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STUDY OF THE RELEASE OF LIMONENE PRESENT IN CHITOSAN FILMS ENRICHED WITH BERGAMOT OIL IN FOOD SIMULANTS

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Abstract

Chitosan films containing different concentrations of bergamot oil (BO) were obtained and the migration of limonene, the major oil component, to five liquid food simulants (aqueous solutions with 0, 10, 50 and 95 % of ethanol and isooctane) was studied at 20ºC. The losses of BO and limonene during the film drying were also quantified. The release kinetics of limonene from chitosan matrix was described using an empirical model which relates the reduced concentration loss of limonene and the square root of time. The results show that kinetic constants for all films increased exponentially when the ethanol concentration increased in the aqueous system and were slightly greater when the film thickness was lower. Composite films remain intact in isooctane CH-BO and no release of limonene was observed. Hydration of the film to promote molecular mobility was essential to ensure the compound release.

Keyword: citrus oil, release, polarity, solubility.

1. Introduction

Active food packaging appears as a promising technology with increasing applications due to its advantages over traditional methods. It is an interesting alternative to the use of chemical preservatives. Natural antimicrobial and antioxidant agents can be incorporated into biodegradable materials to increase shelf life and quality of food products.

Different biopolymers have been widely studied as polymeric matrix. Among polysaccharides, chitosan presents excellent film forming ability (Li et al., 1992). This non-toxic compound obtained by deacetylation of chitin, a structural component present in the shell of some crustaceans, exhibits interesting antimicrobial properties.

Citrus essential oils (EO) appear as interesting natural compounds with great potential use in foodstuffs preservation. *In vitro* studies have revealed significant antimicrobial effects of these natural compounds. Fisher and Phillips (2006) reported the effectiveness of oils and vapours of lemon, sweet orange and bergamot against 5 common foodborne pathogens, *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli* O157 and *Campylobacter jejuni*. Viuda-Martos et al. (2008) evaluated the effect of lemon, mandarin and orange oils on the growth of moulds associated with food spoilage. Orange and mandarin essential oils were the most effective against *Aspergillus niger* and *Aspergillus flavus* respectively. Even if the essential oils' mechanism of action is not clearly described, it seems that the antimicrobial activity is essentially due to their hydrophobicity. Terpens, the major compounds of essential oils, have the ability to disrupt and penetrate the lipid structure of the cell wall of bacteria, leading to denaturation of proteins and destruction of the cell membrane (Turina et al., 2006).

Previous *in vitro* studies reported promising results with chitosan-essential oils composite films. The incorporation of essential oil into chitosan films offers the

54 possibility not only of imparting antimicrobial activity, but also of improving the film's
55 physicochemical properties. A decrease in water vapour permeability was reported by
56 several authors (Sánchez-González et al., 2010a; Sánchez-González et al., 2010b;
57 Zivanovic et al., 2005).
58 Determination of active compounds diffusion rates will be interesting to design efficient
59 active packaging. However release kinetics of antimicrobial substances from
60 biodegradable films to food products has been little explored. Antimicrobial release is
61 dependent of the food product characteristics. For this reason, it is interesting to
62 evaluate substance migration in different food-simulating solvents. European Food
63 Safety Authority (EFSA) recommendations in terms of food simulants are contained in
64 the European Commission Directive 97/48/EC. Aqueous and acidic foodstuffs are well
65 simulated by distilled water and 10% - 50% ethanol. Concerning fatty foodstuffs the
66 choice of the food simulant is more complicated. Food oil was recommended by
67 European directive but migration measurement in oil is a technical challenge due to the
68 numerous components present. For this reason, it is interesting to use substitute fatty
69 simulants such as isooctane or 95% ethanol (McCort-Tipton and Pesselman, 2000).
70 The aim of this work is to study the release behaviour of limonene, the major
71 component of the bergamot essential oil (Moufida and Marzouk, 2003) from chitosan-
72 bergamot oil composite films in five different liquid food simulants (aqueous solutions
73 containing 0, 10, 50, 95 % of ethanol and isooctane). Losses of BO and limonene during
74 the film drying are quantified and kinetics of limonene release in food simulants was
75 described.
76

77 **2. Materials and methods**

78 *2. 1. Materials*

Medium molecular weight chitosan (CH) (Ref. 44887, Batch MKB130566, Sigma-Aldrich Chemical Co., St Louis, USA), 98% glacial acetic acid (Ref. 45726, Batch 0001427866, Sigma-Aldrich Chemical Co., St Louis, USA) and bergamot essential oil supplied by Herbes del Molí (Alicante, Spain) were used to prepare the film-forming dispersions.

