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Green Extraction of Carotenoids from Bee Pollen Using Sunflower Oil: Evaluation of Time and Matrix-Solvent Ratio

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Bee pollen has been recently highlighted as a potential source of fat-soluble bioactive compounds, in particular carotenoids. However, these compounds are trapped or linked to other components in both the inner pollen grain structure and the exine, which might hinder their bioavailability. The solvent-extraction of carotenoids from plant matrixes using edible oils has been profusely investigated, but there are no previous references to the use of bee pollen. In this work, the extraction kinetics of carotenoids from bee pollen was studied, using sunflower oil as an edible solvent, at different matrix-solvent ratios, in order to obtain carotenoid-rich oily extracts. Firstly, extractions were made by continuous stirring pollen and oil at room temperature, at ratios between 5 and 60 %wt during 24 h. Total carotenoids in the resulting extracts varied as a function of pollen ratio from 40.5 ± 1.7 mg β-carotene/kg oil (5 %wt) to 968.8 ± 4.2 mg β-carotene/kg oil (60 %wt). Subsequently, extractions were followed during 16 days at the three ratios that allowed for higher content of carotenoids (40%, 50%, and 60%wt). Concentration of carotenoids in the extracts increased significantly along the extraction time until day 12, following a second-order kinetic trend (R^2>0.999). The content of carotenoids in the extracts at day 12 was significantly higher for higher matrix-solvent ratios, being 471.7 ± 10.1, 691.4 ± 17.7 and 1010.8 ± 11.6 mg β-carotene/kg oil, for ratios 40 %wt, 50 %wt and 60 %wt, respectively. These findings indicate that, for a better extract quality, it is necessary to use 60%wt, or higher ratios, of bee pollen. Also, at room temperature, 12 days of extraction are required to guaranteeing the maximum extraction yield, regardless the matrix-pollen ratio. This work evidences the industrial feasibility of using bee pollen as a raw material for obtaining carotenoid-rich extracts, with potential as natural food colorants and bioactive ingredients, as suggested by previous reports. Furthermore, it is a rationale for future studies on the use of assisted-extraction techniques, such as microwaves and ultrasounds, for this purpose.
Key-words: bee pollen, carotenoids, green extraction, sunflower oil

1. Introduction

Bee pollen is a natural product made by worker bees when they collect nectar and pollen. Floral pollen is mixed with salivary secretions and nectar. It is harvested by beekeepers through traps located in the entrances of the hives (Kieliszek et al. 2018) and typically subjected to drying and cleaning processes to be used as a food ingredient or dietary supplement. This product presents high concentration of several nutrients, including proteins with contents between 7.5-40 %, carbohydrates between 15-60 %, lipids between 0.5-20 %; minerals like potassium, calcium, magnesium, sodium, copper, zinc, iron, manganese, phosphorus, vitamins (water- and fat-soluble) and bioactive compounds (Claudia Salazar-González and Díaz-Moreno 2016). In particular, this product has been recently highlighted as a potential source of carotenoids (Gasparotto Sattler et al., 2015).

Carotenoids are used as natural food colorants in the red-yellow-orange hues, as well as pro-vitamin A precursors (Saini, Nile, and Park 2015) and antioxidants (Delgado-Vargas, Jiménez, and Paredes-López 2010), among others. Their consumption has been associated with beneficial health effects, mainly for lutein and zeaxanthin, like macular carotenoids (Kim et al. 2016).

