Requena-Peris, R.; Vargas, M.; Chiralt A. (2017). Release kinetics of carvacrol and eugenol from poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV) films for food packaging applications. European Polymer Journal. 92:185-193. doi:10.1016/j.eurpolymj.2017.05.008

# Release kinetics of carvacrol and eugenol from poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV) films for food packaging applications.

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**Abstract**

Poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV) films, plasticized with PEG, incorporating 13 wt. % of active compounds (carvacrol-CA, eugenol-EU) were obtained by spraying the active between PHBV layers and their subsequent adhesion. Release kinetics of CA and EU in food simulants of different polarity was analysed and the films' antimicrobial activity was predicted, taking the minimal inhibitory concentration against some foodborne pathogens into account. Overall migration values were also determined. At equilibrium, an almost total release of both CA and EU occurred in 50% ethanol, about 20 and 50 % of CA and EU, respectively, was delivered in the more aqueous simulants and 65-70 % in fatty systems. The release rate increased when the polarity of aqueous simulants decreased, but it fell markedly in fatty systems. EU was released faster than CA in the less polar simulants, but more slowly in the more aqueous systems.

**Keywords**

Release kinetics, carvacrol, eugenol, diffusion, antimicrobial effect, partition coefficient.

## 1. Introduction

The use of active films for food packaging applications represents a good option for the purposes of lengthening the food shelf life while maintaining quality [1,2]. The use of active films mitigates the drawbacks associated with the direct application of the antimicrobials on the food products, usually carried out by spraying or dipping, such as the rapid neutralization of active compounds or the fast diffusion from the surface into

the product [3]. The use of films as carriers of antimicrobials allows for a progressive release of the active into the product surface where it effectively acts for longer, thus improving the antimicrobial effectiveness and enhancing food safety and quality throughout the storage [4,5].

Biopolymers present several advantages over oil-based polymers, such as their biodegradability and the use of renewable resources [6]. In this context, the polyhydroxyalkanoates are a promising option in the food packaging field; specifically polyhydroxybutyrate-co-hydroxyvalerate (PHBV), which leads to less brittle and more stretchable materials than polyhydroxybutyrate [7,8].

Of the antimicrobial compounds that can potentially be used in the active film formulations, natural substances, such as the essential oils (EO); enzymes, such as lysozyme; bacteriocins, such as nisin or organic acids, such as sorbic acid, can be found [3]. EOs have exhibited antimicrobial activity against many foodborne pathogens, which have often been attributed to their main components. Oregano essential oil (OR) and clove essential oil (CLO) are two of the most effective EOs at controlling microbial growth [9]. The antimicrobial effectiveness of both OR and CLO, as well as their respective main components, carvacrol (CA) and eugenol (EU), have been demonstrated in different biodegradable matrices [10-14]. However, the effectiveness of the films as carriers of antimicrobial compounds does not only depend on the nature of the active compounds, but also on the capacity of the film to release an adequate concentration of the active to the food at a determined contact time and at equilibrium (partition coefficient). This, in turn, depends on the active's interactions with the polymer matrix and its solubility in the food system. The release kinetics of the active compound into the food throughout the storage time is, therefore, a crucial factor when guaranteeing antimicrobial effectiveness and food safety [15,16]. In this sense, several mathematical models, such as first order kinetics [17-19], Peppas and Weibull models [20], or the Fickian model [19,21-23] have been used to determine the compound release rate from the films, and the concentration reached in the food system. In this way, the time needed to reach a concentration level of active in the food greater than the minimum inhibitory concentration (MIC) must be predicted in order to ensure the food safety, thus providing useful information about the active packaging's ability to exert the antimicrobial function in real foodstuffs.

PHBV thermoprocessed films with 15 wt % CA, EU or oregano or clove EOs have exhibited effective antimicrobial activity against GRAM + and GRAM – bacteria in *in vitro* studies with tryptic soy broth medium, thus indicating the effective release of actives in this polar substrate [24]. Nevertheless, in order to be applied in different real foods, the active release kinetics should be known in systems with different polarities, simulating different kinds of foods. In this sense, the use of normalised food simulants [25] for the purposes of analysing release kinetics, allows for adequate predictions of release in different real foods.

Thus, the aim of this work was to analyse the release kinetics of CA and EU from PHBV active films obtained by compression moulding in polar and non-polar food simulants and to model the experimental data by fitting different kinetic models. Likewise, predictions of the active concentration in a model packaged liquid food with different polarity were made in order to predict the effectiveness of active films, according to the MIC values for several foodborne pathogens. The overall migration of film components in the different simulants was also determined in order to know how the film fits the legal limits as a function of food polarity.

## 2. Materials and methods

### 2.1. Materials

Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) 8% (PHBV) was supplied in pellet form by NaturePlast (Caen, France). Polyethylenglycol 1000 (PEG100), used as plasticizer; carvacrol (CA), eugenol (EU); and UV grade solvents: methanol, ethanol, acetic acid and isooctane were from Sigma-Aldrich (Sigma–Aldrich Chemie, Steinheim, Germany).

### 2.2. Film preparation

2.2.1. PHBV monolayer films

PHBV monolayer films were prepared by melt blending and compression-moulding as described by Requena [24]. Briefly, PHBV was mixed with PEG1000 (10% w/w) in a two-roll mill (Model LRM-M-100, Labtech Engineering, Thailand) at 180 $^{o}$C and compression-moulded using a hydraulic press (Model LP20, Labtech Engineering, Thailand) at 10 MPa and 180 $^{o}$C for 4 min.