Isooctane (2,2,4-Trimethylpentane, Ref. 360597, Batch 26196 PK, Sigma-Aldrich Chemical Co., St Louis, USA) and 95% ethanol pure or diluted with distilled water (Ref. 32294, Batch 91240, Sigma-Aldrich Chemical Co., St Louis, USA) were used to prepare liquid food simulants.

2.2. Preparation of the film-forming dispersions

Chitosan (1% w/w) was dispersed in an aqueous solution of glacial acetic acid (0.5% w/w) at 25ºC. After the dissolution of the polysaccharide, bergamot essential oil (BO) was added to CH solution to reach final concentrations of 0.5, 1, 2 and 3% (w/w). CH-BO mixtures were emulsified at room temperature using a rotor-stator homogenizer (Ultraturrax DI 25 basic-Yellowline, Janke & Kunkel, Staufen, Germany) at 13,500 rpm for 4 minutes.

2.3. Preparation of films

A casting method was used to obtain films; film-forming dispersions (FFD) were poured onto framed and levelled Teflon Petri dishes (90x110 mm, Welch, USA) and dried 48 hours under atmospheric conditions (20ºC, 54% RH). Film thickness was controlled by pouring the amount of FFD that will provide a surface density of total solids (CH and BO) of 56 g/m^2 in the dry films in all formulations, by considering the solid concentration of the FFD. A manual micrometer (Messmer, London, England, ±

104 0.002 mm) was used to measure film thickness at least ten different points of the same
105 sample.
106 BO losses of the films during drying were evaluated through a mass balance, by
107 considering the theoretical amount of solids poured from the FFD, the final weight of
108 the dried film and its equilibrium moisture content under the drying conditions (20ºC
109 and 54% relative humidity) obtained from the film's water sorption isotherm (data not
110 shown) (Table 2).
111
112 *2.4. Limonene release from the films to the simulants*
113 *2.4.1. Kinetics of release*
114 Liquid food simulants with different polarity were used: isooctane and aqueous
115 solutions containing 0, 10, 50 and 95 % of ethanol. Pieces of films (8 cm^2) were
116 introduced into 11 mL vials, containing 2 ml of each food simulant, which were
117 hermetically closed and slightly stirred during storage. Four vials were prepared for
118 each film to obtain data in quadruplicate. Some characteristics of these solvents are
119 reported in Table 1. The quantity of limonene release in the food simulant was analysed
120 by headspace chromatography (Arab-Tehrany and Desobry, 2007) each day periodically
121 during 4 storage days at 20ºC. Samples (100 μL of simulant solution) were taken with a
122 syringe through a septum in each vial. These samples were introduced in the headspace
123 vial (11 ml volume) of the chromatograph and submitted to a heating period (120ºC) of
124 10 min to release the amount of volatile solved in the sample before the sampling for
125 the injection in the chromatograph. A calibration curve for peak areas was constructed
126 using limonene standard solutions of different concentrations, by using the same
127 procedure. From the values of the limonene amount determined in the simulant at each
128 time, the residual concentration in the film was calculated by taking into account its
129 initial concentration after drying. This concentration was determined by analysing the

