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Fractional Separation and Characterization of Cuticular Waxes Extracted from Vegetable Matter Using Supercritical CO_2

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Abstract: Cuticular waxes can be used in high-value applications, including cosmetics, foods and nutraceuticals, among the others. The extraction process determines their quality and purity that are of particular interest when biocompatibility, biodegradability, flavor and fragrance are the main features required for the final formulations. This study demonstrated that supercritical fluid extraction coupled with fractional separation can represent a suitable alternative to isolate cuticular waxes from vegetable matter that preserve their natural properties and composition, without contamination of organic solvent residues. Operating in this way, cuticular waxes can be considered as a fingerprint of the vegetable matter, where C_{27}, C_{29} and C_{31} are the most abundant compounds that characterize the material; the differences are mainly due to their relative proportions and the presence of hydrocarbon compounds possessing other functional groups, such as alcohols, aldehydes or acids. Therefore, selectivity of supercritical fluid extraction towards non-polar or slightly polar compounds opens the way for a possible industrial approach to produce extracts that do not require further purification steps.

Keywords: cuticular waxes; extraction; fractional separation; supercritical CO_2; gas chromatography-mass spectroscopy

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1. Introduction

Cuticular waxes are compounds ubiquitously present on the surface of all kinds of vegetable matter. They cover leaves, flowers, seeds and other vegetable structures, exerting the main functions of (i) controlling the perspiration, (ii) insulating the plant from external water and (iii) protecting it from pathogens [1,2], biotic and abiotic stresses and plant-insect interaction [3–5]. A cuticular wax is a complex mixture of long-chain alkanes, alkenes, alcohols, aldehydes, alkyl esters, fatty acids and other compound families [2,4,5]; although the large majority is represented by long-chain hydrocarbons [2]. Depending on the plant species, the total amount and composition of cuticular waxes can vary widely [4,6]: i.e., every vegetable species (and even organs from the same vegetable) can exhibit a unique composition. Cuticular waxes are not only interesting from an analytical point of view; they can have industrial applications in the field of cosmetic formulations and healthcare products [7,8], since they show a very large affinity with human skin thanks to the prevalence of odd long-chain hydrocarbons with respect to the analogous products coming from fossil feedstocks [8,9].

The current methods for extracting natural waxes from vegetable matter use large quantities of toxic organic solvents [10]. Guo and Jetter [11] studied cuticular waxes coming from potato leaves and other potato organs, after extraction using chloroform; the samples were extracted twice for 30 s. The same procedure was adopted by Jetter et al. [12] to process *Prunus laurocerasus* L. leaves. Cheng et al. [1] extracted cuticular waxes from rose petals and leaves using chloroform as the extraction solvent, in which the samples were immersed three times for 30 s. Trivedi et al. [2] used the same organic solvent to extract cuticular waxes from bilberry fruits; the immersion was 1 min long. Pimentel et al. [13]

processed *Croton* leaves using three consecutive immersions of 30, 20 and 10 s duration in dichloromethane. They systematically identified C_{19} to C_{33} alkanes and C_{18} to C_{34} alcohols.

Therefore, the extraction of cuticular waxes is carried out, as a rule, by liquid solvent extraction and chloroform is the most frequently used solvent. Moreover, the process is performed in a very fast manner to minimize the co-extraction of other undesired compounds [14]. However, when other extraction techniques are used, as in the case of Soxhlet method that can last some hours, other compounds and intracuticular waxes can be also extracted and the authors generally do not give indications about these co-extracts. In particular, cuticular waxes represent interfering compounds that are extracted together with the desired ones, since the target compounds generally have a biological/industrial interest, such as essential oil, coloring matter, antioxidants and active principles for pharmaceutical applications [8]. However, they are systematically co-extracted during solvent extraction, as previously discussed [15], and during alternative processes [16], like ultrasound assisted extraction (UAE), microwave assisted extraction (MAE), pressurized liquid extraction (PLE) and supercritical fluid extraction (SFE). Therefore, they have to be eliminated by post-processing procedures, such as the so-called winterization [17], in which the extract, dissolved in the organic solvent, is cooled at very low temperatures (e.g., from −10 °C to −40 °C) for several hours to precipitate cuticular waxes that are subsequently separated by filtration [18].

