

Article

Fractionation of Marigold Waxy Extract Using Supercritical CO₂

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Abstract: Marigold oil is a product of great industrial interest thanks to its wide range of medicinal and wound-healing properties. In this work, supercritical carbon dioxide was used to recover marigold essential oil from the hexane solvent extract of marigold flowers, the floral “concrete”. This starting material was mixed with synthetic paraffinic waxes to heighten its melting point and viscosity, thus, improving material processability. Supercritical fluid extraction and fractionation of the modified marigold “concrete” was carried out, and the effect of pressure and CO₂ mass flow rate was studied. The pressure was varied from 80 to 180 bar, keeping the temperature constant at 40 °C: the higher the pressure, the larger the CO₂ solvent power and extraction yield (up to 9.40% w/w). Nevertheless, the optimum between productivity and process selectivity was found at 100 bar. By changing the CO₂ mass flow rate (from 1.20 to 1.50 kg/h), we noted that mass transfer resistance was located externally. GC-MS analysis showed that the most abundant compounds in the oil were δ-cadinene (25%), γ-cadinene (16%), τ-muurolol (6.5%), and α-muurolene (6%). Moreover, the traces of oil and waxes showed no mutual contamination between lighter species and waxes, meaning that the fractionation step was successful.

Keywords: *Calendula officinalis* L.; fragrance oil; paraffins; supercritical fluid extraction; selectivity; mass transfer



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1. Introduction

Calendula officinalis L. (marigold) is a plant that grows in Europe, China, and India, and its properties have been the subject of several studies, especially in the field of pharmaceuticals and cosmetics. Marigold contains various families of compounds, such as volatile oil, coumarins, terpenoids, quinones, and flavonoids [1]. Most of these compounds are associated with medicinal and wound healing properties, immune-stimulants, and spasmolytic, anti-inflammatory, anti-edematous, and antioxidant activity [2–6]. For instance, marigold extracts have been incorporated into bio-composite scaffolds, showing remarkable antibacterial activity in view of tissue engineering applications [7]. Moreover, some constituents of *Calendula officinalis* L. exhibit anti-cancer and anti-viral activities [8]. Marigold extracts can protect target cells against HIV [9] and are active against several tumoral cell lines [10].

Several studies are available on the extraction of compounds of industrial interest from marigold flowers. Ak et al. [11] obtained marigold essential oil (EO) via hydrodistillation; they analyzed the product obtained from both dried flowers and leaves. The most abundant compound in both flower and leaf EOs was α-cadinol (respectively, 31.3% and 32.3%). On the other hand, only one monoterpene was detected (i.e., α-pinene); it was contained in up to 3.8% of flower EO and was completely absent in the product obtained from leaves. These results indicate that, differently from many other vegetable materials, *Calendula officinalis* L. contains small amounts of monoterpenes and oxygenated terpenes. High-molecular-weight compounds (e.g., pentacosane, heptacosane, and phytol) were detected in the product; therefore, the extraction was not selective for fragrance species. Furthermore, homogenizer-assisted (HAE), maceration (MAC), Soxhlet (SOX), and ultrasound-assisted (UAE) extraction were used to recover compounds of interest from marigold flowers,

leaves, and roots [12]. Methanol was used as the extraction solvent; however, this is a toxic substance that can compromise the final utilization of the extracts. Preethi et al. [13] obtained an ethanolic extract from *Calendula* flower tops after an extraction time of two weeks; these authors evaporated the mixture to recover 1.1 g of extract from 700 g of starting material. Fonseca et al. [14] also attempted to obtain a marigold ethanolic extract. In this case, the extraction time was 5 days; the material obtained from the maceration was resuspended in a hydroalcoholic solution and stored at $-20\text{ }^{\circ}\text{C}$ to avoid problems in the extract stability. Marigold extracts were also obtained using other organic solvents, such as *n*-hexane, dichloromethane, ethyl acetate, and *n*-butanol [15].

Supercritical fluid extraction (SFE) is an eco-friendly, flexible, and selective extraction technique. Due to the modularity of solvent power and selectivity [16], supercritical carbon dioxide (SC-CO₂) has been successfully used in the extraction of several vegetable matrixes [17–19]. Generally speaking, SFE can represent a valid alternative to conventional extraction techniques since it can overcome the problems of low selectivity and organic solvent elimination [20]; however, it suffers from the problem of wax co-extraction even at low solvent densities.