130 limonene concentration in the dried film by directly placing it in the headspace vial of
131 the chromatograph and heating at 160ºC for 10 min.
132 Concentration of limonene in the BO was also determined by the same technique by
133 introducing 0.1, 0.2 and 0.4 μL of oil in the headspace vial of the chromatograph.
134
135 *2.4.2. Chromatographic analyses*
136 The chromatographic measurements were made using a Perichrom Sarl model PR 2100
137 automatic Headspace Sampler on the Gas Chromatograph with flame ionization
138 detector. A fused silica capillary column (Sigma Aldrich Co., USA) was employed
139 (SUPELCOWAX 10, 60 m x 0.32 mm).
140 The carrier gas was N_2 at a flow rate of 1 ml/min. The analysis was performed using the
141 following temperature program: oven temperature from 60 to 150ºC at the rate of
142 3ºC.min^{-1}, and isotherm at 150ºC during 10 min. Injector and detector temperatures
143 were both held at 250ºC.
144
145 *2.4.3. Kinetic analysis*
146 An empirical model (Eq. 1) was applied to determine release kinetics of limonene to
147 food simulants from the films, since release involves overlapped phenomena to different
148 degrees, depending on the solvent used: film hydration and swelling, diffusion of
149 limonene through the film and dissolution in the food simulant. The degree and rate of
150 film hydration, which greatly facilitates molecular mobility and limonene diffusion, will
151 be greatly dependent on the water content of the simulant, while solubility of limonene
152 in the simulant will increase when its polarity decreases, since it shows very low
153 polarity (Table 1).
154 $$\frac{C_t - C_0}{C_0} = k.t^{0.5} \qquad \text{(Eq.1)}$$

155 Where C in the concentration of the compound in the film at the initial time (C_0) and t
156 (C_t) and k is the kinetic constant.
157
158 *2.5. Statistical analysis*
159 Results were analysed by multifactor analysis of variance with 95% significance level
160 using Statgraphics®Plus 5.1 (StatPoint Technologies, Inc., Warrenton, VA).
161
162 **3. Results and discussion**
163 *3.1. Estimation of limonene lost during film formation*
164 Table 2 shows some characteristics of the films (film thickness and moisture content)
165 and the concentrations of BO and limonene (expressed per g of CH) theoretically
166 present in the film (as deduced from the FFD concentrations) and those determined
167 experimentally in dried films. Limonene concentration in the BO was 94 % ± 4 and this
168 value was used to estimate the theoretical limonene concentration in each film, from the
169 BO content (per g of CH) in the FFD. Losses of limonene were deduced from the
170 theoretical values and those experimentally determined in each film by headspace
171 chromatography. Table 2 shows the BO and limonene losses, in percent with respect to
172 the theoretical value, for each Compound losses were between 39 and 99%, for the
173 different amounts of BO incorporated in the film forming dispersions. Nevertheless, the
174 losses of BO oil were slightly lower, which is associated with the minor losses of the
175 less volatile constituents of the oil. Monedero et al. (2010) also reported important
176 aroma losses during film formation. In fact, more than 50% of *n*-hexanal incorporated in
177 soy protein isolate-lipid films were lost.
178 Losses of limonene and bergamot oil greatly increased when the CH:BO ratio in the
179 FFD decreased. This agrees with the fact that the amount of polymer in the film
180 decreases when the CH:BO ratio decreases in the FFD, since a constant amount of total