2.2.2. Active PHBV bilayer films

PHBV bilayer films with different active compounds were obtained by spraying a constant amount (15 g of active per 100 g polymer matrix) of each active compound as reported by Requena et al., 2016. Thus, PHBV monolayers were sprayed with CA or EU, covered with another monolayer and compressed together using the hydraulic press. In this way, three kinds of films were obtained: bilayer films without active compounds (PHBV), as a control, and films with the different active compounds (PHBV-CA and PHBV-EU).

**2.3. Analysis of the retention of active compound in the films**

Two different methods were carried out to quantify the CA and EU content in PHBV films. Thus, the weight loss of the films after the bilayer compression was assessed to estimate the mass loss occurring in the process attributed to losses of actives caused by volatilization. Additionally, the CA and EU retention in the PHBV bilayer films was determined by methanol extraction followed by spectrophotometric quantification. To this end, film samples were kept under stirring for 24 hours at 20$^{o}$C, using a methanol: film ratio of 1:10. The extract was filtered and quantitatively diluted to measure the absorbance using a UV-visible spectrophotometer (Evolution 201, Thermo Scientific). In this way, the CA and EU content in PHBV films could be determined by the absorbance measurements at 275 and 282 nm, respectively, using the methanol extract of active-free PHBV bilayer films as blank solution. All analyses were carried out in samples from five different positions of five different films, in order to analyse the degree of homogeneity of the active distribution throughout the film.

**2.4. Kinetics of CA and EU release in food simulants**

In order to determine the release rate of CA and EU from the PHBV bilayer films into different food systems, four types of food simulants were considered. Thus, A (ethanol 10 % (v/v)) and B (acetic acid 3 % (w/v)) food simulants were selected to imitate aqueous food and aqueous food with pH values lower than 4.5, respectively, whereas D1 (ethanol 50 % (v/v)) was selected to imitate alcoholic food and oil-in water emulsions and D2 (isooctane) was used to simulate food with a fatty continuous phase [26]. In this way,

film samples of 500 mg were weighed and placed in flasks with 100 mL of the corresponding simulant. Thereby, each film formulation-food simulant system was kept under stirring at 20 $^{o}$C throughout the assay time. After the different contact times up to equilibrium, the samples were taken from the flasks, and the absorbance was measured. Thus, the CA and EU profile concentration in each simulant over time could be determined by the absorbance measurements using the corresponding standard calibration curve. All analyses were performed in triplicate for three different flasks containing the different film samples. The liquid phase in contact with the active-free PHBV films was used as blank for the absorbance measurements, for each simulant and time.

2.4.1 Mathematical modelling of CA and EU release.

The Peleg model [27] was applied to the data regarding the CA or EU content in the food simulant at the different times in order to estimate the amount of active released at equilibrium ($M_\infty$), as well as the partition coefficient of the active compounds in the different food simulant. Eq. 1 relates the data of the active concentration in the simulant and time

$$\frac{t}{M_t} = k_1 + k_2 t \quad \text{(Eq. 1)}$$

where:

$M_t$: mass of active compounds released into the simulant after contact time t

$k_1$ and $k_2$ are the model constants, where $k_1$ is inversely related with the release rate and $k_2$ with the asymptotic value of the curve or mass of active released at equilibrium ($M_\infty = 1/k_2$)

Likewise, experimental data were also fitted using the Korsmeyer–Peppas model [28] (Eq. 2), to investigate the mechanisms involved in the active release process and 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

$$\frac{M_t}{M_\infty} = kt^n \quad \text{(Eq. 2)}$$

Where $M_t$ / $M_\infty$ is the fraction of active compound released at time t, *k* is the rate constant incorporating characteristics of the matrix 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 [28].

Lastly, Fick's second law was considered to model the diffusion process of CA and EU in the PHBV bilayer films towards the food simulants. Film samples can be considered as infinite plane sheets with the half thickness as a characteristic dimension, where the active compound diffuses only in an axial direction. The diffusional long-time equation for an infinite plane sheet [29] with ten terms was used to determine the values of diffusion coefficient (D) of CA and EU into the different solvents (Eq. 3), by using the Solver tool (Microsoft Excel 2013®) to optimize the D values, by minimizing the Sum of Squared Errors (SSE), and considering the following boundary conditions:

$$t = 0 \qquad 0 < x < L \qquad c = c_0$$

$$t > 0 \qquad x = 0 \qquad x = L \qquad c = 0$$

$$M_t = M_\infty \left( \frac{8}{\pi^2} \sum_{n=0}^{\infty} \left[ \frac{1}{(2n+1)^2} exp \left\{ \frac{-\pi^2 D (2n+1)^2 t}{L^2} \right\} \right] \right) \qquad \text{(Eq. 3)}$$

where:

Mt: is the mass of compound released at time t

$M_\infty$: is the mass of compound released at equilibrium

L: half thickness of film

2.4.2. Prediction of antimicrobial action of the films from release kinetics.

Along with the obtained parameters, Peleg´s equation, was used to predict the amount of CA or EU released throughout time into different types of foodstuffs, simulated by

the considered simulants; this was compared with the MIC values reported for different bacteria in order to determine whether these values are reached in the food system in a reasonable time. To this end, a theoretical mass ratio of active packaging and food of 12:1000 was considered, corresponding to a 1 kg of product packaged in a 15x10x6 cm pack with a film area of 450 $cm^2$. In this way, the length of time necessary to reach the active's MIC of some foodborne bacteria has been predicted depending on the kind of packaged food.