SC-CO₂ extraction has also been attempted on marigold flowers. Danielski et al. [21] explored the effect of temperature and pressure on the extraction yield. However, the higher the CO₂ density, the higher the extraction yield and the lower the extraction selectivity. It is noteworthy that, even if an extraction yield of 17% was reached, marigold extracts contained a low amount of sesquiterpenes (i.e., acetyl eugenol), whereas higher-molecular weight compounds were abundant (i.e., hexacosane, tetracosane, etc.). The product collected was, thus, a so-called “oleoresin”. Campos et al. [22] performed a CO₂-assisted extraction on marigold flowers, using pressures ranging from 120 bar to 200 bar and temperatures from 26 to 40 °C. The obtained extract was, also in this case, an oleoresin. However, some of the used operating conditions did not assure supercritical conditions of the solvent; but liquid CO₂ was used.

To isolate the fragrance from waxes, inevitably co-extracted during the SFE process, a double-step separation system can be adopted [23]. The low solubility of paraffinic waxes in SC-CO₂ at low temperatures (i.e., around 0 °C) turns out to be the solution to avoid contamination of the fragrance oil. Indeed, using a separator in which the overall extract is cooled down (at least) to 0 °C, paraffins precipitate; then, SC-CO₂ and terpenic extract flow to a second separator (operated at lower pressures), where CO₂ returns to the low-density gaseous state, and the volatile oil can be collected. This process arrangement has been successfully used to fractionate fragrance oils from waxes during the extraction of vegetable materials [24,25]. Even though *Calendula officinalis* L. products were extracted using SC-CO₂, to the best of our knowledge, only one attempt of fractionation was carried out [26]. The marigold extract obtained in that case still contained higher molecular weight compounds, such as methyl linoleate and methyl eicosanoate.

A possible industrial improvement of supercritical technology applied to vegetable matter (flowers, leaves, roots, seeds, etc.) can be used to process a semi-finished product, the so-called “concrete”, instead of the raw vegetable matter. Indeed, fresh vegetable matter needs to be stored at low temperatures to preserve the stability of target compounds; moreover, it is bound to the seasonality of crops. In addition, fresh vegetable matter requires very large process and storage volumes. Consequently, high capital costs are involved for small quantities of extract. The solution for these problems can be the utilization of a vegetable “concrete” as the starting material; it is obtained by hexane extraction of the flowers and subsequent solvent elimination. The product obtained is a waxy material that contains the compounds of interest in a concentrated form. To summarize, by coupling supercritical technology with the intrinsic advantages of using a floral “concrete”, high-quality extract and very small process volumes can be obtained. This means reduced capital and operating costs and higher productivity. Some attempts to perform supercritical extraction and fractionation of other “concretes” are reported in the literature [27–29].

As a result of the literature analysis, in this work, the processability of a marigold “concrete” was assessed for the first time. The effect of the variation of the operating conditions was studied (i.e., pressure and CO₂ flow rate) to understand thermodynamic limits and mass transfer mechanisms controlling the process. The extract composition was analyzed. Moreover, the attention was focused on the effect of pressure on the wax’s chemical profile to confirm the connection between CO₂ density, its solvent power, and the kind of molecules co-extracted.

2. Materials and Methods

2.1. Materials

Marigold dry extract obtained by hexane extraction followed by solvent elimination (“concrete”) was purchased from Bulgarian Rose (Karlovo). Prior to extraction experiments, it was mixed with glass beads (3 mm diameter). The relative amounts of “concrete” and glass beads were obtained, fixing the “concrete”/spheres ratio at 20% *v/v*. It resulted that 22 g of “concrete” were loaded into the extraction vessel, together with 334 g of glass beads. Synthetic paraffin waxes (PW) were supplied by Sigma Aldrich (CAS 8002-74-2). CO₂ was purchased from Morlando Group Srl (Sant’Antimo, NA, Italy).