181 solids are poured in the plate to obtain the dried film. The lower amount of polymer
182 implies a reduction in its encapsulating capacity of the oil, which made its volatilization
183 easier, in part due to the fact that evaporation occurs in the same surface area in every
184 case. The oil losses also implied a reduction of the film thickness (Table 2) since, at the
185 end of drying, lower amounts of total solids per surface unit are present in the film. The
186 reduction of the final BO or limonene concentration in the films when the CH:BO
187 decreased can also be related to the fact that the encapsulating effect of CH will be
188 relevant when the polymer concentration in the aqueous phase reaches a critical
189 viscosity at a determined level of water evaporation in the film and, at this moment,
190 volatilization of a great part of the free oil has occurred when it is present at high ratio
191 in the formulation. Oil evaporation will be promoted by steam distillation during water
192 evaporation such as in the distillation of immiscible liquids. From the obtained results, it
193 is obvious that no more than 1:1 CH:BO ratio is recommendable to obtain bioactive
194 films since the remaining concentration of EO after the film drying is very low,
195 although greater than the limonene content, which is probably lost to a greater extent
196 due to its higher volatility. The remaining compounds can also be active against
197 microorganisms, since in a previous paper (Sánchez-González et al., 2010b), CH films
198 containing the initial 1:3 CH:BO ratio showed the greatest antimicrobial activity against
199 *Penicillium Italicum*, as compared with films with 0.5:1, 1:1 and 1:2 ratios. Similar
200 effect of the CH:BO ratio was observed in the antimicrobial activity against *Escherichia*
201 *coli* and *Listeria monocytogenes* in another study (Sánchez-González, 2010c). It is
202 important to consider that with at the lower ratios, an antimicrobial effect can be found
203 since the minimal inhibitory concentration (MIC) for most of the microorganisms is
204 very small (Fisher and Phillips, 2006). On the other hand, the use of lower BO
205 concentration reduces the cost and leads to low sensorial perception of the oil flavour, in
206 some cases the latter being an inconvenience.

3.2. Limonene release

Limonene release was studied as a function of time in five different food simulating liquids (aqueous solutions with 0, 10, 50 and 95 % of ethanol and isooctane). Other terpenes present in bergamot oil also diffused but in minor concentrations and their detection was not possible with the sensitivity of headspace chromatography equipment. Only at the longest storage time did some small peaks from other compounds appear in the chromatogram. The concentrations of limonene in the films (expressed as μL limonene per g of CH of the film) at the different times are shown in table 3 for each simulant, except for isooctane where no limonene was detected in the solvent at any time with the technique used. So, no significant release of this compound could be deduced from the obtained results, despite the low polarity of this solvent and so the high expected solubility of limonene.

According to Buonocore et al. (2003) the release of an active compound from a polymeric network takes place in several steps. First solvent molecules (e.g. water) diffuse from the outer solution to the polymer matrix leading to network weakening. These changes in the film structure allow the diffusion of the active compound through the polymer matrix into the outer solution until thermodynamic equilibrium is achieved. Thus, limonene release is dependent on different factors such as liquid migration to the chitosan matrix, the polymer solubility and diffusion of the active compound through polymer matrix to the food simulating liquid. This last phenomenon is not only due to mass transfer but it is the result of different factors such as the specific interactions between the volatile compound and the matrix.

According to the described steps for the release of compounds from a polymer matrix, the observed behaviour in isooctane can be explained by non significant diffusion of

isooctane in the highly polar polymer matrix and so the maintenance of the closed structure of the polymer which efficiently encapsulates the limonene, inhibiting its diffusion and release to the simulant. Thus, it can be concluded that in contact with non-polar systems, i.e. isooctane, the polar polymeric systems remained intact throughout storage, preventing limonene release.

Concerning water solutions, a tendency to the progressive decrease of limonene concentration in the films throughout the storage time was observed in all cases, this being more intense when the ethanol concentration increased in the solution. The only exception to this behaviour was the thinnest film containing the lowest residual amount of limonene (CH:3BO formulation) which showed a very fast release during the first storage day in pure water, the concentration then decreasing slowly during the rest of the period. This could be attributed to the fast hydration of the thin film in pure water, which promotes the fast release of the limonene at the very beginning of the process.

So, the highest limonene release was observed in all films for 95% ethanol aqueous solutions. This observation is in line with the expected results. Limonene, a cyclic terpene, has higher solubility in ethanol than in water, according to the lower ethanol polarity. The increase of the limonene release when the ethanol concentration rises in the food simulant agrees with the decrease in the solvent polarity (Table 1) and the subsequent increase of the chemical affinity and solubility of limonene with the solvent. Aqueous solubility of limonene is limited to 41 μM at 23ºC (Li et al., 1998), which implies that at equilibrium, 11.2 μg (13.3 μL) of limonene could be present in the water solution taking into account the solvent volume used. In this sense, it is remarkable that amounts released in water after 4 storage days (ranging between 0.05 and 0.13 μL) are very far from the limonene solubility or equilibrium concentration. The equilibrium concentration of limonene in the simulant will be higher as the ethanol concentration increases and, so, the release process driving force will be greater. Nevertheless, the