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Regarding carotenoid composition, Colombian bee pollen (503.6 mg β-carotene equivalents/kg) (Salazar-González, Céspedes, and Díaz-Moreno 2013) exceeds that of bee pollens from China (271.6 mg/kg) (Xu et al. 2011), Brazil (252.6 mg/kg) (Gasparotto Sattler et al. 2015), Spain (20.40 mg/kg) (Domínguez-Valhondo et al. 2011) and Italy (24.7 mg/kg) (Gardana et al., 20181). The higher carotenoid content in Colombian bee pollen, highlights it as a potential industrial natural source of these compounds. However, carotenoids in pollen are known to be trapped inside the bee pollen grain structure, or even chemically bonded to macromolecules, which might hinder their bioavailability (Domínguez-Valhondo et al. 2011). A recent work evaluated the possibility to extract polyphenols from Colombian bee pollen using emerging technologies, like ultrasound and microwaves; the authors found that microwaves are better to obtain polyphenols-rich extracts (Rodríguez-González, Ortega-Toro, and Díaz 2018). There are not studies on the extraction of carotenoids from bee pollen. In this work, a simple conventional process, using continuous stirring vessels and sunflower oil as a green solvent, for extraction of carotenoids from bee pollen, was studied. The effect of bee pollen:oil ratio and the extraction kinetics were evaluated, providing useful information for process design aimed at obtaining bee pollen carotenoid-rich extracts with potential as natural food pigments and bioactive ingredients.

2. Materials and methods

2.1 Bee pollen

Bee pollen was collected from an apiary in the geographic region of the Colombian high Andean forest (2.800 y 3.200 meters above sea level) in Boyacá. It was subjected to a drying and cleaning process according to Durán-Jiménez (2014). Commercial sunflower oil from local supermarket was used as the extraction solvent.

2.2 Extraction process

In a first set of experiments, extractions were made by continuous stirring sunflower oil with bee pollen at ratios between 5 and 60 %wt [g pollen/100 g mixture (pollen + oil)] during 24 h at room temperature (17-23°C), in the dark. Stirring was made at 500 rpm for all experiments, which were carried out in triplicate. In a second set of experiments, the three ratios with the highest carotenoid content (40 %wt, 50 %wt and 60 %wt) were selected, and extraction was performed and monitored during 16 days for the kinetic study.

2.3 Carotenoid determination

Total carotenoids were determined spectrophotometrically with a UV-Vis spectrophotometer (Thermo Fisher Scientific, USA). A known weigh of β-carotene standard (Sigma Aldrich, Germany) was added in a known volume of sunflower oil (Ordóñez-Santos, Pinzón-Zarate, and González-Salcedo 2015). From this solution, six aliquots were taken to build-up a calibration curve by measuring the absorbance of each dilution at 463 nm against sunflower oil as blank, and plotting it vs. the β-carotene concentration (µg β-carotene/ g oil).
Total carotenoid content in sunflower oil extracts was calculated using the equation:

$$Carotenoid\ concentration\ in\ oil\ \left(\frac{mg\ \beta-carotene_{eq}}{kg\ oil}\right)=\left[\frac{\mu g\ \beta-carotene_{eq}}{g\ oil}\right]*DF \quad (1)$$

where DF is the dilution factor. The carotenoid yield was calculated using the equation:

$$Yield\ (\%)=\frac{EC}{PC}*100 \quad (2)$$

where EC (mg β-carotene$_{eq}$/kg pollen) corresponds to the carotenoids extracted from the pollen, determined dividing the carotenoid concentration in oil (1) by the amount of pollen used for the extract (kg), and PC (741 ± 33.5 mg β-carotene$_{eq}$/ kg pollen) is the concentration of carotenoids in the pollen used, i.e., the maximum amount of extractable carotenoids, determined spectrophotometrically after successive extractions with acetone, following the method described by Salazar-González et al. (2013).

2.4 Kinetics

There are many models to adjust kinetic data in extraction processes. In this work, a second-order model was chosen, represented in the following equation:

$$C_t = \frac{k * t * C_e^2}{1 + k * t * C_e} \quad (3)$$

Where C_t is the carotenoid content at time t in mg β-carotene/kg oil, k is the second-order extraction rate constant (kg oil/g*min), and C_e is the extraction capacity (concentration of carotenoids at saturation in g/kg oil) or the equilibrium concentration in the liquid extract.

2.5 Statistical analysis

Descriptive statistics using Excel® software was used for the analysis of the first part. For the second part, the effect significance of both matrix-solvent ratio and extraction time, was evaluated using an analysis of variance - ANOVA and Tukey tests (95%confidence level) with Matlab® software.

3. Results and Discussion

3.1 Effect of matrix-solvent ratio

Extraction experiments of carotenoids using sunflower oil were carried out varying the amount of bee pollen used 5 to 60%wt in the extraction mixture. Average of total carotenoids present in the extracts and extraction yields after 24 h, with their standard deviations, are shown in Figure 1.

