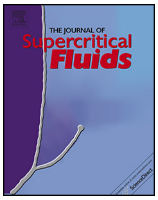
J. of Supercritical Fluids 122 (2017) 18–26

Contents lists available at ScienceDirect

The Journal of Supercritical Fluids

journal homepage: www.elsevier.com/locate/supflu



Supercritical CO_2 iof LDPE films with terpene ketones as biopesticides against corn weevil (*Sitophilus zeamais*)

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ARTICLE INFO

Article history:
Received 7 July 2016
Received in revised form
29 November 2016
Accepted 30 November 2016
Available online 1 December 2016

Keywords:
Supercritical fluid impregnation
Biopesticides
Thymoquinone
Pulegone
Sitophilus zeamais
Crop protection

ABSTRACT

In this work, the supercritical carbon dioxide impregnation of low density polyethylene (LDPE) films with a mixture of two terpenic ketones (thymoquinone and *R*-(+)-pulegone) with biopesticide activity is investigated, as a strategy for developing a packaging material or delivery device for protecting seeds, kernels and derivatives during storage and transport. Impregnation runs were performed according to a fractional factorial experimental design in order to evaluate the effect of four process variables at two levels (pressure: 10–15 MPa; depressurization rate: 0.5–2.0 MPa/min; time: 2–4 h; initial ketone mole fraction: 0.0017–0.0025) on impregnation yield and selectivity between ketones. Operational temperature was constant for all experiments and equal to 45 °C. The impregnated films were analyzed by infrared spectroscpy (FTIR) in order to confirm the presence of both ketones and determine their relative ratio in the final product. ANOVA test of the results indicate that contact time and ketone concentration are the only factors with significant effects on impregnation yield (ranging between 2.25 and 5.59%), while no factor seems to affect significantly the loading selectivity, with thymoquinone/pulegone ratios in the final product between 0.39 and 0.99. The insecticidal activity of impregnated film samples was evaluated in fumigant assays against corn weevil (*Sitophilus zeamais* Motschulsky), a major pest of stored kernels. The films showed a high activity level during the first 2 days (~100% mortality), which gradually decreased until showing no toxicity after 7 days.

1. Introduction

Biopesticides are recognized components of sustainable and environmentally friendly pest management systems, with increasing market as customers and regulation agencies are setting lower residue limits for synthetic pesticides or directly encouraging the use of nonsynthetic alternatives [1]. The growing of organic agriculture and the change in consumers dietary habits towards safer food products also open an opportunity for researching, developing and registering new biopesticides and innovative formulations [2]. According to the current regulations, biopesticides may include naturally occurring botanical substances and microorganisms that control pests as well as pesticidal substances produced by plants containing added genetic material [1,3].

Among phytochemical biopesticides, terpenes and terpene-rich essential oils are the most studied group of compounds [4–6]. These compounds are a part of the natural defense mechanisms of many plant species, usually showing high selectivity against target pests with low toxicity for mammals, birds and fish, besides being biodegradable. Due to these properties, they are generally considered « low-risk » products [7]. Although only few of them have reached application at commercial scale so far, many terpenes have great potential in the control of disease-vector insects and pests of economic relevance.

One of these potential applications is the protection of crops and seeds, which can be seriously damaged by insect attack and infestation during storage and transport, causing important economic losses every year [8]. A major crop pest is the corn weevil *Sitophilus*

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http://dx.doi.org/10.1016/j.supflu.2016.11.017
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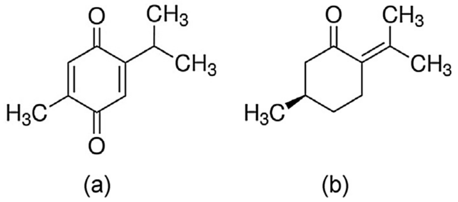

Fig. 1. Chemical structure of (a) thymoquinone and (b) *R*-(+)-pulegone.

zeamais Motschulsky (Coleoptera: Curculionidae), which mainly attacks corn kernels but also other cereal crops such as rice, wheat, barley, oats and cottonseed, as well as derived products. Several studies have shown that *S. zeamais* is sensitive to several essential oils and terpene compounds, specially oxygenated derivatives such as ketones, aldehydes and epoxides [9–11].

In previous studies, Herrera et al. [12,13] screened several terpene ketones and ketone-rich essential oils in order to assess their contact and fumigant toxicity against corn weevil adults. It was found that the lower lethal concentrations corresponded to thymoquinone and *R*-(+)-pulegone. This activity was correlated with the inhibition of acetylcholinesterase, suggesting that this was the main toxicity mechanism. A quantitative structure-activity relationship model (QSAR) suggested that the *p*-menthane structure and the presence of α-β insaturations improve the toxicity of ketones against *S. zeamais*. These ketones are naturally occurring compounds, being produced by several plants. For instance, thymoquinone is the main active constituent of *Nigella sativa* oil, and it is also present in other genera such as *Monarda* and *Juniperus*. As for *R*-(+)-pulegone, it is present in the essential oil of several aromatic species from the genus *Mentha* (mainly *Mentha* × *piperita*). Their chemical structure is shown in Fig. 1.

Nevertheless, the effective application of terpenic compounds as biopesticides has to overcome some limitations, such as their high volatility and reactivity with the ambient. In this sense, supercritical fluid technologies can contribute to the formulation of active materials allowing their protection and a more controlled release [14]. Supercritical carbon dioxide (scCO_2) has been proposed and studied as a green solvent for the extraction of active compounds from plants (e.g., pyrethrins, rotenone, terpenes), as well as an antisolvent for their recovery from organic solutions and the preparation of micronized powders and capsules [14]. Within this field, the impregnation of polymeric materials with biopesticides using scCO_2 is a promising and environmentally friendly strategy for the design and preparation of active packaging and/or controlled release devices. The main features and advantages of supercritical fluid impregnation of polymers have been extensively reviewed by several authors [15–17].