### 2.5. Overall migration of active PHBV bilayer films

An important issue in the field of food contact materials is the migration of the packaging constituents into the food. The OM2 overall migration test was carried out, according to Regulation 10/2011/EC [26], in order to determine the overall migration of the PHBV films with and without the different actives (CA or EU). The OM2 test determines the migration of a specific packaging material in A, B and D2 food simulants for 10 days at 40$^oC$; this simulates a long food storage period at room temperature or lower, including heating at 70$^oC$ for 2 hours or heating at 100$^oC$ for 15 min. To this end, five film samples of 23 mm diameter were placed in tubes with 69 mL of corresponding solvent and kept at 40$^oC$ for 10 days. Afterwards, the solvent was transferred to cups and evaporated at 105 °C until a constant weight was reached. Thus, the overall migration of each film formulation was determined as the weight of residue after drying and expressed as mg/$dm^2$ of film, according to the regulation. All analyses were carried out in duplicate.

## 3. Results

### 3.1. Concentration of active compounds in the films

The final mass ratio of the active compounds in the films after compression moulding was estimated through the weight loss of the films in this step. It was about 2.5% with respect to the initial mass of the film, regardless of the type of active. The weight loss of the films can be attributed to the active compound volatilization and moisture loss (0.9%), which represents a final content of active in the films of about 11-12 g of active/100 g film, and a retention percentage with respect to the amount initially

incorporated of nearly 90%. Nevertheless, the methanol extraction of CA and EU from PHBV-CA and PHBV-EU films and the subsequent quantification by the spectrophotometric method yielded 10.3 ± 1.0 and 5.7 ± 0.9 g per 100 g of film, respectively for CA and EU, without significant differences between samples from different films and film zones. Thus, a reproducible and homogenous distribution of the actives in the film can be assumed, while, as compared with the theoretical active content in the films (13 g per 100 g film), the retention percentages would be 80 ± 6% and 43 ± 4% for CA and EU, respectively. Nevertheless, taking into account the similar boiling points of CA (234-236 °C, [30]) and EU (253 °C, [31]), no total extraction could be carried out from the active PHBV films in methanol, especially for EU, due to an inadequate partition coefficient associated with the compounds' particular interactions with the polymer matrix and their solubility in the extraction solvent. So, about 11-12 g of active/100 g film could be assumed in the films with CA or EU.

### 3.2. Release kinetics of CA and EU in food simulants

Release mass of CA and EU at different contact times with simulants have been determined and Figure 1 shows the mean values, referred to the maximum value (at equilibrium) for each case. This ratio represented the fraction of active released at each time with respect to the final amount released at equilibrium in each simulant. Table 1 shows the maximum values ($M_\infty$), referred per mass unit of the initial film, and estimated by applying Peleg's model to the experimental data for CA and EU release. The values of $1/k_1$ parameter, related to the release rate, were also included in Table 1. A good fitting of the model was achieved in all cases ($R^2 > 0.97$). Both release rate and asymptotic value were greatly affected by the polarity of the food simulant, yielding different values for each active. The fastest release of both CA and EU was observed in 50% ethanol (D1 simulant), whereas the slowest delivery occurred in the non-polar solvent (D2: isooctane). Likewise, the maximum active release occurred in D1 simulant for both compounds, without any significant differences in the $M_\infty$ values. Similar amounts of both compounds were also delivered at equilibrium in the non-polar simulant (D2). This behaviour agrees with that reported by other authors for the EO compounds' delivery, which increased when the ethanol ratio rose in the food simulant,

683 according to the promotion of the active compound solubility in the aqueous system
684 when the ethanol ratio rose [19,23,23,32]. In the more polar simulants of different pH
685 (A and B), greater amounts of EU than CA were released, since EU is more soluble (2460
686 mg/L, [33]) than CA (1250 mg/L, [33]) in water. Nevertheless, EU was released at a
687 slower rate. Thus, the maximum expected release of CA and EU will occur in less polar
688 foodstuffs, such as alcoholic beverages or oil-in-water emulsions (sauces, dressings or
689 high fat dairy products), whereas in more aqueous foods a lower release would be
690 expected. Table 1 also shows the maximum delivery ratio ($M_\infty/M_0$) for each compound,
691 related with its partition coefficient (defined as the mass of active released at
692 equilibrium in the simulant ($M_\infty$) with respect to the corresponding residual mass of the
693 active in the film ($M_0$-$M_\infty$)). This ratio was referred to the $M_0$ value of the theoretical
694 amount incorporated in the initial film and also to the amount determined by methanol
695 extraction. Values higher than 100% can be observed for the second approach, thus
696 indicating that methanol extraction was not complete, especially for EU, as previously
697 commented on. A practically total release of the retained compound occurred in films
698 in contact with D1 simulant, where about 95 % of the theoretical amount incorporated
699 was released. Then, only about 5 % of the incorporated active compounds were lost
700 during the film thermocompression process, as deduced from the weight loss analyses,
701 and a final concentration of 11-12 g of active/100 g film could be assumed.