2.2. Supercritical Fluid Extraction

Supercritical fluid extraction was carried out in a laboratory-scale extraction and fractionation plant, which is reported elsewhere [30]. The extraction vessel (300 cm³) was set up to the desired temperature using heating bands. Cooling of the first separator was carried out using a thermostatic bath (Julabo, mod. F38-EH, Seelbach, Germany) loaded with polyethylene glycol. Temperature in the first separator was set at 0 °C for all the experiments. The second separator was set at 35 bar and 30 °C, to favor the release of lighter compounds from CO₂. A membrane high-pressure pump (Lewa, mod. LDB1 M210S, Rho (MI), Italy) was used to pump the liquid CO₂ through the plant lines to reach the desired pressure in the extraction vessel. A thermostatic oven was located immediately after the high-pressure pump to guarantee CO₂ heating and the transition to supercritical conditions. Waxes precipitated in the first separator were collected at the end of the extraction experiment, whereas the fragrance oil was collected from the second separator at specific time intervals during the experiment. Fragrance was weighed, and extraction yield (g extract/g “concrete”) was calculated. CO₂ flow rate was monitored continuously using a calibrated rotameter (ASA, mod. N.5-2500, Serval 11502, Sesto San Giovanni (MI), Italy) located after the second separator. CO₂ flow rate was monitored by associating the rotameter plug to a specific value of mass flow rate through a calibration curve. This calibration curve was obtained by knowing how much CO₂ exited the line in a fixed time interval; in this way, it was possible to have an association between rotameter plug position and CO₂ flow rate. Pressure and temperature along the extraction plant were checked continuously by test gauges and thermocouples, respectively. Temperature was controlled using PID controllers (Watlow, mod. 93). Pressure in the extraction vessel was controlled manually using a micro-metering valve (Milli-Mite 1300 Series HOKE, CV 0.010 in); pressure in the second separator was controlled manually using a back-pressure valve (Tescom, mod. 23-1700). Every extraction experiment was performed twice.

2.3. GC-MS Analysis

The collected products were characterized by gas chromatography–mass spectroscopy (GC-MS) using a Varian 3900 apparatus (Varian, Inc., San Fernando, CA, USA) equipped with a fused-silica capillary column (mod. DB-5, J & W, Folsom, CA, USA). The capillary column was 30 m long with a 0.25 mm internal diameter and a 0.25 µm film thickness. It was connected to a Varian Saturn Detector 2100T (Varian, Inc., San Fernando, CA, USA). Helium was used as the carrier gas, fixing the flow rate at 2 mL/min. The temperature in the column was set at 40 °C, and kept constant for 6 min. After, it was heated up to 270 °C, with a ramp of 2 °C/min. Once the temperature reached the value of 270 °C, it was held

for 10 min. The injection was performed using 1 μL of a 1:10 *n*-hexane solution in split mode. The injector was set at a temperature of 280 $^{\circ}\text{C}$. MS used an ionization voltage of 70 eV in the range of 40–650 a.m.u. at a scanning speed of 5 scans/s. The detected species were identified using NIST databook. A semi-quantitative analysis was carried out; the relative abundance of the compounds was calculated as the percentage area under the chromatographic peaks. Every analysis was performed at least twice.

3. Results

The first step of the work consisted of the analysis of the starting material. The marigold “concrete” was diluted in *n*-hexane and analyzed by GC-MS. Figure 1 reports the result of this analysis. The GC-MS trace of the “concrete” was complex. However, three major families of compounds can be evidenced, namely: monoterpenes and oxygenated monoterpenes, sesquiterpenes and oxygenated sesquiterpenes, and high-molecular-weight compounds, such as paraffines and long-chain alcohols. Overall, GC-MS trace highlighted that marigold “concrete” contained very low amounts of lighter compounds (i.e., C_{10} , terpenes, and oxygenated terpenes). This observation is coherent with some results of the scientific literature, according to which *Calendula officinalis* L. contains relatively low quantities of monoterpenes, whereas it is richer in sesquiterpenes [11,26], differently from many other flowers [31–36]. Looking at this result, an ideal fractionation should be performed by collecting all terpenic compounds in the second separator (a + b families in Figure 1) and all paraffinic compounds in the first one (c family in Figure 1).