259 increase in the ethanol concentration in the solution limits the film hydration and the
260 weakening effect of the polymer network, which will make the limonene diffusion
261 through the film matrix difficult.
262 Due to the difficulty of separating all of these overlapped phenomena, which control the
263 limonene release, an empirical model was fitted to the kinetic data in order to obtain a
264 kinetic constant which involves the different factors: the changes in the film thickness,
265 associated to the film hydration rate and differences in the process driving force, which
266 is defined by the initial concentration in the film and its equilibrium concentration, the
267 latter being controlled by the limonene solubility in the solvent medium.
268
269 *3.3. Kinetic constant determination*
270 Equation 1 was applied to concentration data as a function of time. Taking into account
271 the diffusional nature of the limonene release and the "short times" involved in the
272 process, since, at 4 storage days, the systems are far from the equilibrium conditions
273 (solvent saturation in limonene) in all cases, a linear relationship between the reduced
274 concentration $((C_t-C_0)/C_0)$ and the square root of time can be expected, according to the
275 simplified "short time" solution of Fick's second law for a plane sheet geometry, with
276 constant boundary conditions and uniform initial concentration (Crank, 1975).
277 Figure 1 shows the experimental points and the fitted straight lines of the model. For all
278 films and solvents a close fit of the model can be observed, except for the simulant
279 containing 95 % of ethanol, where an initial period (t_0) was required to reach the linear
280 behaviour, and for the thinnest film (CH:BO 3 formulation) in pure water, where a very
281 fast release of limonene occurs during the first day (12 % of the initial concentration)
282 before reaching the linear behaviour. This particular behaviour can be due to different
283 causes. In solvent with 95 % ethanol, the low amount of water makes the film hydration
284 difficult. This limits the increase in the molecular mobility permitting the limonene

285 diffusion through the matrix and only after a critical time (t_0), was the film sufficiently
286 hydrated to allow limonene diffusion. The t_0 values were estimated from the plot in
287 about 20 h for the thickest films (CH:BO 0.5 and CH:BO 1 formulations) and 3-10 h for
288 the thinnest films (CH:BO 2 and CH:BO 3 formulations). For the thinnest film (CH:BO
289 3) the fast hydration in the pure water leads to a very fast release of the limonene during
290 the first storage day, probably associated with the water solubilisation of the limonene
291 fraction located near to the film surface. Afterwards, the linear behaviour was observed
292 for the release of the limonene fraction deeply encapsulated in the film.
293 Figure 2 shows the values of the kinetic constants (k) as a function of the ethanol
294 content of the food simulant, where an exponential increase in their values can be
295 observed in line with the increase of ethanol concentration in the food solvent. For the
296 thicker films with similar thickness and initial concentration of BO (CH:BO 0.5 and
297 CH:BO 1), no notable differences were observed for k values in a determined solvent,
298 while the decrease in film thickness slightly promote the k values, although for the
299 CH:BO 3, the k value in 95% ethanol was lower than that expected which can be due to
300 the variability in the experimental points and its impact on the intercept and slope
301 values of the fitted straight-line. The influence of film thickness agrees with that
302 reported by Mastrometteo et al. (2009) for the release of active compounds (thymol)
303 from zein polymeric matrix.
304 Molecular mobility of limonene within the hydrophilic polymeric network was decisive
305 to ensure the compound release to the external medium and so, only in foods of high
306 water activity (fresh cut fruit and vegetable, meat or fish), such as in low ethanol
307 content aqueous solutions, the film application may provide active compounds for food
308 preservation. In reduced water activity foods such as dried fruits, the compound release
309 requires a critical time to promote film hydration; as it has been observed in the high
310 content ethanol solutions. The solubility of limonene in the food phase will accelerate