702 Table 2 shows the diffusion values and the Kormeyer&Peppas parameters for CA and EU
703 release in each simulant. The values of n coefficient were not significantly higher than
704 0.5 in any case for the active compound release and so, Fickian or quasi-Fickian diffusion
705 can be predicted for both actives in the PHBV matrix, as reported by other authors for
706 different essential oil compounds from different matrices. Particularly, a diffusional
707 mechanism has been reported for thymol release in different polyester films, such as
708 poly(butylene succinate) [22] and poly(lactic acid) [19], lemongrass essential oil in
709 sodium alginate films [20] and *Satureja hortensis* essential oil in alginate microparticles
710 [34]. Therefore, the relaxation of the polymer in contact with the solvents was not
711 coupled with the compound diffusion and three steps can be assumed for the release
712 process: a) the solvent diffusion into the polymer matrix, b) the network relaxation in
713 line with solvation and plasticization, and c) the diffusion of the compound through the

relaxed polymer network until the thermodynamic equilibrium between phases (polymer/food system) is reached. At equilibrium, the compound affinity with the solvated polymer and its solubility in the liquid food system will determine the partition coefficient for the delivered compound. The compound diffusion through the matrix will be affected by the solvent impregnation into the polymer network and the interactions established among the components.

Figure 1 shows the experimental points in terms of the ratio of released compound with respect to the equilibrium value and the fitted Fick's model for CA and EU diffusion, in each simulant. The good fitting of the model can be observed in all cases (SSE< 0.04) as well as the different pattern of the curves depending on the simulant and the released compound. Whereas the CA release in aqueous simulants (A and B) was significantly faster than that of EU, significantly slower CA release occurred in the less polar simulants (D1 and D2), as compared to EU. The values of diffusion coefficients (Table 2) were coherent with the commented release rates of each compound in the different simulants. In this sense, it is remarkable that diffusion of EU in the matrix when it is in contact with the most polar simulants was greatly reduced, with respect to that of CA. This suggests stronger interactions of EU with the solvated PHBV matrix, which reduced its migration rate through the network, although higher amounts were delivered at equilibrium in these simulants where this compound is more soluble. On the contrary, when solvent polarity was reduced, and a less polar solvent is entrapped in the matrix, the EU diffusion, and its release rate, increased with respect to that of CA, the matrix releasing similar amounts of the compounds at equilibrium, near to the initial total content of the film (13 g/100 g film). Tawakkal et al. [19] and Petchwattana and Naknaen [22] also reported an increase in the diffusion coefficient of thymol in poly(lactic acid) and poly(butylene succinate) films, respectively, when the polarity of the simulant decreased (higher ethanol/water ratio), in agreement with that observed for EU in PHBV films, but contrary to CA behavior. Nevertheless, the obtained D values were in the range of those obtained by other authors [19,22] for thymol release from polyester films to water/ethanol mixtures, at similar temperatures ($0.1\text{-}3.0\cdot10^{-13}$ $m^2s^{-1}$). The differences can be explained in terms of the respective interactions of the active with the solvated polymer matrix and its solubility in the liquid phase, depending on its polarity.

745 The slowest diffusion of both active compounds was obtained in the least polar system
746 (isooctane), probably due to the fact that the polymer matrix swells to a lower extent
747 with this solvent, which supposes a reduction in the free volume of polymer chains, thus
748 limiting the diffusion process, as reported by other authors [21-23]. A more closed
749 network implies a more restricted compound mobility, thus inhibiting molecular
750 diffusion.

751 Table 1. Parameters of Peleg's model: amount of active compound released at equilibrium in the simulant ($M_\infty$) and its release rate ($1/k_1$), and maximum
752 release ratio ($M_\infty/M_0$): mass of active released at equilibrium in the simulant related to the initial mass of the active in the film (expressed with respect to the
753 theoretical incorporated amount ([1]) and with respect to the amount determined by methanol extraction ([2]).

| Active | Simulant | $1/k_1$ (µg act./s) | $M_\infty=1/k_2$ (g act./100 g film)* | $M_\infty/M_0$ (%)[1] | $M_\infty/M_0$ (%)[2] | $R^2$ |
|---|---|---|---|---|---|---|
| **Carvacrol** | **A** | 3.5±1.1$^{c}$ | 2.9±0.2$^{e}$ | 22±2$^{e}$ | 28±2$^{f}$ | 0.980 |
| | **B** | 2.8±1.0$^{c}$ | 2.9±0.8$^{e}$ | 23±6$^{e}$ | 29±8$^{f}$ | 0.997 |
| | **D1** | 7.2±0.7$^{b}$ | 12.5±0.2$^{a}$ | 96±2$^{a}$ | 122±2$^{c}$ | 0.995 |
| | **D2** | 0.15±0.02$^{e}$ | 8.4±0.4$^{c}$ | 65±3$^{c}$ | 82±4$^{e}$ | 0.973 |
| **Eugenol** | **A** | 1.9±0.3$^{cd}$ | 6.1±0.3$^{d}$ | 47±2$^{d}$ | 107±5$^{d}$ | 0.999 |
| | **B** | 2.0±0.7$^{cd}$ | 6.7±0.1$^{d}$ | 52±1$^{d}$ | 118±2$^{c}$ | 0.999 |
| | **D1** | 19.0±2.0$^{a}$ | 11.9±0.2$^{a}$ | 92±2$^{a}$ | 210±4$^{a}$ | 0.977 |
| | **D2** | 0.30±0.03$^{de}$ | 9.3±0.3$^{b}$ | 71±3$^{b}$ | 163±6$^{b}$ | 0.982 |

