0	4	70±59 ^{A,a}	1013±45 ^{B,a}	903±38 ^{C,a}
		25	131±79 ^{A,a}	66±22 ^{B,b}	576±89 ^{C,b}
		32	78±79 ^{A,a}	13±2 ^{B,c}	287±62 ^{C,c}
	1	4	65±63 ^{A,a}	121±17 ^{A,d}	207±4 ^{B,d}
		25	82±39 ^{A,a}	258±31 ^{B,e}	212±10 ^{B,d}
		32	57±43 ^{A,a}	158±29 ^{B,d}	268±31 ^{C,c}

Values are given as mean ± standard deviation. Values having the same superscript capital (A,B,C,..) and lowercase (a,b,c...) letters are not significantly different at p level = 0.05 respectively for the same line and column.

Biopolymer-Phenolic acid interactions



Active films based on gelatin & chitosan



Food simulant (Aqueous)

Packaging-Food interface

Temperature and salt content of simulant affect differently the release parameters