CO_2 at supercritical conditions (SC-CO_2) is largely used to extract the compounds of interest from vegetable matter. In particular, above its critical point (P_c = 73.8 bar and T_c = 31 °C), CO_2 shows a liquid-like density and a gas-like diffusivity that favor the extraction of chemically affine compounds from solid matrices. Several studies [19–24] reported in the scientific literature describe the main advantages of using this green technique instead of the traditional ones, such as lower operative temperatures and higher selectivity. Moreover, post-processing steps, adopted to purify the extracts from cuticular waxes, are not required when winterization is performed in series to the extraction process in the same operative plant. In particular, Reverchon and co-workers demonstrated in several papers [25–27] that, by using SC-CO_2 extraction coupled with high-pressure fractional separation, it is possible to separate cuticular waxes during the extraction process by the selective precipitation from the overall extract [28]. Specifically speaking, the compounds of interest for extraction are generally located well inside the vegetable structure; whereas cuticular waxes are located on the surface of the vegetable material and show a non-negligible solubility in SC-CO_2 [28]. For this reason, they are inevitably co-extracted at all SC-CO_2 processing conditions due to the overlap of mass transfer limitations and thermodynamic (solubility) limits [28]. However, since they are generally considered as an interfering matter that reduces the quality (purity) of the extracts, a procedure has been developed that allows the selective separation of cuticular waxes from the extract by cooling the mixture CO_2 plus overall extract at the exit of the extractor down to temperatures lower than 0 °C [25–27,29,30]. Operating at these process conditions, the solubility of cuticular waxes reduces to near zero in SC-CO_2 and, therefore, they can be precipitated in a separator before the final collection of the extract of interest.

Therefore, according to the previous discussion of the literature, the scope of this work is to attempt, for the first time, a systematic analysis of cuticular waxes extracted by SC-CO_2 plus fractional separation from several vegetables. After performing the specific SC-CO_2 extraction processes, several high-resolution gas chromatography-mass spectroscopy (GC-MS) identifications are carried out on cuticular waxes obtained from more than ten different vegetable species, to analyze their composition and dependence on the vegetable tested, and to show that their composition can be specific for the different vegetable species and tissue analyzed.

2. Materials and Methods

2.1. Materials

Basil leaves (*Ocimum basilicum* L.), cannabis inflorescence (*Cannabis sativa* L.), chamomile flower heads (*Chamomilla recutita* L. Rausch.), clove buds (*Eugenia caryophyllata* Thun.), ginger rhizomes (*Zingiber officinale* Roscoe), lavender inflorescence, marjoram leaf (*Origanum Majorana* L.), rosemary leaf (*Rosmarinus officinalis* L.), tangerine peels and tobacco leaves were supplied by Planta Medica srl (Pistrino di Citerna (PG), Italy). Jasmine concrete (*Jasminum grandiflorum* L.) was supplied by Chauvet (Seillans, France). Vegetable materials (except for jasmine concrete) were dried and ground using an electric stainless-steel grinder (KYG, mod. 304, China); mean particle size was determined by mechanical sieving. Carbon dioxide (CO_2, 99.9% purity, Morlando Group srl, Naples, Italy) was used to carry out SFE processing.