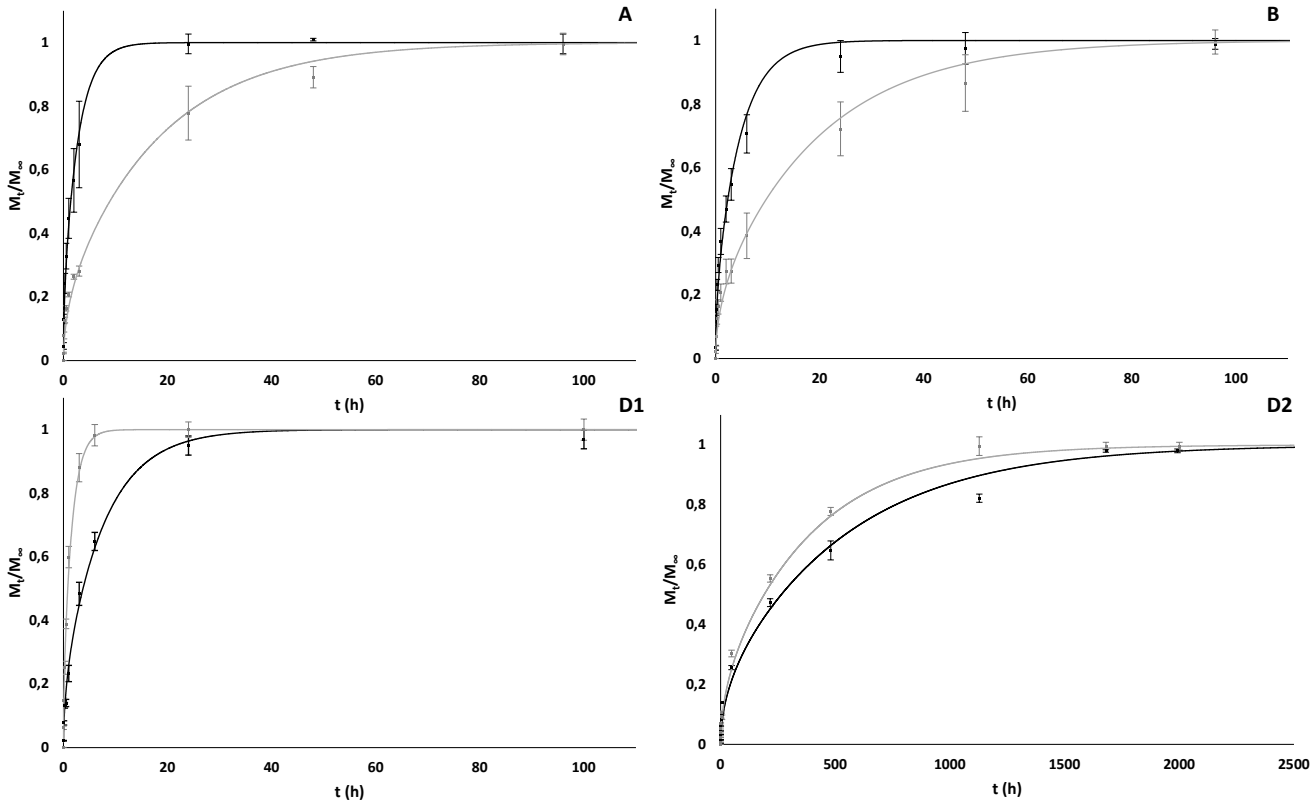
754 a-g: different letters in the same column indicate significant differences (P<0.05) between samples
755 *: in 100 mL of simulant.

756

Table 2. Diffusion coefficient (D) and parameters of the Korsmeyer–Peppas model (rate constant (k) and diffusional exponent (n))

| Active | Simulant | $Dx10^{13}$ ($m^2/s$) | n | k ($h^{-n}$) | $R^2$ |
|---|---|---|---|---|---|
| **Carvacrol** | **A** | $3.2\pm0.4^{d}$ | $0.450\pm0.020^{d}$ | $0.27\pm0.02^{bc}$ | 0.990 |
| | **B** | $4.8\pm0.4^{e}$ | $0.354\pm0.008^{a}$ | $0.37\pm0.03^{cd}$ | 0.999 |
| | **D1** | $1.2\pm0.2^{b}$ | $0.508\pm0.006^{e}$ | $0.25\pm0.02^{bc}$ | 0.975 |
| | **D2** | $0.017\pm0.002^{a}$ | $0.510\pm0.014^{e}$ | $0.060\pm0.001^{a}$ | 0.989 |
| **Eugenol** | **A** | $0.50\pm0.10^{ab}$ | $0.383\pm0.006^{ab}$ | $0.17\pm0.06^{ab}$ | 0.982 |
| | **B** | $0.50\pm0.12^{ab}$ | $0.390\pm0.030^{b}$ | $0.20\pm0.03^{ab}$ | 0.992 |
| | **D1** | $5.5\pm0.4^{d}$ | $0.549\pm0.012^{f}$ | $0.43\pm0.03^{d}$ | 0.996 |
| | **D2** | $0.023\pm0.0013^{a}$ | $0.383\pm0.004^{ab}$ | $0.068\pm0.003^{a}$ | 0.997 |

a-f: different letters in the same column indicate significant differences ($P<0.05$) between samples




760

761 Figure 1. Ratio of the active compound released in each food simulant, with respect to that of
762 the equilibrium value, as a function of contact time (points) and fitted Fick's model (lines). CA
763 (—) and EU (—). A: ethanol 10 % (v/v), B: acetic acid 3 % (w/v), D1: ethanol 50 % (v/v), and D2:
764 isooctane.