311 the release, but at the same time/also, the internal diffusion of the compound in the food
312 could inhibit the antimicrobial effect, since microbial contamination is usually prevalent
313 in the food surface (Kristo et al., 2008). In highly non-polar foods, such as fatty
314 products, the lack of film hydration may inhibit the active compound release and
315 preservation effect of the film may be not observed.
316
317 **4. Conclusion**
318 When the ratio of BO in the CH FFD increased, losses of bergamot oil and limonene
319 were higher. This observation calls into question the incorporation of higher quantities
320 of essential oil into biodegradable films. An optimum ratio has to be found between the
321 initial incorporation of essential oil and retention percentage during drying process and
322 storage.
323 Limonene release in food simulants is a complex phenomenon which involves different
324 factors such as film structure, solvent and migrant polarities and solubility. Kinetic
325 constants increased with the increase of the ethanol percentage in the aqueous solutions
326 due to the increase of the solubility of limonene in the simulant (increase of the process
327 driving force), while the decrease in the film thickness also promotes the release
328 kinetics, (despite the lower initial limonene concentration in the film) due to the
329 enhancement of the film hydration rate.
330 For possible future applications of these films based on chitosan and bergamot oil,
331 antimicrobial effectiveness could be improved by promoting higher water content in the
332 films since it favours the active compound diffusion to the product surface. In contact
333 with non polar foods, such as fats, the active compounds will be released very slowly
334 and their effectiveness could be limited, although the active non-polar compound (e.g.
335 limonene) solubilisation in the food is more feasible.
336

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394

Figure Captions

Figure 1. Limonene release data at 20ºC for different CH-BO composite films (Δ CH-0.5BO, □ CH-1BO, ● CH-2BO, ■ CH-3BO), as a function of the square root of time, in food simulants: (a) Ethanol 0%, (b) Ethanol 10%, (c) Ethanol 50%, (d) Ethanol 95%. Experimental points (mean values and standard deviations) and fitted model (lines).

Figure 2. Values of the kinetic constants (k) *vs.* percentage of ethanol of food simulant for the different composite films (□ CH-0.5BO, ΔCH-1BO**,** ▲ CH-2BO, ■ CH-3BO).

Figure 1

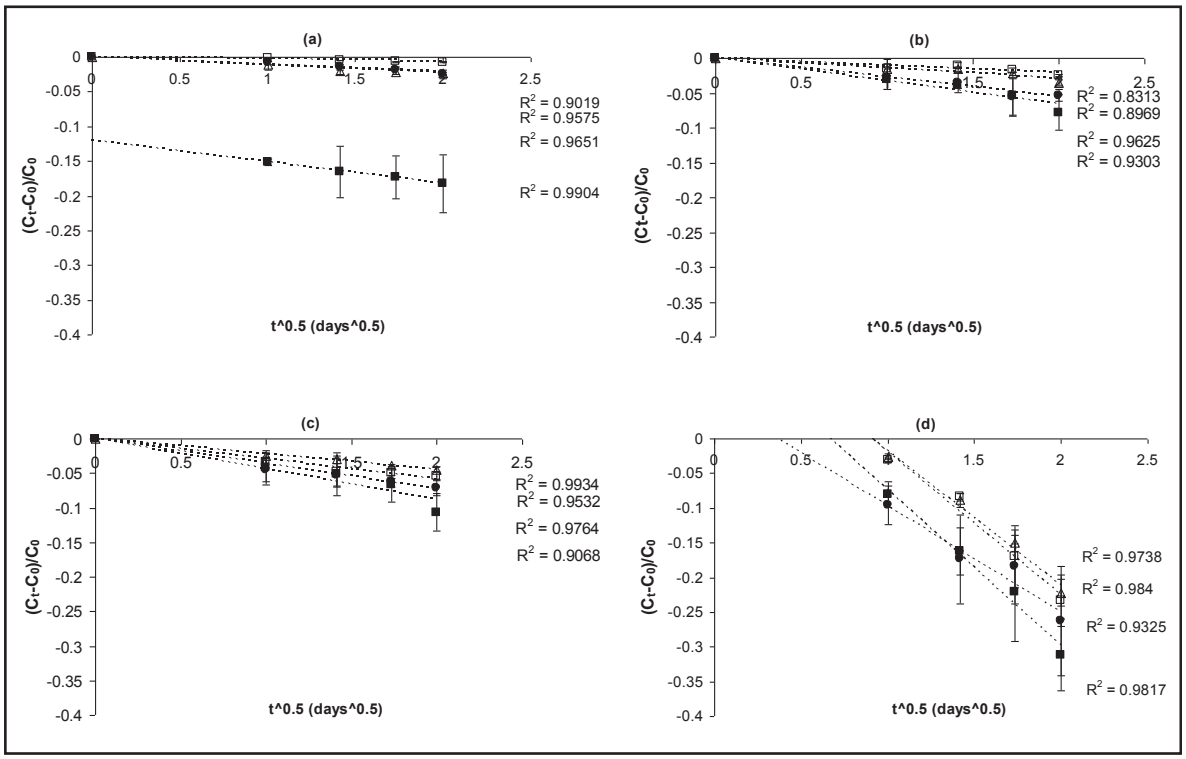
(a)
(b)
(c)
(d)
(Ct-C0)/C0
t^0.5 (days^0.5)
R2 = 0.9019
R2 = 0.9575
R2 = 0.9651
R2 = 0.9904
R2 = 0.8313
R2 = 0.8969
R2 = 0.9625
R2 = 0.9303
R2 = 0.9934
R2 = 0.9532
R2 = 0.9764
R2 = 0.9068
R2 = 0.9738
R2 = 0.984
R2 = 0.9325
R2 = 0.9817