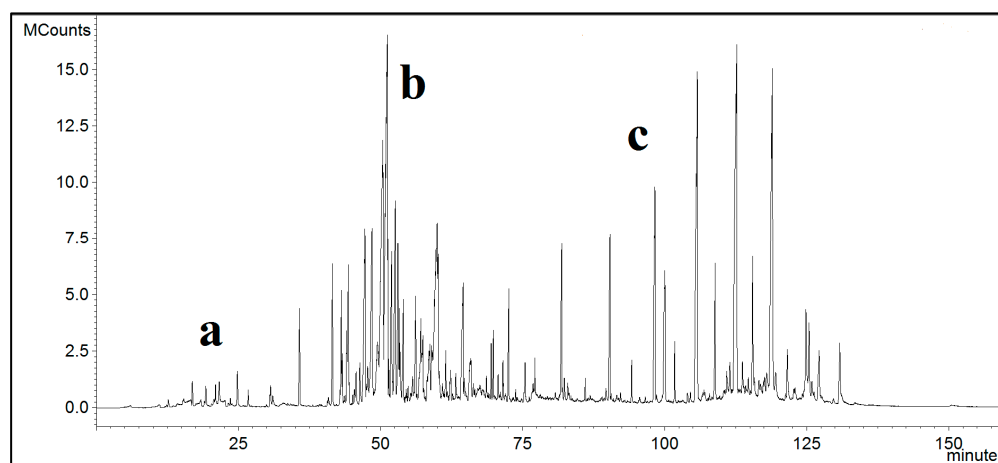


Figure 1. GC-MS trace of the starting marigold “concrete”: (a) monoterpenes and oxygenated monoterpenes; (b) sesquiterpenes and oxygenated sesquiterpenes; (c) waxes.

When the vegetable matrix was processed, the choice of SFE operating conditions was influenced by the internal structure of the material. When working with “concretes”, it is not necessary to overcome large internal mass transfer resistances. Therefore, since “concretes” are semi-solid materials, previous papers in the literature [27–29] mentioned that they have to be mixed with packing materials to avoid channeling and caking phenomena. Consequently, in this work, 3 mm glass beads were chosen and mixed with the starting vegetable matrix since spheres with a larger diameter produced a decrease in the exposed area, and smaller beads produced a larger thickness of the active material on the beads, possibly causing larger internal mass transfer resistances. Moreover, the material could pack inside the extraction vessel, and the extraction became less effective since SC-CO_2 could not diffuse into the semi-solid material. Other types of packings (e.g., Raschig rings, Berl saddles) were also discarded since the deposition of the “concrete” on their internal surfaces would produce a non-homogenous distribution of the waxy material.

A sample of the purchased marigold “concrete” as it was, is reported in Figure 2. It showed a cream-like consistency, and no heating was necessary to mix the material with glass spheres.

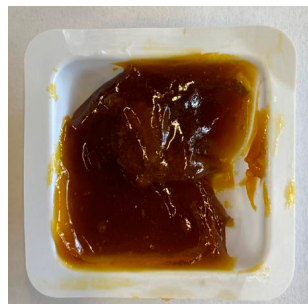


Figure 2. Sample of marigold “concrete”.

A first set of experiments was carried out on the marigold “concrete” plus 3 mm glass beads, operating at 80 bar and 40 °C ($\rho_{\text{CO}_2} = 0.28 \text{ g/cm}^3$), to assess the feasibility of the process. The CO_2 mass flow rate was set at 1.20 kg/h. The extraction process was partly unsuccessful: when the extraction vessel was opened at the end of the process, part of the material was still in the vessel and moved irregularly inside the whole volume. The corresponding yield was very low, as shown in Figure 3.