765

766 **3.3. Active release prediction over the storage time.**

767 On the basis of the kinetic analysis, the active release concentration *vs*. time was
768 predicted assuming bulk diffusion into the food mass (e.g packaged liquid food), for a
769 packaged food with a food-film mass ratio of 1000:12, (e.g. 1 kg product in a 15x10x6
770 pack with 450 $cm^2$ area) and the kinetic equations obtained for the different simulants,
771 using both film formulations, PHBV-CA and PHBV-EU. Figures 2 and 3 show the
772 concentration values of CA and EU, respectively, reached in the food system as a
773 function of time, where the range of values for the MIC for several foodborne pathogens
774 were also shown. As deduced from kinetic analysis, CA would be quickly delivered in
775 aqueous foods, achieving a limited maximum concentration after 24 hours. On the
776 contrary, the CA release will be higher and more gradual in non-polar systems (D2).

Almost 44 hours will be needed for fatty foods to reach the required concentration for the antimicrobial action, taking into account the MIC values of CA against some foodborne pathogens such as Staphylococcus aureus ($1.7 \cdot 10^{-4}$ g/ml), *Bacillus cereus* ($1.8 \cdot 10^{-4}$ g/ml), *Salmonella typhimurium* ($2.2 \cdot 10^{-4}$ g/ml), *Escherichia coli* ($2.2 \cdot 10^{-4}$ g/ml) and *Listeria monocytogenes* ($3.7 \cdot 10^{-4}$ g/ml) [35]. In contrast, in aqueous foods, MICs for most bacteria would be reached after short contact times (between 2 and 24 hours depending on the bacteria) and, therefore, a fast microbial growth inhibition could be expected. In contrast, for foods with alcohol content higher than 20% or oil in water emulsions (D1 simulant), only 1 hour would be required to achieve these MIC values.

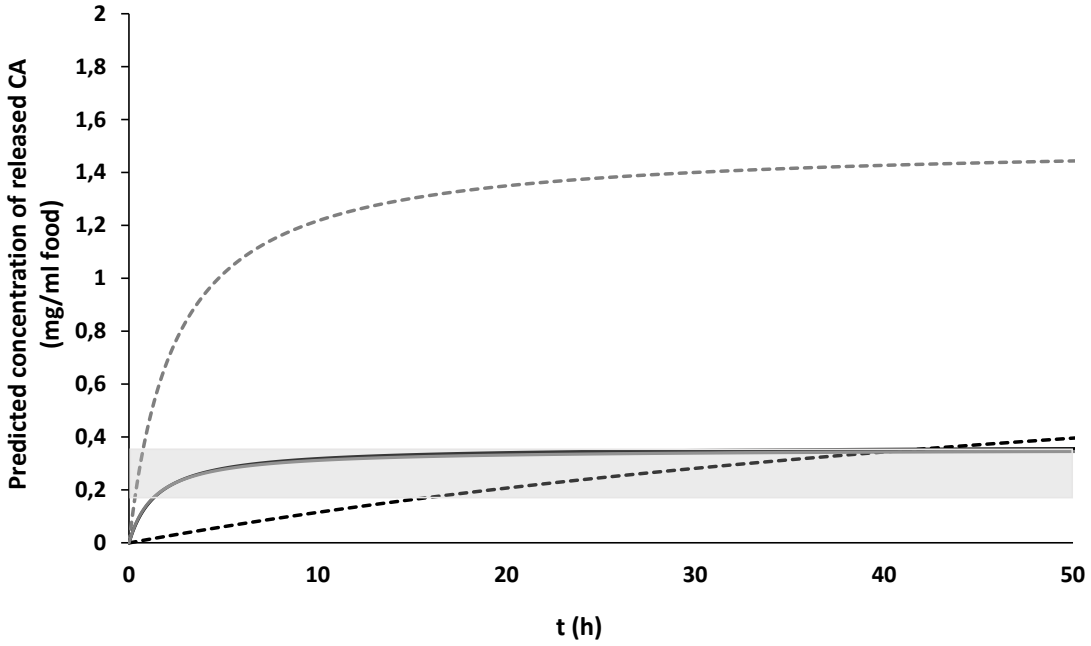



Figure 2. Predicted concentration of released CA versus time in different types of food or food simulants: 10% ethanol (—), 3% acetic acid (—), 50% ethanol (– – –) and isooctane (– – –). Shaded area corresponds to the range of minimum inhibitory concentration of CA against different bacteria.

Figure 3 shows the predicted concentration of EU released in the different types of foods (simulants) compared with the MIC values of several bacteria. Since the bacteria show different sensitivity to EU than to CA, different EU amounts will be necessary to inhibit their microbial growth. The reported MIC values of EU were $1.6 \cdot 10^{-5}$ g/ml *for B. cereus* [36], $1 \cdot 10^{-4}$ g/ml for *S. aureus* [37], $5 \cdot 10^{-4}$ g/ml for *S. typhimurium* [35], $1 \cdot 10^{-3}$ g/ml for *E. coli* [35] or $1 \cdot 10^{-3}$ g/ml for *L. monocytogenes* [35]. Thus, in some cases, longer contact

times between the active film and the foodstuffs would be required, according to the EU release rates. Then, whereas in aqueous foods the MIC of EU would be achieved after 5 min-8 hours contact time, for some of the above mentioned bacteria, the MIC for *E. coli* and *L. monocytogenes* would not be reached at any time, since the maximum expected EU release ranges between $7 \cdot 10^{-4}$ g/ml - $8 \cdot 10^{-4}$ g/ml in the polar food model. On the contrary, all the MIC values would be reached in less polar foodstuffs at different times, depending on the food continuous phase. Thus, in oil-in-water foods, such as some dairy products, only 2 hours would be necessary to reach the MIC of EU against *E. coli* and *L. monocytogenes*, while 15 days would be required in foodstuffs with a fatty continuous phase.