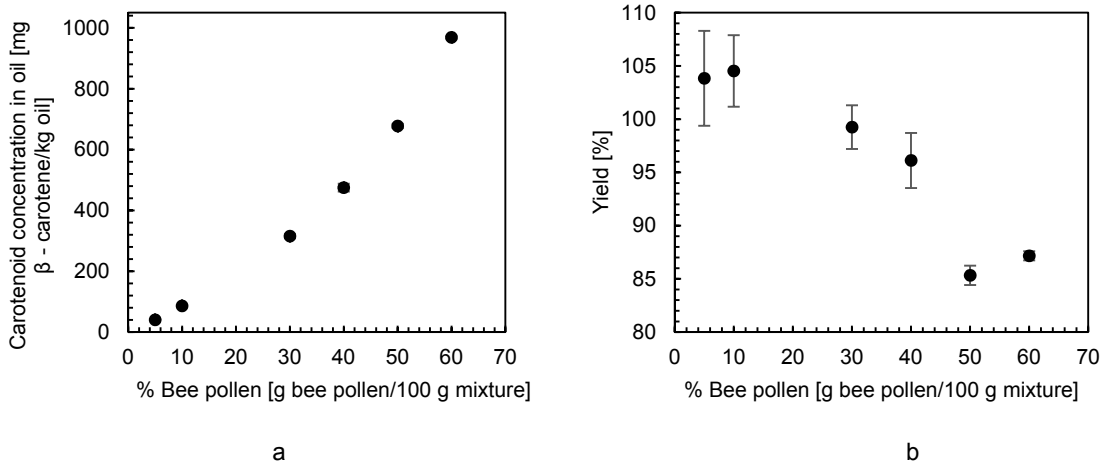

Figure 1: a) Carotenoid total content in extracts after 24 h using different bee pollen:oil ratios. b) Carotenoid extraction yield in extracts after 24 h using different bee pollen:oil ratios.

Vegetable oils are considered as green solvents in extraction processes of food-relevant compounds due to their non-toxicity, the fact that they produce non-denatured extracts with minimum contamination, and their ability to protect them against oxidative degradation (Goula et al. 2017). Depending on their composition and fatty acid profile, their consumption might even represent benefits to human health, and their status of food ingredients eliminates the need of a separation step. Nonetheless, such absence of separation processes, means that, for oil extracts of fat-soluble compounds to be useful as food products, ingredients or additives, they need to have higher concentrations of the extracted compounds.

The results presented in Figure 1 show that, after 24 h, higher pollen concentrations allow for carotenoid-richer extracts, but with significantly lower extraction yields. The increasing trend of carotenoid concentration in the extracts with respect to the matrix concentration indicates that pollen carotenoids are not near to the saturation point in the sunflower oil, even at the highest proportion evaluated. On the other hand, the decreasing trend of the extraction yield with respect to the matrix concentration, which varied from nearly 100% at the lower ratios to 85-87% at the higher ratios, is a typical behaviour of diffusion-like extractions of compounds with limited solubility. It is due to the fact that a greater solute:solvent ratio generates a greater concentration gradient (greater mass transfer driving force) in the more diluted mixtures during the migration of the compounds from the solid matrix into the solvent bulk and, therefore, a faster diffusion rate of

carotenoids in the extract (Goula et al. 2017). This effect will be more easily noticeable in high-viscosity solvents, as in the case of edible oils at low temperatures.