Besides the specific properties of the carrier matrix and the active substance, the efficiency of the impregnation process and the final characteristics of the material depend on several factors: scCO_2 density, depressurization conditions (fast or slow), temperature (above or below the polymer glass transition temperature), concentration of active compounds in the fluid phase (saturated or below saturation). As it is a batch process, the final amount of solutes incorporated into the polymer may also depend on the contact time, especially if equilibrium is reached slowly.

When the effect of several operation factors is to be studied simultaneously, it is often advantageous to use a design of experiment (DOE) approach, which provides a statistical basis for the analysis of the effects and allows to save experimental runs [18]. This approach can be used for screening as well as optimization purposes [19] and has been recently applied to supercritical fluid extraction and precipitation processes [20,21].

In this work, a DOE approach is applied to study the impregnation of low-density polyethylene films (LDPE) with an active mixture of thymoquinone and *R*-(+)-pulegone using scCO_2 as solvent. Preliminary studies of the toxicity of these ketones revealed a synergistic action when used together, hence a mixture of both ketones was used. LDPE was chosen as the polymeric matrix because it is a widely used, low cost, highly available and recyclable material, suitable for food and crop packaging. In a previous study, LDPE films were impregnated with eugenol, showing that a good impregnation yield, along with minimal changes in the polymer thermal and mechanical properties, can be achieved if the operation conditions are properly set [22]. Based on these facts, the specific goals of the present work are: a) the study of the effect of four operation parameters (pressure, depressurization rate, contact time and ketone concentration) on the impregnation performace; b) a preliminary assessment of the insecticidal activity of the impregnated films against *S. zeamais* in order to evaluate its potential application as a packaging material and/or a delivery device for the protection of grains under storage or transport.

2. Materials and methods

2.1. Materials

R-(+)-pulegone (purity: 97%, MW: 152.2 g/mol, bp: 224 °C) and thymoquinone (purity: 99%, MW: 164.2 g/mol, mp: 45 °C) were purchased from Sigma-Aldrich (Steinheim, Germany). Industrial extra-dry carbon dioxide (water content ≤ 10 ppm v/v, Linde, Argentina) was used as the impregnation solvent. Commercial low density polyethylene film (LDPE, M_w: 229300 g/mol, M_n: 22500 g/mol, MFI: 0.6 g/10 min at 190 °C/2.16 kg, density: 921 kg/m^3, thickness: 130 ± 20 μm, Dow-Polisur, Argentina) was used as polymeric matrix in all impregnation tests. Paraffin oil (medicinal grade, density: 870 kg/m^3, Sanitas S.A., Argentina) was used as solvent in the FTIR analysis calibration. Commercial food-grade ethanol (96% v/v, Porta Hnos., Argentina) was used for cleaning the experimental equipment.

2.2. Experimental setup and supercritical CO_2 impregnation experiments

The impregnation runs were performed in a high pressure apparatus described in a previous work [22]. In brief, the system consists in a 50 cm^3 stirred vessel with temperature control connected to a CO_2 delivery system and provided with a micrometering valve for controlled depressurization. In each run, the film samples were placed into the vessel using a metallic mesh support, which allows to maintain them separated and in vertical position, avoiding precipitation and/or deposition of ketone droplets or particles onto the film surface during depressurization. The corresponding amount of *R*-(+)-pulegone and thymoquinone (in 1:1 mass ratio, equal to 1.08 mol$_{pul}$/mol$_{thym}$) was added, the vessel was closed and CO_2 was delivered until the desired value for the pressure was achieved. The system was kept at the desired temperature (45 °C for all runs) and pressure conditions for a period of time, after which the CO_2 was released at constant depressurization rate. The films were removed from the vessel and gently dried with tissue paper in order to remove residual quantities of ketones from the surface. The mass of ketones impregnated into the films was calculated gravimetrically by measuring the mass increase of the films after the assay in a precision balance (±0.0001 g). Then, the impregnation yield (*Y*%) was defined as follows (Eq. (1)):

$$Y\% = \frac{m_f - m_0}{m_0} \times 100 \quad (1)$$

where m_o and m_f are the original and the final mass of the film, before and after impregnation, respectively.

In order to assess the possible effect of the extraction of additives (such as phthalate-type plasticizers and antioxidants) or short polymer chains by CO_2 during impregnation, preliminary blank runs were performed following the same impregnation procedure but without the addition of ketones. Film samples showed no mass decrease after treatment with CO_2 at 15 MPa and 45 °C during 4 h, concluding that the extraction of additives and short polymer chains at process conditions is negligible. This is in agreement with the observations of Arias et al. [23], which suggest that the extraction efficiency for this type of compounds from LDPE is low (under 30% recovery) at pressures below 25 MPa and 40 °C. These preliminary tests also provided information about the time required for the complete release of dissolved CO_2 from the films after depressurization, which can also affect the gravimetric measurements. It was observed that the films recovered their original weight within a period of 10 min. Therefore, in the impregnation runs (with ketones), the samples were weighed after waiting at least 10 min after depressurization.

2.3. Fourier transformed infra-red spectrometric analysis (FTIR)

FTIR analysis was performed in order to confirm the impregnation of pulegone and thymoquinone, as well as for quantifying their relative concentration in the films. Absorbance spectra were obtained in an infrared imaging microscope (Nicolet iN10 Mx, Thermo Fisher Scientific, USA) in transmission mode, with a resolution of 4 cm^{-1}, in a wavenumber range of 400–4000 cm^{-1} with 16 scans, at room temperature and using a KBr disc. Spectra of the pure ketones, original LDPE film and impregnated film samples were acquired and normalized in order to identify characteristic absorbance peaks and quantify their relative absorbance values. Absorbance was measured at least at three different positions of each film, including points near the edges (upper and lower), and far from the edges ("centre"). Background spectra were acquired before each test for air humidity and carbon dioxide correction.