Figure 2

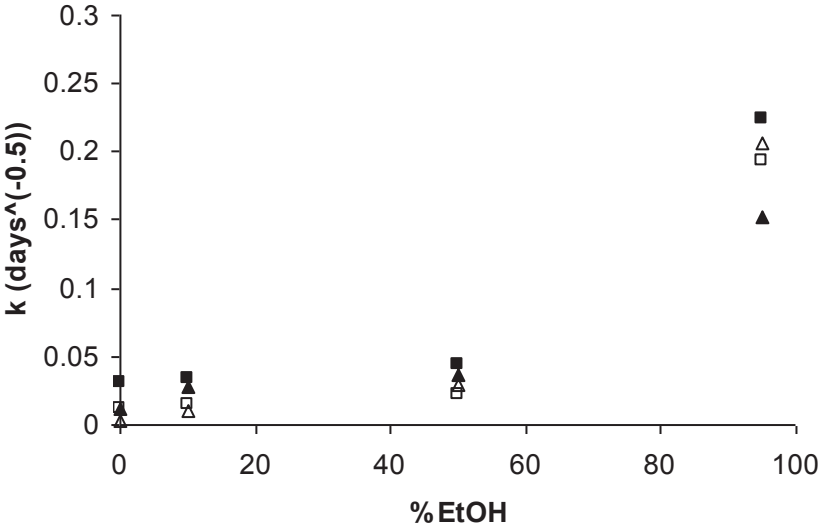

Table 1

Physico-chemical properties of food simulants and migrant (limonene).

Name	pH	Polarity[1]
Distilled water	3.99 (0.08)[a]	16.00*
Ethanol 10 %	3.87 (0.02)[b]	14.40*
Ethanol 50 %	4.04 (0.02)[a]	10.82*
Ethanol 95 %	5.73 (0.03)[c]	8.80*
Isooctane	-	0*
Limonene	-	0.97*

[a,b,c] Different letters in the same column indicate significant differences among formulations ($p < 0.05$).
[1]Hansen polarity
*Data from Molecular Modeling Pro

Table 2

Values (mean values and standard deviations) of thickness and equilibrium moisture content at a_w=0.54 and 20ºC (We) of the films and concentration of BO and limonene (per g of CH) in the film forming dispersion (FFD) (before drying) and in dried films. Losses (%) of BO and limonene during film drying with respect to the initial concentration are also shown.