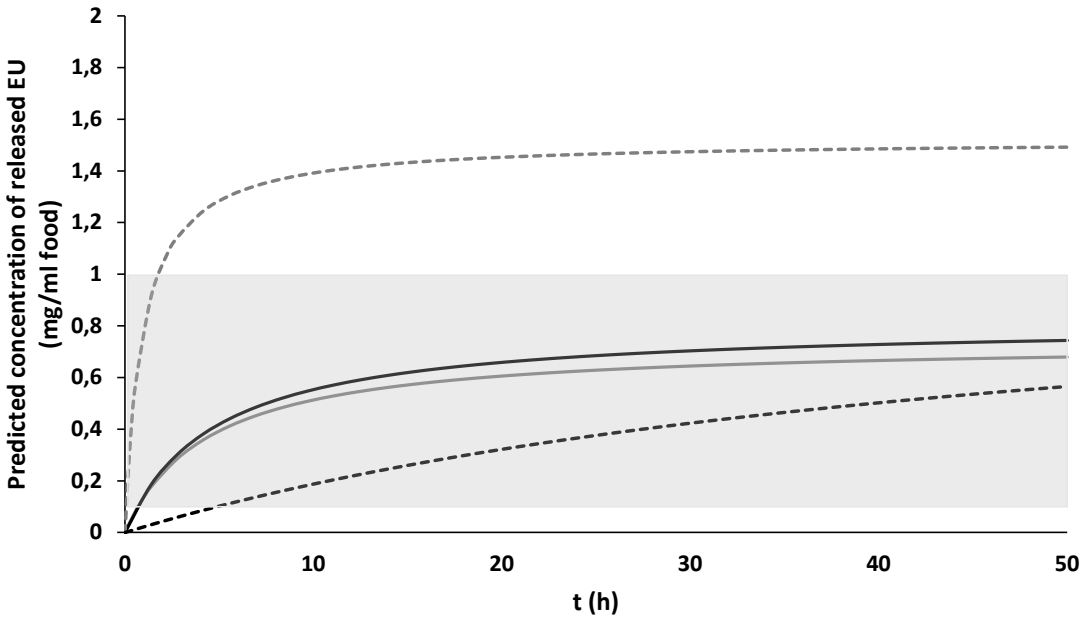



Figure 3. Predicted concentration of released EU versus time in each different type of food or food simulants: 10% ethanol (—), 3%acetic acid (—), 50% ethanol (- - -) and isooctane (- - -). Shaded area corresponds to the minimum inhibitory concentration of EU against different bacteria.

### 3.4. Overall migration of active PHBV bilayer films

Overall migration of the obtained films was also assessed to determine how they fit the European Regulation in terms of the overall migration limit (OML). All the films maintained their integrity after the contact time at 40 $^{o}$C in all the food simulants tested.

According to the Regulation 10/2011/EC [26], the OML for plastic materials and articles must not exceed 10 mg of total constituents delivered per $dm^2$ of food contact surface. Nevertheless, also according to the regulation for active materials intended to come into contact with foodstuffs, the active substances released should not be included in the overall migration [38]. Moreover, as reported by Balaguer et al. [39], the volatile substances, such as the EO and their main compounds, are not considered in the overall migration values, since the possibly migrated compounds evaporate with the solvents and do not contribute to the final residue weight. This is especially true when aqueous solvents are used and the steam drag effect favors the compound evaporation. Then, the determined migration values do not include the active compounds and it is assumed that they correspond to a fraction of the polymer matrix (polymer plus plasticizer).

Figure 4 shows the values of the overall migration, expressed in $mg/dm^2$ of the film, for the different film samples and simulants. Active-free PHBV films exceeded the OML in both neutral and acid polar simulants, without significant differences due to pH, while no migration occurred in isooctane. Most of the residue found in polar solvents could be mainly attributed to plasticizer migration, due to its more hydrophilic nature and water solubility, since, in all cases, the overall migration values were lower than the PEG concentration in the films (107 $mg/dm^2$).

However, PHBV films with active compounds showed significantly lower overall migration values than active-free PHBV films in both neutral and acid polar simulants. This suggests that interactions between actives and plasticizer could lead to linking reactions (e.g. PEG can act as Lewis acceptor of phenolic protons through the oxygen electron pairs of ether groups), thus reducing the water solubility of PEG, which contributes to its greater retention in the matrix. No significant differences in overall migration in 10 % ethanol and 3% acetic acid were observed for PHBV films containing CA or EU. On the contrary, in non-polar solvents such as isooctane, PHBV films with the active compounds led to slightly higher migration values than the active-free PHBV films, although in every case the values of the overall migration were lower than in polar simulants and below the OML. When using this solvent, the evaporation of actives before determining the weight of residue could occur to a lesser extent, since no steam drag effects would occur in the absence of water. Then, the potentially released active compounds could contribute to the total amount of migrated mass determined.