The ratio in which both ketones were loaded into the films was assessed, as a measure of the selectivity of the process, by comparing the relative absorbance values between the characteristic peaks for each compound. For that purpose, a calibration curve was prepared using different ketone mixtures dissolved in paraffin oil (in order to obtain a molecular environment similar to LDPE) [24] with thymoquinone:pulegone mass ratios ranging from 25:75 to 75:25. The total ketone concentration in all the mixtures assayed was constant and equal to 5% (w/w). The absorbance spectra of this set of solutions were acquired, and the relationship between the concentration ratio of thymoquinone and pulegone and their relative absorbance was adjusted with a linear function (Eq. (2)).

$$\frac{A_{thym}}{A_{pul}} = a\left(\frac{C_{thym}}{C_{pul}}\right) + b \tag{2}$$

Where A_{thym} and A_{pul} are the absorbance values for the characteristic peaks of thymoquinone and pulegone, respectively, as well as C_{thym} and C_{pul} are their mass concentrations in the ketone mixtures. In this way, results are independent of the optical path length. The parameters a and b were determined by linear regression. The calibration curve was performed by duplicate, and each measurement was replicated four times.

In this sense, the selectivity of the impregnation towards thymoquinone (TS) was defined as the mass concentration of this compound divided by the mass concentration of pulegone impregnated into the films (Eq. (3)).

$$TS = \frac{C_{thym}}{C_{pul}} \tag{3}$$

Table 1
Experimental variables for two-level factorial design.

Factor	Variable	Low level (−)	High level (+)
A	Pressure (MPa)	10	15
B	Depressurization rate (MPa/min)	0.5	2
C	Time (h)	2	4
D	Initial ketone mole fraction	0.0017	0.0025

2.4. Experimental design

For the design of experiments, four factors were selected at two levels: (A) pressure, (B) depressurization rate, (C) contact time, and (D) initial ketone concentration. The corresponding high and low values are presented in Table 1. A 3/4 fractional factorial screening design (12 runs) was used. This resolution V arrangement allows to estimate all main factor effects and two-factor interactions aliased only with three-factor or higher order interactions [25].

Pressure has a strong influence on CO_2 density and viscosity, and therefore it is expected to affect the polymer swelling and the diffusional properties, as well as the solute partition coefficient between the fluid phase and the polymer. Two levels were compared (10 and 15 MPa), corresponding to pure CO_2 density values of 498 and 742 kg/m^3, respectively (at 45 °C, according to the NIST database [26]).

Besides, it is known that the depressurization rate can be a key parameter in order to control the solute precipitation and retention inside the polymer. The CO_2 desorption rate during depressurization may also affect the morphological and thermomechanical properties of the polymer. Therefore, fast (2 MPa/min) and slow (0.5 MPa/min) depressurization conditions were compared.

As it is a diffusion-controlled process, impregnation efficiency is also likely to depend on the concentration of ketones in the fluid phase. In this case, there are two possible approaches. One consists in loading an excess amount of ketones and therefore keeping the fluid phase concentration constant (corresponding to the thermodynamic solubility value) along the whole process. This strategy is generally preferable regarding the process efficiency, as the driving force for mass transfer is maintained constant and maximum. However, the solute concentration in the fluid phase is automatically fixed at given temperature and pressure conditions. The other approach consists in the complete dissolution of ketones in the fluid phase (either by setting the initial concentration at a value below solubility or by operating above the mixture critical pressure). In this case, the fluid phase concentration (and therefore the mass transfer rate) decreases as the impregnation proceeds, which is an important disadvantage. However, in this work the second strategy was adopted, which allowed to set independently the pressure and concentration values, and therefore to determine the effect of both factors separately. For that purpose, phase equilibrium conditions leading to complete CO_2-ketone miscibility were selected. To the best of our knowledge, there is no information regarding the high pressure phase equilibrium behavior of the system CO_2 + pulegone. Therefore, estimations were performed from data reported by several authors for the system CO_2 + carvone, a closely related terpene ketone [27–29]. These data suggest that the system critical pressure at 45 °C is around 10 MPa. In the case of thymoquinone, there is a single report limited to the determination of the system critical point at 50 °C, which occurs at a pressure of 10.3 MPa and a thymoquinone mole fraction of 0.0162 [30]. In this case it can also be assumed that the system will be homogeneous above 10 MPa at 45 °C. Therefore, for both ketones we can consider that there are no solubility limitations at pressure values above 10 MPa (extending this assumption to the ternary system). However, in order to ensure complete miscibility, the ketone concentration was set below the solubility value at the closest available conditions. Gamse et al. [29]

Table 2
Experimental design of impregnation conditions and corresponding yield and selectivity results. All impregnation runs were performed at T = 45 °C.

Run no.	Pressure (MPa)	Depressurization rate (MPa/min)	Time (h)	Initial ketone mole fraction	*Y%*[*] (% w/w)			*TS*[**] (w/w)		
1	10	0.5	2	0.0017	2.40	±	0.57^a	0.84	±	0.08^a
2	10	0.5	2	0.0025	3.06	±	0.83^a	0.58	±	0.02^a
3	10	2.0	4	0.0017	4.08	±	0.70^a	0.52	±	0.05^a
4	10	2.0	4	0.0025	5.59	±	0.19^a	0.97	±	0.05^a
5	15	0.5	4	0.0017	2.25	±	0.05^a	0.52	±	0.04^a
6	15	0.5	4	0.0025	4.43	±	0.80^b	0.80	±	0.07^b
7	15	2.0	2	0.0017	2.99	±	0.38^a	0.39	±	0.04^a
8	15	2.0	2	0.0025	3.52	±	0.49^b	0.40	±	0.03^b
9	10	0.5	4	0.0017	4.51	±	0.19^a	0.97	±	0.08^a
10	15	2.0	4	0.0017	3.91	±	0.28^b	0.86	±	0.08^b
11	15	0.5	2	0.0025	4.90	±	0.24^b	0.90	±	0.03^b
12	10	2.0	2	0.0025	4.69	±	0.32^b	0.99	±	0.09^b

Mean values ± standard deviation with ^{a} $n = 4$, ^{b} $n = 6$.