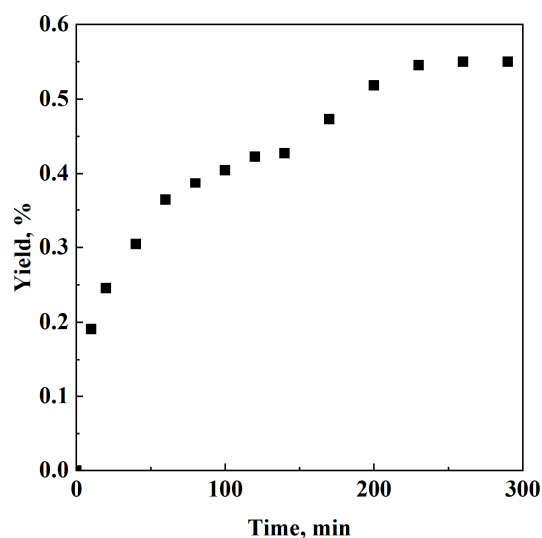


Figure 3. Yield vs. time experimental points of the marigold concrete working at 80 bar and 40 °C.

The yield vs. time experimental points were not particularly coherent with the typical exponential shape of the extraction curves [20,24]. Moreover, the extraction vessel, when opened at the end of the process, confirmed that the solid material melted during the process and moved down in the apparatus. In Figure 4, an image of the bottom of the vessel is reported. Marigold “concrete” was evidently not stable at the operating conditions adopted, and the resulting contact surface between the material and the extraction fluid was irregular. In conclusion, marigold “concrete” was not processable as it was—its consistency and viscosity needed to be adjusted.

To resolve this problem, the viscosity of the starting material was increased. In particular, high molecular weight paraffins were added to the marigold “concrete” and had less solubility in SC-CO_2 at the selected operating conditions and a larger melting temperature that could avoid the previously encountered processing problems. It was relevant to add compounds that were chemically similar to the starting vegetable material as they increased the waxy-like consistency and could not interfere with the overall characteristics of the

marigold “concrete”. Therefore, in a second series of experiments, marigold “concrete” was preliminary mixed with 3 g of melted synthetic paraffin wax, with a molecular weight of up to 520 g/mol. In this way, a higher melting point of the “concrete” could be obtained, allowing the processability at supercritical conditions.

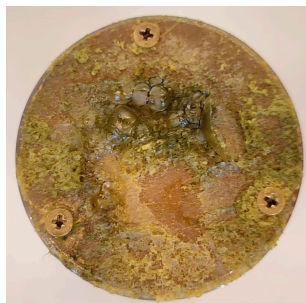


Figure 4. Bottom of the extraction vessel, after working at 80 bar and 40 °C.

As hypothesized, the addition of paraffinic waxes stabilized the “concrete”—they did not melt during the process. Therefore, other extraction experiments were carried out using the same methods previously described, and four pressure values were tested (namely, 80, 90, 100, and 180 bar), keeping the temperature constant at 40 °C. The CO₂ mass flow rate was set at 1.20 kg/h.

Figure 5 reports the collection of extraction yield vs. time experimental points (each related to a different operating pressure) obtained by processing the marigold “concrete” mixed with synthetic paraffinic waxes.

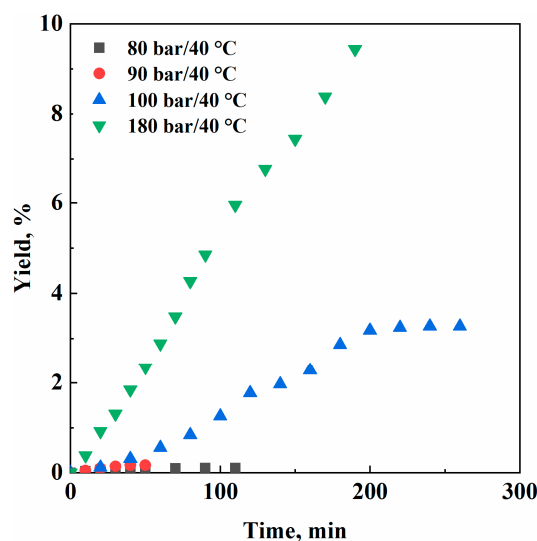


Figure 5. Yield vs. time experimental points at different pressures (80, 90, 100, 180 bar), with the addition of PW.

When the pressure increases, CO₂ density (and, consequently, its solvent power) increases as well. This consideration can have an immediate effect on the extraction yield value obtained at the end of the process, i.e., operating at 80 and 90 bar, extraction was substantially unsuccessful since CO₂ density was not sufficient to extract larger molecular weight compounds.