Film	Thickness (µm)	We (g H_20/g d.m.)	BO concentration in FFD (g/g CH)	BO concentration in dry film (g/g CH)	Limonene concentration in FFD (g/g CH)	Limonene concentration in dry film (g/g CH)	BO loss (%)	Limonene loss (%)
CH-0.5BO	42 (3)[a]	0.112	0.5	0.334	0.442	0.266	33	39
CH-1BO	42 (2)[a]	0.104	1	0.435	0.884	0.302	56	65
CH-2BO	32 (2)[b]	0.075	2	0.297	1.768	0.038	85	97
CH-3BO	20 (2)[c]	0.074	3	0.327	2.652	0.019	89	99

[a,b,c] Different letters in the same column indicate significant differences among formulations ($p < 0.05$).

Table 3

Limonene concentration in the films (μL/g CH) at different times (0, 1, 2, 3 and 4 days) in contact with food simulants (aqueous solutions with 0, 10, 50, and 95% of ethanol), at 20ºC, for the different CH-BO composite films. Mean values and (standard deviations).

	Films			
	CH-0.5BO	CH-1BO	CH-2BO	CH-3BO
C_0	317 (9)^{aw}	359 (5)^{bw}	46.2 (1.7)^{cw}	23 (2)^{dw}
Ethanol 0%				
C_1	314 (2)^{aw}	359.26 (0.19)^{bw}	45.88 (0.12)^{cw}	19.93 (0.12)^{dw}
C_2	311.1 (1.4)^{aw}	358.32 (0.15)^{bw}	45.59 (0.16)^{cw}	19.5 (0.8)^{dw}
C_3	310.70 (1.07)^{aw}	357.4 (0.3)^{bw}	45.3 (0.2)^{cw}	19.4 (0.7)^{dw}
C_4	310.1 (0.4)^{aw}	357.3 (0.2)^{bw}	45.1 (0.2)^{cw}	19.1 (0.9)^{dw}
Ethanol 10%				
C_1	312 (2)^{aw}	354 (4)^{bw}	44.9 (0.7)^{cw}	22.7 (0.3)^{dw}
C_2	312.30 (1.06)^{aw}	356.1 (1.5)^{bw}	44.6 (0.7)^{cw}	22.57 (0.12)^{dw}
C_3	310.3 (0.5)^{aw}	353.8 (1.2)^{bw}	43.7 (1.2)^{cw}	22.2 (0.6)^{dw}
C_4	306.30 (1.15)^{aw}	351.0 (0.5)^{bw}	43.8 (0.3)^{cw}	21.63 (0.02)^{dw}
Ethanol 50%				
C_1	309.5 (1.2)^{aw}	347 (2)^{bx}	44.2 (0.7)^{cw}	22.4 (0.5)^{dw}
C_2	307.8 (1.8)^{aw}	342 (4)^{bxy}	43.8 (0.7)^{cw}	22.2 (0.7)^{dw}
C_3	305.1 (0.6)^{awx}	341 (2)^{by}	43.4 (0.4)^{cw}	21.9 (0.6)^{dw}
C_4	303.3 (1.0)^{ax}	340 (2)^{by}	42.9 (0.5)^{cw}	20.9 (0.6)^{dw}
Ethanol 95%				
C_1	309.2 (1.1)^{aw}	348.79 (1.0)^{bw}	41.8 (1.2)^{cwx}	21.5 (0.4)^{dwx}
C_2	289 (3)^{ax}	329.7 (1.9)^{bx}	38 (4)^{cwx}	19.6 (0.7)^{dxy}
C_3	269 (3)^{aby}	298 (7)^{by}	37 (5)^{cx}	18.2 (1.6)^{dyz}
C_4	246 (6)^{az}	275 (8)^{bz}	34 (3)^{cx}	16.1 (1.2)^{dz}

^{a,b,c} Different superscripts within a file indicate significant differences among samples for the same time($p<0.05$).
^{w,x,y,z} Different superscripts within a column indicate significant differences between times for the same sample ($p<0.05$).