[*] Impregnation yield (Eq. (1)).

[**] Impregnation selectivity (Eq. (3)).

report a mole fraction of 0.003 for carvone at 51 °C and 10 MPa, while Corazza et al. [28] report a value of 0.0046 at 50 °C and 10.5 MPa for this compound. Based on this, the initial concentration of ketones in the fluid phase was set at 0.0017 and 0.0025 (mole fraction).

Finally, the amount of ketones incorporated into the polymer may also depend on the contact time before reaching equilibrium. Based on literature reports [27,28] and our previous works [22], impregnation runs was performed during 2 and 4 h.

Although temperature affects the solvent density, the solubility and the polymer properties, it was not analyzed here as a factor, and it was set at a constant value of 45 °C. On one hand, solvent density was controlled only by means of the operation pressure, and ketone concentration was kept below the solubility limit, as mentioned above. On the other hand, the mild temperature conditions required when supercritical CO_2 is used in the processing of thermolabile natural compounds are well above the glass transition temperature of LDPE ($T_g < -100\,°C$) and below its crystallization temperature ($T_c = 110\,°C$) [22], therefore its effect on the polymer morphology is expected to be minimal. Besides, the mixture of ketones is liquid at this temperature.

Two responses were evaluated: (a) the total amount of ketones incorporated into the LDPE films per unit weight of material (as a measure of the impregnation *yield*), and (b) the mass fraction ratio of thymoquinone and pulegone incorporated into the films (as a measure of the process *selectivity*). These were calculated according to Eqs. (1) and (3), respectively.

All impregnation experiments were duplicated and 2–3 films were used at each run, considering all the films obtained at the same operational conditions as replicas in the statistical analysis. The effect of each factor and all two-factor interactions on both responses was statistically determined by analysis of variance (ANOVA) using the software Statgraphics© (StatPoint Technologies, Inc.) [18]. The effect of the factors on the different responses was considered significant for $p < 0.05$.

2.5. Toxicity assays

The toxicity of ketone-loaded films was evaluated using the methodology described by Herrera et al. [12], with some modifications. Unsexed adults of *S. zeamais* were used. The colony was maintained in the laboratory for one year without exposure to insecticides, and the insects were reared in brood chamber under controlled humidity and temperature conditions (60–70% and 28 °C, respectively) under light/dark 12:12 h cycles [12,31]. Briefly, ten weevils and some corn kernels (1.5 ± 0.05 g) were placed in 30 mL glass vials, hermetically sealed with a screw cap. The impregnated films were placed inside the caps, supported by a metal mesh to avoid direct contact with the weevils. Non-impregnated films were used in the same conditions for control purposes. The vials were kept at room temperature and protected from direct light. After 24 h, the mortality was determined by counting the dead insects and subsequently, the screw cap containing the film was removed and placed in a new vial (with new weevils and kernels). This procedure was repeated over 7 days in order to determine the residual toxicity of the impregnated films every 24 h, under headspace renewal conditions. Each impregnated film was considered a replica ($n = 3$).

3. Results and discussion

3.1. Supercritical fluid impregnation

Supercritical CO_2 impregnation of active ketones into LDPE films was assessed as described above. The experimental design with the conditions corresponding to each impregnation run and the values obtained for both responses are presented in Table 2.

Original and impregnated LDPE films, as well as pure ketone samples, were analyzed by infra-red spectroscopy using a FTIR microscope, as described in Section 2.3. This technique allowed to confirm the presence of both ketones, thymoquinone and pulegone, in the impregnated films. As an example, the absorbance spectra of the pure ketones, together with original and impregnated film samples, are shown in Fig. 2. Thymoquinone showed a characteristic absorbance peak at approx. 1238 cm^{-1} (typical for C=C double bonds) [32], while a characteristic peak for pulegone was detected at 1208 cm^{-1} (assigned to the vibration of C—H bonds in the >CH—CH_3 group) [33]. The absorbance peak at 725 cm^{-1} was assigned to LDPE and used as reference to normalize the spectra [34]. As mentioned, absorbance was measured at different positions of the films (near the center and the edges) and both compounds, pulegone and thymoquinone, were always detected.

Results show that the process was effective for the impregnation of active ketones into LDPE films, with impregnation yield values ranging from 2.25 ± 0.05 to 5.59 ± 0.19% (w/w), operating at 45 °C and different combinations of pressure, depressurization rate, time and initial ketone concentration. These results are in agreement with a previous work [22], where LDPE films were impregnated with eugenol, at similar operational conditions ($T = 45\,°C$, $P = 10–15$ MPa, and $t = 4$ h), achieving loading values from 1 to 6% (w/w) approximately. Other authors also reported comparable results for the impregnation yield of compounds with similar chemical structure or molecular weight into LDPE films, using supercritical CO_2 impregnation. Torres et al. [35] reported the