Table 1 shows that by operating at larger SC-CO₂ densities, extraction yield was largely modified. It is noteworthy that when pressure was low (i.e., 80 and 90 bar), the overall amount of fragrance oil collected was near zero. The explanation of this result lies in the fact that, operating at these conditions, only the monoterpene fraction was soluble in SC-CO₂,

whereas sesquiterpenes and oxygenated sesquiterpenes showed a reduced solubility in it [37,38].

Table 1. Pressure, SC-CO₂ density, and extraction yields, working at 40 °C and using a CO₂ flow rate of 1.20 kg/h.

Pressure (Bar)	SC-CO ₂ Density (g/cm ³)	Extraction Yield (%)
80	0.28	0.55
90	0.48	0.62
100	0.62	3.27
180	0.81	9.44

Keeping a constant temperature at 40 °C and CO₂ mass flow rate at 1.20 kg/h for every extraction experiment, the pressure was changed (namely, from 80 to 180 bar, as reported in Table 1). Marigold “concrete”, as aforementioned, contains small traces of monoterpenes; this explains the low extraction yield when working at 80 bar and 40 °C, and 90 bar and 40 °C. The extraction trend changed when SC-CO₂ density increased. Sesquiterpenes became much more soluble in the extraction fluid and were more effectively depleted from the starting material. When the pressure was set at 180 bar, again, the extraction yield was successful—much more oil was collected, up to about 9.50% by weight. However, operating at 180 bar, large quantities of undesired compounds (likely heavier than sesquiterpenoids) were co-extracted. Therefore, a pressure of 100 bar was selected as the best compromise between extraction yield and process selectivity.

Then, the CO₂ mass flow rate was changed (namely, values of 1.20 and 1.50 kg/h were tested, using 100 bar and 40 °C as extraction operating conditions) to understand the mass transfer phenomena driving the process. Fixing the SC-CO₂ density at 0.62 g/cm³, it can be noted from Figure 6 that the velocity at which the yield curve approached the asymptote largely depended on the CO₂ flow rate.

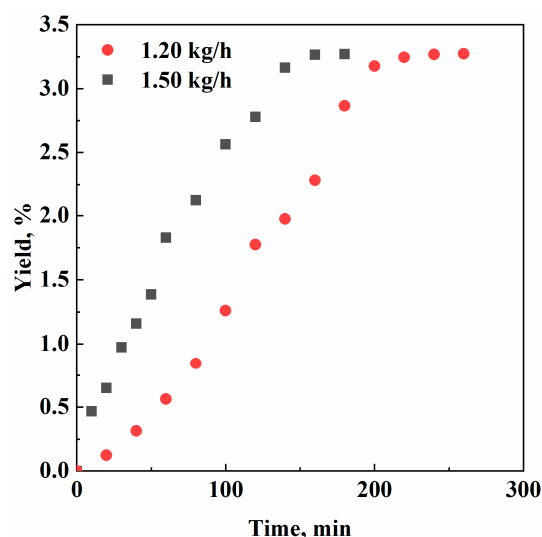


Figure 6. Yield vs. time experimental points at two different CO₂ mass flow rates (1.20 and 1.50 kg/h), working at 100 bar and 40 °C.

A smaller time was needed to reach the asymptotic yield value using larger CO₂ flow rates. This result means that the mass transfer resistance related to the extraction was mainly external; indeed, by fixing the ratio “concrete”/spheres at 20% *v/v*, the “concrete” shell thickness around the spheres was about 15 μm (as the result of a calculation reported elsewhere [39]). Therefore, internal mass transfer resistance can be substantially neglected. Information on the CO₂ flow rate is important for a possible scale-up of the process. Indeed, knowing the solvent mass flow rate and the extractor volume, it is possible to calculate

the CO₂ residence time in the vessel. The contact between the extraction fluid and the “concrete” determines the effectiveness of the extraction process; the larger the volumes, the larger the CO₂ flow rate to keep the residence time constant.

Once we collected the experimental points, a simplified mathematical model was applied to describe the process; a modified Chapman–Richards equation was used [40,41]. This equation was successfully used in a previous work [39] to fit the experimental points when CO₂ mass flow rate was changed. Once again, this equation showed how to correctly describe the overall trend of the yield vs. time experimental points, since values of R² were around 0.99 for both curves reported in Figure 7.