Overall, these results demonstrate that sunflower oil, despite its high viscosity and the limited solubility of some carotenoids, in particular xanthophylls (Borel et al., 1996) in trilinoleins, is suitable for carotenoid extractions from pollen, even at room temperatures. This is in agreement with previous reports on pomegranate wastes (Goula et al. 2017), carrot (Li et al. 2013) and chontaduro (Ordóñez-Santos, Pinzón-Zarate and González-Salcedo 2015). Indeed, for the highest ratio evaluated (60 %wt), carotenoid concentration in extract was 968.8 ± 4.2 mg β-carotene/kg oil, which is almost three times as high as that that reached by Li et al. (2013) in extractions of carotenoids from carrot using sunflower oil (363.9 mg β-carotene/kg oil), two orders of magnitude higher than the highest concentration reached by Goula et al. (2017) in extractions of carotenoid from pomegranate wastes using sunflower oil (1.35 mg β-carotene/kg oil), and twice as high as the highest concentration reached by Ordóñez-Santos, Pinzón-Zarate and González-Salcedo (2015) in extractions of carotenoids from chontaduro peels using sunflower oil (443 mg β-carotene/kg oil). However, higher matrix-solvent ratios and/or longer extraction times are required for obtaining carotenoids-richer extracts with better extraction yields.

3.2 Extraction kinetics over longer periods

Extraction processes were followed during 16 days at the three ratios that presented higher content of carotenoids, namely 40 %wt, 50 %wt and 60 %wt. Results are shown in Table 1. As expected, longer contact time between the matrix (bee pollen) and the solvent (oil) allowed for a greater interaction between the particles and hence, a higher concentration of carotenoids in the extracts (Mahfoudhi et al. 2015). The extraction kinetic curves at the three matrix-solvent ratios presented the same form, with three distinguishable regions in the time-increase carotenoid concentration: linear increase, deceleration and plateau (Figure 2), which is typical of diffusion-like processes. This can be attributed to the fact that the extraction process has two stages: the first stage, characterized by a rapid migration, involves the penetration of the solvent into the cellular structure followed by the dissolution of the soluble constituents in the solvent; while the second stage involves the external diffusion of the soluble constituents through the porous structure of the residual solids and their transfer from the solution in contact with the particles to bulk solution (Goula et al. 2017). Greater contact times will allow the second extraction stage to be carried out, whereby greater quantities of compounds will be present in the final extraction solution. The plateau value was reached between days 8 and 12, regardless the matrix-solvent ratio. This means that at these pollen-oil ratios, the concentration of carotenoids in the extracts increased significantly along the extraction time only until day 12, with higher concentrations for higher matrix-solvent ratios.

Table 1: Total carotenoid content in extracts along 16 days of extraction.

Time (days)	40 %wt	50 %wt	60 %wt	40 %wt	50 %wt	60 %wt
	mg β-carotene/kg oil			mg β-carotene/kg bee pollen		
1	416.2 ± 4.1	601.6 ± 12.1	857.2 ± 17.5	624.1 ± 6.2	601.6 ± 12.2	571.5 ± 11.7
4	437.4 ± 3.6	653.5 ± 10.2	930.9 ± 8.7	656.0 ± 5.3	653.3 ± 10.2	620.7 ± 5.9
8	450.2 ± 12.4	663.8 ± 3.5	975.2 ± 19.3	675.1 ± 18.5	663.7 ± 3.4	650.2 ± 12.9
12	471.7 ± 10.2	691.4 ± 17.7	1010.8 ± 11.6	707.4 ± 15.2	691.3± 17.6	673.9 ± 7.6
16	472.1 ± 14.1	669.0 ± 22.3	1012.1 ± 4.5	707.9 ± 21.1	668.9 ± 22.3	674.9 ± 3.0

Although the yield significantly improved with respect to the 24 h-extractions, in no case it reached 100%, being 95%, 93% and 91%, for 40 %wt, 50 %wt and 60 %wt matrix-solvent ratios, respectively, at day 12. This can be due to a partial loss of carotenoids due to oxidative reactions and other degradation processes, which become more significant over longer periods at room temperature (Borel et al., 1996). In fact, between days 12 and 16 there was a slight overall decrease in carotenoid concentration.

The kinetic behaviour of the carotenoid concentration of the extracts adjusted well to a second-order model (equation 3). The good agreement between experimental and predicted kinetic curves is illustrated in Figure 2, for the three ratios evaluated.