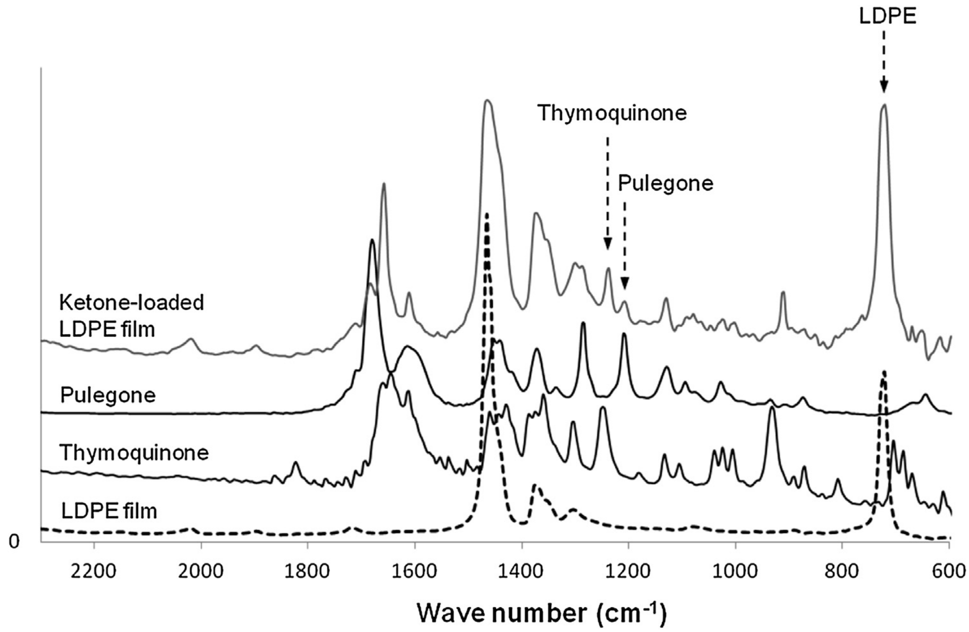

Fig. 2. FT-IR spectra for original LDPE film, pure thymoquinone and pulegone, and ketone-loaded LDPE film (impregnated at T = 45 °C, P = 10 MPa, depressurization rate = 2 MPa/min, t = 2 h and initial ketone mole fraction = 0.0025).

supercritical impregnation of thymol into LDPE films, operating at 40 °C and pressure levels of 9 and 12 MPa, obtaining loading values of 2 and 4% (w/w), respectively. Li and Han [36] reported similar yield values when LDPE films were impregnated with styrene (in absence of the polymerization initiator). Moreover, impregnation yields reported for 2-nonanone were about one order of magnitude lower, with values of 0.3% (w/w), approximately, operating at 40 °C and 12 MPa [37].

Results are comparable and representative of the behavior of semicrystalline polymers when subjected to $scCO_2$ impregnation. The impregnation process is favored by the absorption of CO_2 by the polymer and the resulting swelling effect, which reduce the solute diffusion coefficient into the matrix [38]. In this sense, the CO_2 sorption and swelling degree of different types of polymers have been studied and modeled by several authors [39–46]. In the case of semicrystalline polymers (such as polyolefins), it is generally considered that CO_2 is mainly sorbed into the amorphous parts, while the sorption rate into the crystalline regions (crystallites) is much slower. In turn, these regions impose a mechanical restriction to the CO_2-promoted swelling of the amorphous domains, limiting the volume expansion of the polymeric matrix [40,47]. As a consequence, semicrystalline polymers usually show lower CO_2 sorption, swelling and impregnation degrees than the completely amorphous ones. However, it has to be mentioned that the CO_2 sorption may induce a partial disorganization of the crystallites, reducing the crystallinity degree of the polymer. In our case, the original LDPE film has a crystallinity degree of 44%, which is reduced to approx. 37% after the high pressure treatment, independently of the incorporation of solutes, as observed in a previous work [22].

To the best of our knowledge, there is no information in open literature regarding the CO_2 sorption and swelling behavior of polyethylene at high pressure conditions. However, Lei et al. [46] have studied the system CO_2 + polypropylene (PP), another semicrystalline polyolefin, reporting sorption values of approx. 0.05 g/g and a volume increase of ~5% at 10 MPa and temperatures in the range of 40–50 °C. Similar values have been reported for semicrysalline polytetrafluoroethylene (PTFE) in the range 10–15 MPa and 50 °C [40].

In the case of amorphous polymers, these values are usually higher. For example, Pantoula et al. [43] have reported CO_2 sorption values of 0.14–0.15 g/g for polystyrene (PS) at 35 °C and 0.11–0.13 g/g at 51 °C in the pressure range of 10–15 MPa, and swelling values of 10–12% at the same pressure and temperature conditions [44], showing a good agreement with data reported by other authors. Besides, when strong or specific interactions between the polymer functional groups and CO_2 can occur, these values are further increased. In the case of polymethylmethacrylate (PMMA), whose carbonyl groups interact with CO_2, typical sorption values are in the range of 0.20–0.30 g/g with a swelling degree of 20–25%, at pressures of 10–15 MPa and temperatures of 35–60 °C [40,43,44]. In a recent work, Champeau et al. [48] provide a comparison between different crystalline and amorphous polymeric fibers, concluding that a high CO_2 sorption –and the resulting swelling effect– depends on the presence of functional groups capable of interacting with CO_2 and also on the mobility of the polymer chains, which is higher in the amorphous domains.

Furthermore, the ratio in which both ketones were incorporated into the films was assessed by comparing the relative absorbance values of the selected characteristic peaks of each compound. As previously mentioned, a calibration curve was constructed, and a linear response ($R^2 = 0.993$) was obtained between the relative absorbance and the mass ratio of thymoquinone over pulegone, as described in Eq. (2), with regression coefficients $a = 0.75$ and $b = 0.95$. The selectivity of the impregnation towards thymoquinone (TS) showed values ranging from 0.39 ± 0.04 to 0.99 ± 0.09 (w/w), as shown in Table 2. As mentioned before, absorbance measurements were performed at different positions of the films (near the center and the edges), and the inner variation of selectivity in each film ranged from 3 to 10%, showing a fair degree of homogeneity in the impregnation process.