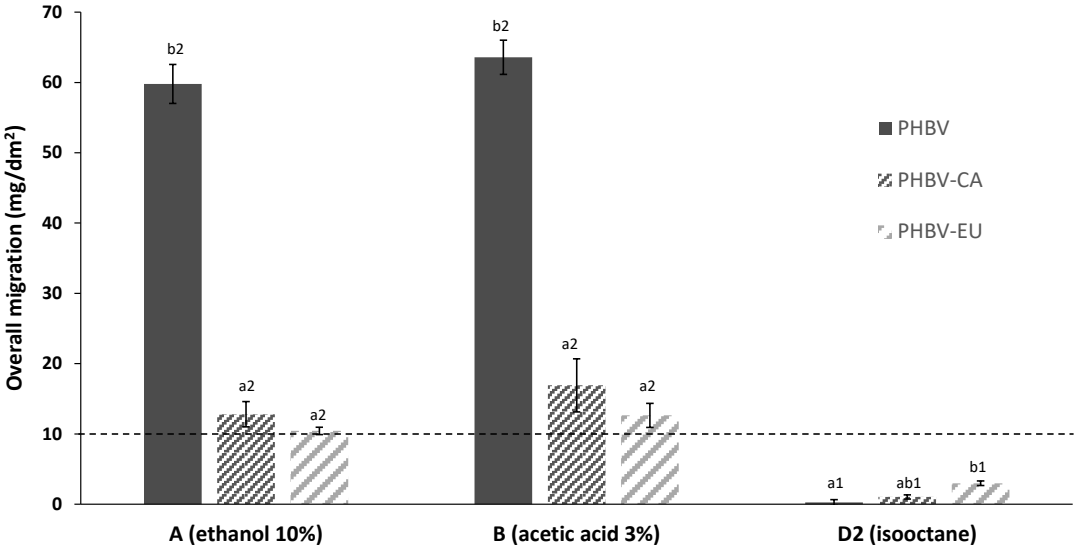



Figure 4. Overall migration in different food simulants of active-free PHBV films and those containing different active components: carvacrol (PHBV-CA) and eugenol (PHBV-EU). Different letters (a, b, ..) indicate significant differences between samples for a determined simulant and different numbers (1,2,..) significant differences between simulants for a determined film sample. Overall migration limit (---).

Under the extreme conditions tested, the water affinity of the plasticizer used (PEG) leads to the OML being exceeded in most of the cases in polar systems. Nevertheless, overall migration values within the permitted range could be achieved in less extreme environmental conditions [39]. Plasticizer seems to be retained in the polymer matrix when the phenolic active compounds are present in the films.

## 4. Conclusion

PEG plasticized PHBV active films with CA and EU could be obtained by incorporating them between two polymer layers by spraying the active, and subsequent compression moulding. Although 15 g of actives per 100 g polymer matrix were incorporated, about 5 % of these were lost during thermal compression and the bilayer films contain about 12 g of actives per 100 g of film. This method simulates incorporating actives and adhesive at the same time during the industrial production of multilayer films. CA and EU diffused through the polymer layers and were effectively released into different food simulants. At equilibrium, a total release of both CA and EU occurred in 50% ethanol

(simulating high fat content, oil-in-water foods), whereas around 20 and 50 % of the content, for CA and EU respectively, was delivered in the more aqueous systems, regardless of the pH. In fatty systems, 65-70 % of the active content was delivered at equilibrium. The release rate was enhanced when the polarity of aqueous systems decreased (50% ethanol), but it fell markedly in fatty systems (isooctane). The delivery of EU from PHBV films plasticized with PEG was slower than that of CA in aqueous systems, but this tendency was inverted when the polarity of the medium decreased. On the basis of the release kinetics, the antimicrobial activity against some foodborne pathogens could be predicted, taking the reported minimal inhibitory concentration of each compound into account. This concentration was reached for CA in every simulant tested at different times, which permits its effective antimicrobial action to be predicted. Nevertheless, EU did not reach the antimicrobial levels of some pathogens in neutral and acid aqueous systems or in fatty foods. However, antimicrobial *in vivo* tests are required to assess the antimicrobial effectiveness of these kinds of materials in real foods.

**Acknowledgements**

The authors thank the Ministerio de Economía y Competitividad (Spain) for the financial support provided through Project AGL2013-42989-R and AGL2016-76699-R. Author Raquel Requena thanks the Ministry of Education, Culture and Sport (Spain) for the FPU (FPU13/03444) Grant.

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**Figure captions**

Figure 1. Ratio of the active compound released in each food simulant, with respect to that of the equilibrium value, as a function of contact time (points) and fitted Fick's model (lines). CA (—) and EU (—). A: ethanol 10 % (v/v), B: acetic acid 3 % (w/v), D1: ethanol 50 % (v/v), and D2: isooctane.

Figure 2. Predicted concentration of released CA versus time in different types of food or food simulants: 10% ethanol (—), 3% acetic acid (—), 50% ethanol (– – –) and isooctane (– – –). Shaded area corresponds to the range of minimum inhibitory concentration of CA against different bacteria.

Figure 3. Predicted concentration of released EU versus time in each different type of food or food simulants: 10% ethanol (—), 3%acetic acid (—), 50% ethanol (– – –) and isooctane (– – –). Shaded area corresponds to the minimum inhibitory concentration of EU against different bacteria.

Figure 4. Overall migration in different food simulants of active-free PHBV films and those containing different active components: carvacrol (PHBV-CA), eugenol (PHBV-EU), oregano essential oil (PHBV-OR) and clove essential oil (PHBV-CLO). Different letters (a, b, ..) indicate significant differences between samples for a determined simulant and different numbers (1,2,..) significant differences between simulants for a determined film sample. Overall migration limit (– – –).