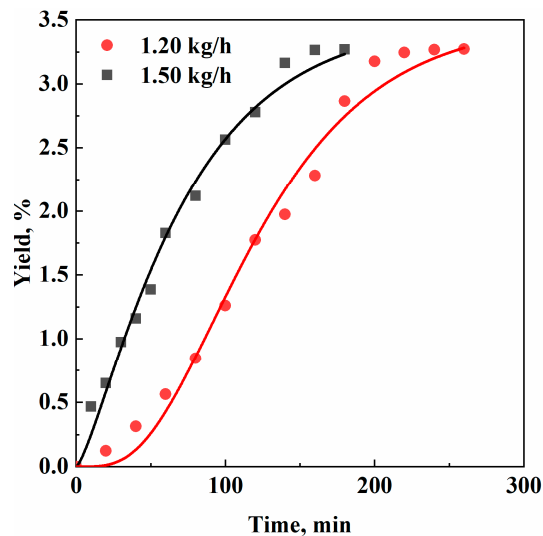


Figure 7. Yield vs. time experimental points fitted using the Chapman–Richards equation.

The second part of this work was dedicated to the analysis of the oil and waxes collected after the extraction experiments. The extract was analyzed by a GC-MS, following the procedure described in the Materials and Methods.

Figure 8 reports the GC-MS trace of fragrance oil collected in the second separator, whereas Figure 9 shows an example of the GC-MS trace of waxes recovered in the first separator after the extraction experiment. A superposition of Figures 8 and 9 shows that the separation of the volatile oil from the waxes was successful since the traces were well-separated according to the family of compounds.

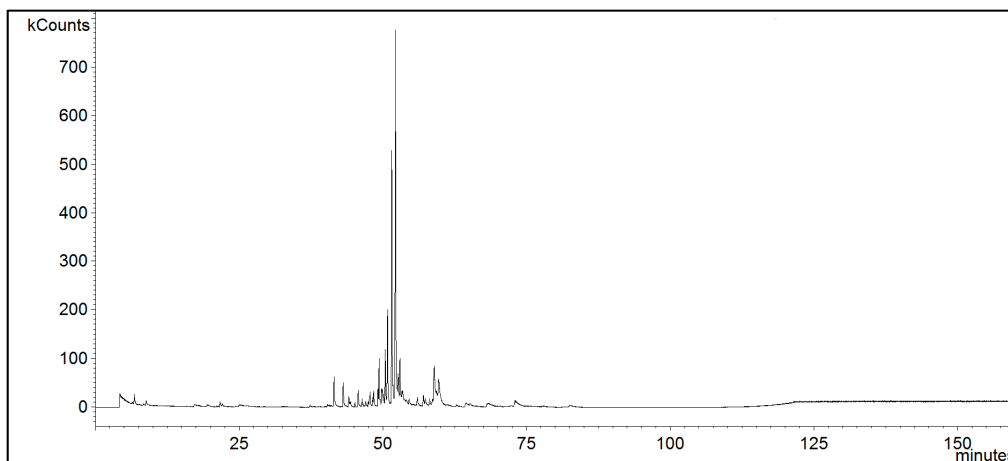


Figure 8. GC-MS trace of the oil collected during the experiment performed at 100 bar and 40 °C.