Selectivity results indicate that thymoquinone is loaded into the films in the same or lower proportion than pulegone in all cases. Considering that the initial ketone ratio loaded into the cell was always 1:1, results suggest that the impregnation process has

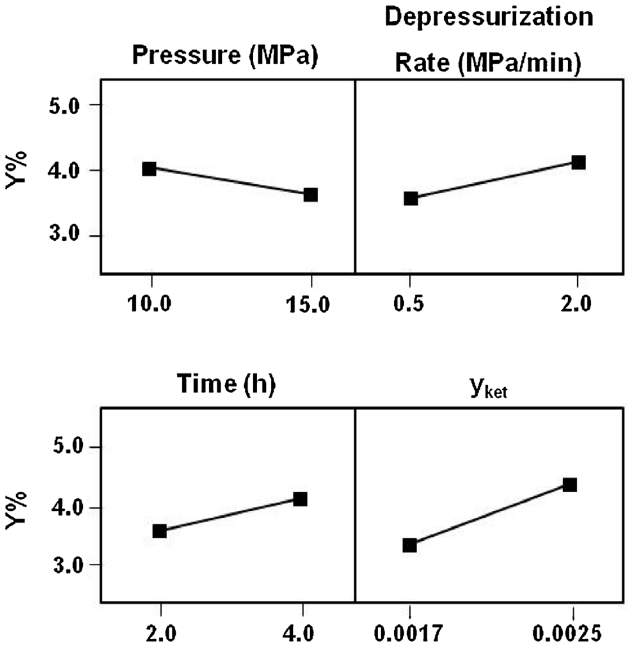

Fig. 3. Single effects of pressure, depressurization rate, time and initial ketone mole fraction (y_{ket}) on impregnation yield (Y%). All impregnation runs were performed at $T = 45\,^{\circ}\mathrm{C}$.

Table 3
ANOVA testing the effects of process variables on impregnation yield for the fractional design model.

Factor	DF	Effect	SS	MS	F	p-value
A	1.00	−0.44	0.93	0.93	1.42	0.248
B	1.00	0.38	0.69	0.69	1.05	0.318
C	1.00	0.80	3.08	3.08	4.73	***0.043***
D	1.00	1.15	5.83	5.83	8.95	***0.008***
AB	1.00	−0.36	0.62	0.63	0.96	0.341
AC	1.00	−0.57	1.57	1.57	2.41	0.138
AD	1.00	0.08	0.03	0.03	0.04	0.844
BC	1.00	0.11	0.06	0.06	0.09	0.772
BD	1.00	−0.26	0.30	0.30	0.46	0.507
CD	1.00	−0.08	0.04	0.04	0.06	0.812
Residual	18.00		11.73	0.65		

DF: Degrees of freedom. SS: Sum of squares. MS: Mean square.

some degree of selectivity towards pulegone. This result may be explained in different ways. The impregnation process involves the distribution of the solutes between the fluid phase and the swollen polymer. However, this equilibrium only occurs instantly at the interface. Inside the polymer, there is a constant diffusion of solutes from the surface, until reaching a uniform equilibrium concentration if the process is carried out for enough time. Afterwards, during depressurization, the equilibrium conditions are changed, the solubility decreases and the $scCO_2$ is desorbed from the polymer, extracting a part of the solutes with it. The observed differences between thymoquinone and pulegone may be connected with all these phenomena. It is possible that the partition equilibrium of both ketones is different: the low selectivity for thymoquinone suggests that it has a higher affinity for the fluid phase than pulegone. These compounds have similar molecular weight and chemical structure, but thymoquinone has a lower polarity (dipolar moment $\mu = 0.3$ D, versus $\mu = 3.27$ D for *R*-(+)-pulegone [49]) as the carbonyl groups oppositely located tend to cancel out, which supports the hypothesis of a higher affinity for $scCO_2$, both during the impregnation and the depressurization steps. Another possible explanation is a difference between the diffusion coefficients of these ketones in the polymer. Although there is no experimental information about it, to the best of our knowledge, an examination of their spatial configuration suggests that thymoquinone molecules have a higher steric hindrance to penetrate and move among polyethylene chains, retarding their diffusion [49].

3.2. Statistical analysis

As previously mentioned, the applied fractional factorial design allows the estimation of the effect of all main factors and two-factor interactions on the process responses. In the case of impregnation yield, these effects are shown graphically in Figs. 3 and 4, respectively. The occurrence of interaction between factors is indicated by non-parallel lines. Table 3 shows the ANOVA results for the effects of process variables and their interactions on impregnation yield. It can be seen that significant effects were only found for factors C and D (contact time and initial ketone concentration, respectively), while none of the interactions showed significant effects, for $p < 0.05$. According to the analysis (Fig. 3), impregnation yield increases both with time and with the initial concentration of ketones, suggesting that the process does not reach equilibrium within the studied range. In a study concerning the impregnation of LDPE films with styrene using supercritical CO_2 as solvent and swelling agent, Li and Han [36] observed that the uptake of styrene increased with time until reaching a constant value of about 0.8 g/g after 24 h (with no polymerization initiator) operating at 35 °C and 12 MPa. This suggests that the impregnation rate is strongly controlled by the diffusion of the solutes into the swollen polymer. Considering that the chemical structure of styrene has some similarity to the ketones studied in this work (basically consisting in a branched C_6 ring), this conclusion may be extended to our case and therefore it is expected that the saturation of the films will occur after longer contact times. However, it has to be noted that the films impregnated in our work have a lower thickness (130 μm vs 400 μm), which can reduce this equilibration time.

A similar enhancing effect of solute concentration on impregnation yield has also been observed by Li and Han in the above mentioned study [36], reinforcing the hypothesis of a diffusion-controlled process, dependent on the concentration gradient between the surface and the bulk of the swollen polymer.