2.2. SFE Plant Description

SC-CO_2 extraction experiments were carried out in a homemade laboratory apparatus equipped with a 490 cm^3 internal volume extractor. One hundred grams of vegetable matter, with a mean particle size of 600 μm, were used in all the experiments. In the case of jasmine concrete, since it was a semi-solid material and can produce undesired caking/channeling phenomena during extraction, it was mixed with glass beads (3 mm diameter) to create an inert core surrounded by a thin shell of jasmine concrete. Extracts were recovered using two separation vessels with an internal volume of 200 cm^3 each, operated in series. The first separator was cooled down to −10 °C using a thermostated bath (Julabo, mod. F38-EH, Milan, Italy); the second one allowed the continuous discharge of the extract using a valve located at the bottom of the vessel. It was operated at 25 bar and 15 °C. A high-pressure pump (Lewa, mod. LDB1 M210S, Leonberg, Germany) pumped liquid CO_2 at the desired flow rate. CO_2 was then heated to the extraction temperature in a thermostated bath (Julabo, mod. CORIO C-B27, Milan, Italy). CO_2 flow rate was monitored by a calibrated rotameter (ASA, mod. d6, Sesto San Giovanni (MI), Italy), located after the last separator, coupled with a volumetric meter (Sacofgas 1927 SpA, mod. G.4, Milan, Italy). Temperature and pressure along the plant were measured by thermocouples and test gauges, respectively. More details about the apparatus and the experimental procedure are published elsewhere [25,27,30,31].

The operative conditions selected for the experiments carried out in this work were 90 bar and 50 °C ($\rho_{CO2} \approx 0.280$ g/cm^3) in the extractor, 90 bar and −10 °C in the first separator and 25 bar and 15 °C in the second one. CO_2 flow rate was fixed at 0.8 kg/h for all the experiments. The first separator, used for cuticular waxes precipitation, was operated at the same extraction pressure and at a temperature lower than 0 °C since, operating in this way, the solubility of cuticular waxes in CO_2 drastically reduced [25,27,28,30,31].

2.3. Characterization of Cuticular Waxes

Gas chromatography-mass spectroscopy (GC-MS) analysis was carried out 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) of 30 m length, 0.25 mm internal diameter and 0.25 μm film thickness, and connected to a Varian Saturn Detector 2100T (Varian, Inc., San Fernando, CA, USA). Helium was used as the carrier gas, at a flow rate of 1 mL/min. Column temperature was set at 120 °C and held for 5 min; then, it was ramped up to 320 °C, at 2 °C/min, where it was held for 10 min. An injection step was performed using 1 μL of a 1:10 *n*-hexane solution in split mode; the injector temperature was set at 320 °C. The mass spectrometer operated at an ionization voltage of 70 eV in the 40–650 a.m.u. range, at a scanning speed of 5 scans/s. The retention indices (RI) were determined considering the retention time (Rt) values of homologous series of *n*-alkanes (C_{21}-C_{40}) obtained at the same operating conditions. The various components were also identified by a comparison of their RI with published data in the scientific literature. Further identifications were performed, by comparison, of the mass spectra with those stored in the

NIST 02 (National Institute of Standards and Technology, Gaithersburg, MD, USA) library. The relative amounts of the components were evaluated as a percentage of normalized peak area.

3. Results and Discussion

As reported in the literature, the major constituents of cuticular waxes extracted by SFE processing plus high-pressure fractionation of the vegetable matter were paraffinic compounds and, among them, heptacosane, nonacosane, hentriacontane and tritriacontane showed the larger percentages [32–38]. Moreover, odd-carbon-atom hydrocarbons were largely more represented than the homologous, even-carbon atoms. This is an interesting characteristic from an applicative point of view; indeed, differently from paraffins coming from fossil fractions, odd-carbon-atom hydrocarbons are largely more compatible with the human skin and can be applied in cosmetics and health care products [9,39–42].

A photograph of the cuticular waxes extracted during SFE processing of cannabis inflorescence is reported in Figure 1. In all cases, the precipitated material looks like a white powder, sometimes with a light smell, similar to that of the starting vegetable species.

Figure 1. Macroscopic and qualitative example of cuticular waxes extracted by SFE processing of cannabis inflorescence.