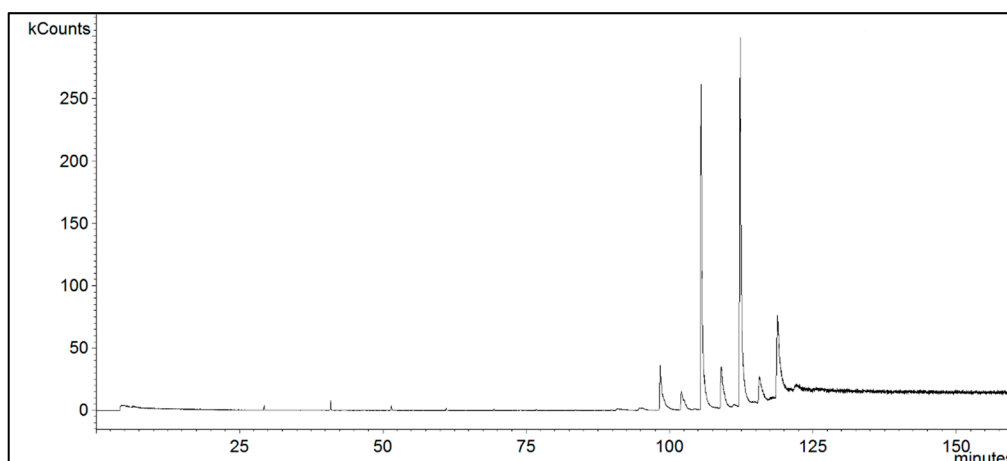


Figure 9. GC-MS trace of the waxes collected in the first separator after the experiment performed at 100 bar and 40 °C; CO₂ flow rate 1.20 kg/h. Paraffin waxes were added to the starting marigold “concrete”.

Table 2 reports the major compounds identified in the marigold oil and waxes (whose GC-MS trace is represented in Figures 8 and 9), collected after the experiment performed at 100 bar and 40 °C, using a CO₂ flow rate of 1.20 kg/h.

Table 2. GC-MS data related to marigold oil and waxes (Operating conditions 100 bar and 40 °C; CO₂ mass flow rate 1.20 kg/h).

Oil	Area, %
β-cubebene	1.5
α-gurjunene	1.5
β-caryophyllene	1
Aristolene	1.5
α-copaene	1
Germacrene	3
Cedrene	2
α-cubebene	4.5
α-murolene	6
γ-cadinene	16
δ-cadinene	25
Cadina-1,4-diene	7
α-cadinene	0.5
Nerolidol	3.5
Viridiflorol	1
Oplopenon	1
Cubenol	1
τ-cadinol	5.5
Waxes	Area, %
Nonadecane	5
Eicosane	1.5
Heneicosane	17
Docosane	2.5
Tricosane	30
Pentacosane	21
1-Hexacosanol	2
Heptacosane	10

Compounds with relative abundance lower than 0.5% are not reported. Identified compounds belong to the family of sesquiterpenes and oxygenated sesquiterpenes. The most abundant species were: δ-cadinene (25%), γ-cadinene (16%), τ-murolol (6.5%),

and α -muurolene (6%). This analysis confirmed the qualitative observation of Figure 8: volatile oil was not contaminated by high-molecular weight compounds (i.e., paraffins added to perform processing and paraffins belonging to the original marigold material). Therefore, the fractionation operated in the first separator allowed a very selective recovery of fragrance-related compounds.

The effect of the operating pressure on waxes composition was also studied. As an example, Table 2 reports also the semi-quantitative analysis of the waxes collected in the first separator, working at 100 bar and 40 °C, and using a CO₂ mass flow rate of 1.20 kg/h. Data reported in Table 2 evidence only waxes with a molecular weight up to around 380 g/mol. This is an indication of the fact that the addition of higher molecular weight paraffins did not interfere with the extraction process: thus, the synthetic waxes were not co-extracted; but they improved marigold material processability. It was also noted that, by increasing the operating pressure (and, therefore, the density of the supercritical fluid), an increase in the percentage of higher-molecular weight paraffins occurred; whereas, for low CO₂ densities, only low molecular weight compounds were collected in the first separator. Moreover, when CO₂ density increased, also more polar compounds were co-extracted (i.e., long-chain alcohols). This result confirms again that the higher was the CO₂ density, the higher was its solvent power and, thus, its capacity to co-extract molecules with a relatively low affinity with the supercritical fluid.

4. Conclusions

In this work, the processability of the marigold “concrete” was verified. In particular, for the first time, a viscosity increaser was added, to guarantee the treatment of the vegetable material. By increasing the operating pressure, the extraction yield increased; however, higher molecular weight compounds were also co-extracted.

Further studies could be needed to assess the effect of the “concrete” thickness around the glass beads, as mass transfer mechanisms and overall bed behavior. The results reported in this work are a further step to the full understanding of “concretes” processing.

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