The statistical analysis indicates that there is no significant difference in operating at 10 or 15 MPa, which may suggest that operation pressure has no effect on impregnation yield. This is in agreement with previous results obtained in the impregnation of LLDPE films with eugenol at 10, 12 and 15 MPa [22]. Nevertheless, other authors have reported a certain relationship between them. Pressure has two opposite effects on the impregnation process: on one hand it enhances CO_2 solvent power and therefore the solutes affinity for the fluid phase; on the other hand it increases the polymer swelling, improving the internal diffusion of the solutes. Li and Han [36] concluded that the first effect predominates at higher pressure levels, while the second one is more important at lower pressure. As a result, the impregnation yield shows a maximum at an intermediate level (around 13 MPa at 35 °C). Shen et al. [50] have studied the impregnation of cellulose acetate (CA) fibers with L-menthol and vanillin at several pressure levels in the range 5–17 MPa, and observed the same behavior. These conclusions are also in agreement with results reported by Torres et al. [35] and Rojas et al. [37], who observed increasing yields from 7 to 12 MPa and decreasing yields from 12 to 22 MPa (with thymol and 2-nonanone, respectively). Therefore, in order to clarify the effect of pressure on impregnation yield further investigation at intermediate pressure levels should be performed. This shows a clear limitation of the two-level experimental design when the effect

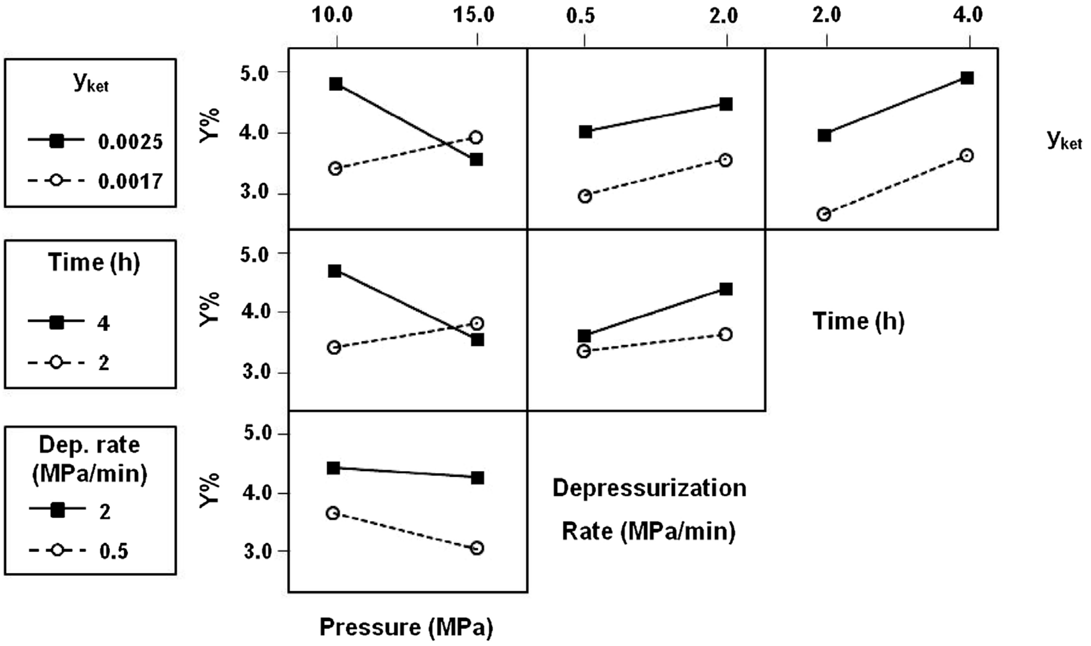

Fig. 4. Interaction plots of the effects on impregnation yield (Y%). Continuous lines represent high values (■), and discontinuous lines represent low values (○) for each variable. All impregnation runs were performed at $T = 45\,°C$. y_{ket}: initial ketone mole fraction.

of a parameter is linked with several undergoing phenomena in a complex way.

Regarding the effects on the impregnation selectivity, ANOVA results are shown in Table 4. Despite the high variability observed, the statistical analysis revealed that the selectivity does not depend significantly on any of the studied operational variables (or two-factor interactions) within the experimental ranges covered in this work. The observed dispersion might be attributed to random effects, to uncontrolled variables (such as temperature oscillations, local variability of the polymer density or crystallinity degree, or small differences in the depressurization rate control), or to a more complex interrelation of the process parameters and the polymer properties.

Table 4
ANOVA testing the effects of process variables on impregnation selectivity for the fractional design model.

Factor	DF	Effect	SS	MS	F	p-value
A	1.00	0.724	0.058	0.058	1.16	0.29
B	1.00	−0.098	0.016	0.016	0.31	0.58
C	1.00	−0.051	0.066	0.066	1.33	0.26
D	1.00	0.105	0.067	0.067	1.35	0.26
AB	1.00	0.106	0.005	0.005	0.09	0.76
AC	1.00	−0.028	0.015	0.015	0.30	0.59
AD	1.00	0.050	0.001	0.001	0.01	0.92
BC	1.00	0.010	0.084	0.084	1.68	0.21
BD	1.00	0.118	0.095	0.095	1.90	0.18
CD	1.00	0.126	0.002	0.002	0.04	0.84
Residual	32.00		1.249	0.050		

DF: Degrees of freedom. SS: Sum of squares. MS: Mean square.

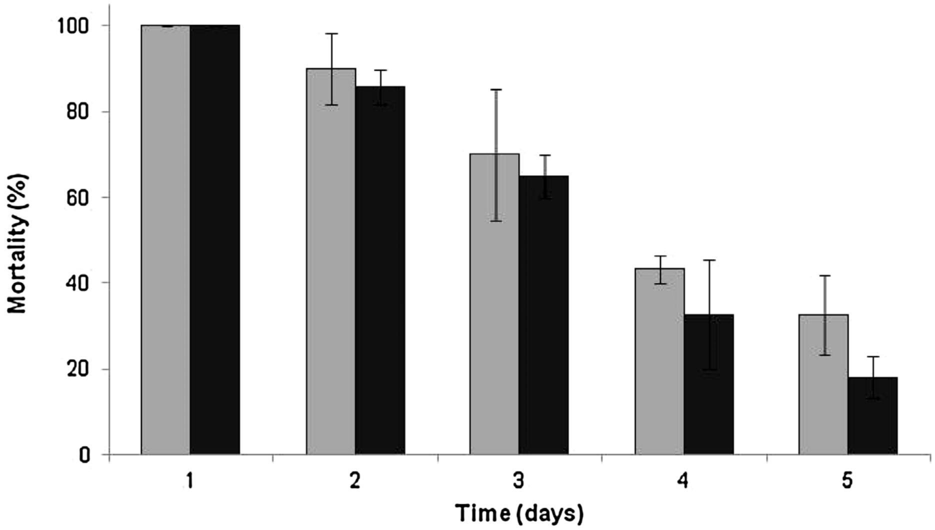

Fig. 5. Toxicity of films impregnated at $P = 10$ MPa, $T = 45\,°C$, $t = 4$ h, $y_{ket} = 0.0025$ and depressurization rate: 0.5 MPa/ min (▨) and 2 MPa/ min (■). Vertical bars represent the standard error.