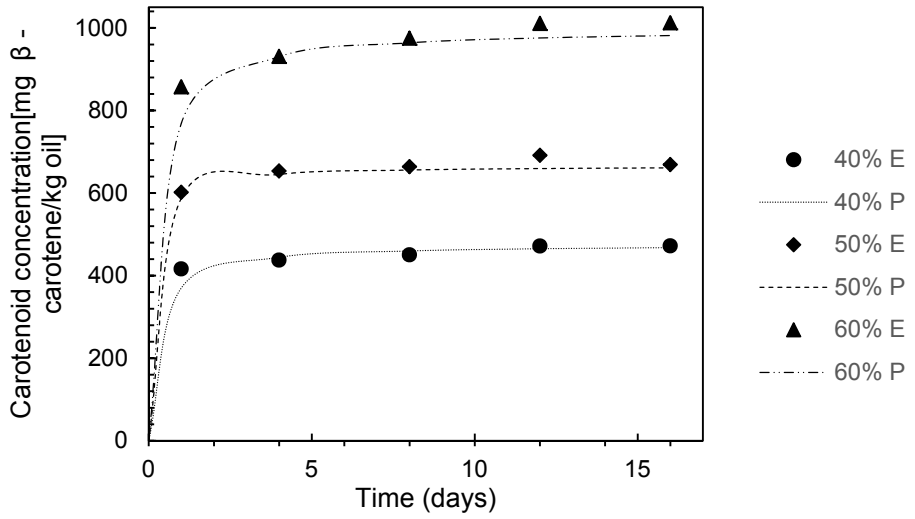

Figure 2: Kinetics and carotenoid contents in extracts along 16 days, at different pollen bee pollen:oil ratios (40%wt; 50%wt; 60%wt). E: Experimental data. P: Predicted data.

Table 2 shows the parameters found for the second-order model and the correlation coefficient for each ratio tested. The constant rate (*k*) in the second order model allows to see the extraction speed, therefore, a greater constant will indicate that the extraction is faster, which favors the operating costs (Kusuma and Mahfud 2016). In this case, the proportion that has the highest constant was 50%. Regarding the extraction capacity, this allows to see the maximum concentration of carotenoids that would allow extracting the process (Kusuma and Mahfud 2016). In this extraction process, the proportion of 60% is the one with the highest carotenoid content. For the selection in a process both factors should be taken into account, the constant rate and the extraction capacity; in the case of a carotenoid-rich extract, it is desired that has the highest amount of carotenoid compounds. For this reason, the 60% proportion is chosen, despite the fact that the extraction rate is the slowest among the three ratios.

Table 2: Second-order parameters

Ratio	**Ce (mg β-carotene/kg oil)**	**k (kg oil/(mg β-carotene*min))**	**h (mg β-carotene/(kg oil*min))**	**R^2**
40 %wt	476.19	0.0074	1666.67	0.9994
50 %wt	666.67	0.0113	5000.00	0.9991
60 %wt	1000.00	0.0033	3333.33	0.9996

4. Conclusion

Continuous stirring for carotenoid extraction from bee pollen allows for obtaining carotenoid-rich extracts. Varying bee pollen:oil ratio, extract concentrations varied between 40.5 ± 1.7 mg β-carotene/kg oil (for 5%wt) and 968.8 ± 4.2 mg β-carotene/kg oil (for 60%wt). Higher matrix-solvent ratios allow for obtaining extracts with better extraction yields. The study of extraction process along time allow to conclude that longer extraction times were required for obtaining carotenoids-richer extracts because at day 12, the total carotenoid content was 471.7 ± 10.1, 691.4 ± 17.7 and 1010.8 ± 11.6 mg β-carotene/kg oil, for 40%wt, 50%wt, and 60%wt; those values are greater than the ones obtained in the first part of the work. The best conditions for a better extract quality and maximum extraction yield were 60 %wt of bee pollen: oil ratio and 12 days of extraction. Second-order model was an accurate model to adjust kinetic data for the extraction process of carotenoids from bee pollen. The results of this study evidence the industrial feasibility of using bee pollen as a raw material for obtaining carotenoid-rich extracts, with potential both as lipophilic natural food colorants and bioactive ingredients, as anticipated by previous characterization studies. Moreover, this is the first attempt to obtain a natural colorant with antioxidant activity. To improve the extraction conditions, studies through assisted technologies such as ultrasound or microwaves are advisable.

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