3.3. Toxicity tests

The samples corresponding to the highest impregnation yield (i.e., $P = 10$ MPa, depressurization rate = 2 MPa/min, $t = 4$ h and $y_{\text{ket}} = 0.0025$) were selected for the toxicity tests against *S. zeamais*. On the other hand, even though the depressurization rate showed no significant effect on the amount of ketones impregnated into the films, it may have an influence on the solutes retention inside the polymer, and therefore on the release profiles and the resulting toxicity of the films along time. In order to investigate this effect, new LDPE samples were impregnated under the same conditions but using a lower depressurization rate (0.5 MPa/min) and the toxicity of these films was also evaluated for comparison purposes. It has to be mentioned that this new set of conditions does not correspond to any point of our previous experimental design.

Fig. 5 shows the toxicity of both films expressed as the percentage of dead weevils (mortality%) at each step of 24 h. Results revealed that the films remained active along 5 days of exposure, after which no mortality was observed. During this period, the mortality decreased in every step, being this reduction faster for the films depressurized at higher rate (2 MPa/min). For both treatments, the mortality of the films is higher than 85% during the first two steps (days 1 and 2). At step 3, the mortality decreased to 65–70% for both treatments, respectively, suggesting that under these experimental conditions the impregnated films released to the flask atmosphere an active compound concentration still higher than the LC_{50} (lethal concentration, i.e., the concentration that is needed for killing 50% of the insects). In the next steps (days 4 and 5), the films remained active but with mortality values of 30–40%. Finally, in the last two stages (days 6 and 7), no mortality was observed.

Comparing both samples, it can be concluded that the depressurization rate seems to affect in some degree the release rate of ketones from the film. Although a slower depressurization allows a more gradual release, this difference is not higher enough to extend the fumigant activity of the films to longer periods. As mentioned before, the same procedure was performed using non-impregnated samples for control purposes, and no mortality was observed for these films.

4. Conclusions

In this work, the impregnation of a mixture of two terpenic ketones (thymoquinone and *R*-(+)-pulegone) with insecticidal activity into LDPE films using supercritical CO_2 was studied. The influence of four process variables (pressure, depressurization rate, contact time and initial ketone concentration) on the impregnation yield and selectivity was investigated by a fractional factorial design of experiments approach. Impregnated films with a final amount of ketones ranging from 2.25 to 5.59% (w/w) were obtained. The statistical analysis indicated that time and ketone concentration are the only factors with significant effects on impregnation yield, both enhancing the final amount of ketones in the impregnated films. This suggests that the impregnation rate is strongly controlled by the diffusion of the solutes into the swollen polymer.

The impregnated samples were analyzed by infrared spectroscopy, not only for confirming the presence of both ketones but also to quantify their final ratio in the impregnated films. In spite of the fact that they are loaded into the cell in a 1:1 ratio in all runs, it was found that the ratio thymoquinone:pulegone in the films was in the range 0.39–0.99, indicating a selectivity for pulegone in most cases. According to the statistical analysis, none of the studied factors had significant effects on this response.

Due to the limitations of a two-level screening design, further experiments should be carried out at intermediate conditions, in order to clarify more deeply the effect of some operation variables −such as pressure– and elucidate the complex interactions that occur between the different physico-chemical processes that affect the impregnation efficiency. However, screening results can provide useful indications for future process optimization studies.

Finally, the fumigant toxicity of the films obtained in the best yield conditions against *S. zeamais* under controlled conditions was evaluated and compared with films obtained under the same conditions but at a lower depressurization rate. The films showed a high activity level during the first 2 days (<85% mortality), which gradually decreased until 20–30% (day 5) and finally showing no toxicity after 7 days.

These preliminary results suggest that supercritical CO_2 impregnation is a suitable technique for producing active films with potential application in the preservation of seeds during storage and transport. According to the case, further research is envisaged in order to enhance the release profile of biopesticides and extend its activity to longer periods. The use of clays dispersed in the polymer (nanocomposites) may be a promising strategy for improving the impregnation yield as well as retarding the release of ketones by a combination of adsorption and diffusion. Another way of modulating the release rate of ketones is the incorporation of the impregnated film into a multilayer packaging material, where one or more polymeric films (inner and/or outer) should provide an additional barrier to diffusion [51].

In any case, the films obtained in this work could be potentially applied as ketone release materials placed in the inner face of silo bags, which are increasingly used for corn storage on field. These films can provide an additional barrier to the development of weevils when the natural modification of the internal atmosphere is not sufficient for creating a lethal environment, or when the bag itself is damaged, constituting an interesting alternative to the fumigation of silo bags with synthetic insecticides.

Acknowledgments

The authors gratefully acknowledge Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina), Universidad Nacional de Córdoba (UNC, Argentina. SECyT 2015 Project), Universidad Tecnológica Nacional (UTN, Argentina, PID 3486 and PID 3458) and Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, Argentina. PICT Startup 2011-0726) for their financial support, as well as Dr. Julio Zygadlo for his technical support. N.A. Gañán, A.E. Andreatta, M.C. Strumia and R.E. Martini are career members of CONICET. M.L. Goñi and J.M. Herrera acknowledge CONICET for their postdoctoral and doctoral fellowships, respectively.

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