Eleven GC-MS traces of the produced cuticular waxes are summarized in Figure 2, for overall comparison purposes. These traces can give a qualitative perspective of the compounds present in the various plants tested and their relative abundance.

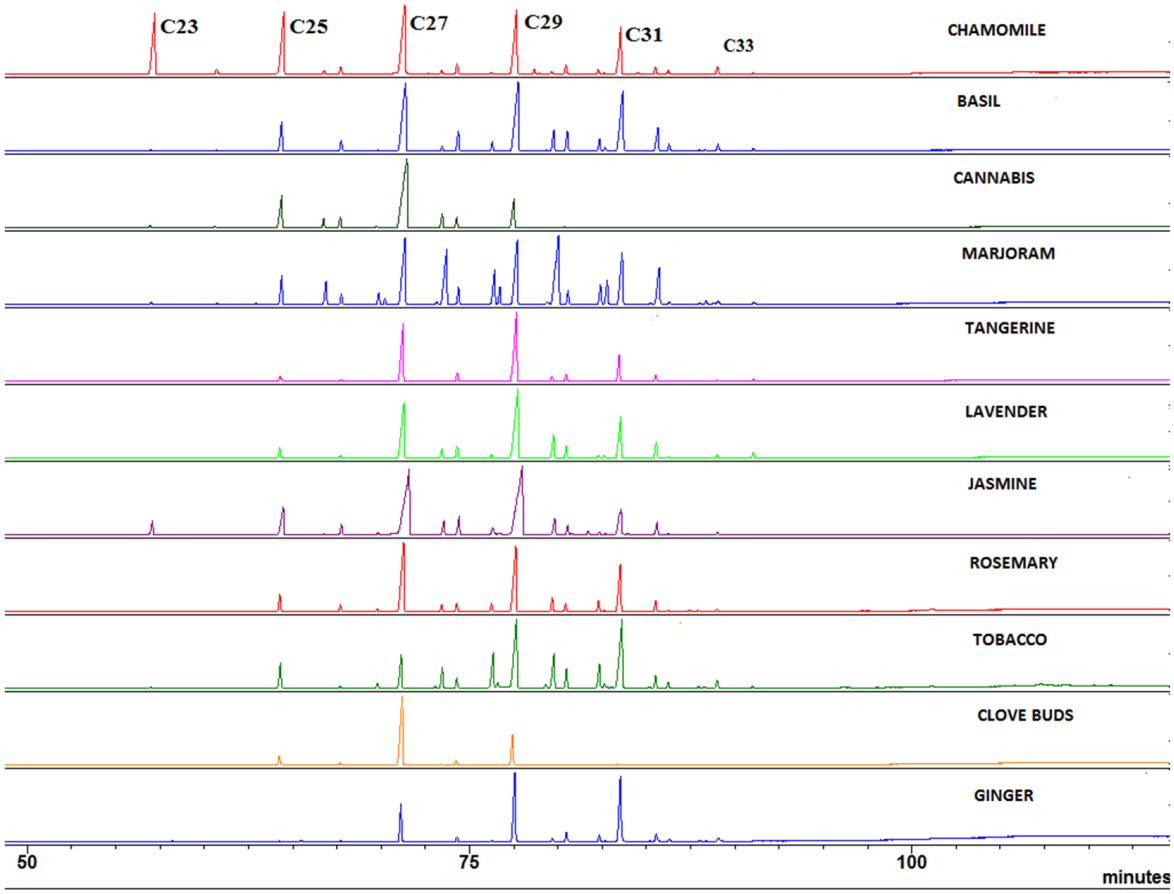

Figure 2. Comparison of GC−MS traces of cuticular waxes extracted by SC−CO_2 plus fractional separation from different vegetable matter, studied in this work.

Extensive identification of the cuticular waxes extracted and analyzed in this work is reported in Table 1. In particular, Figure 2 and Table 1 confirm that the most abundant compounds present in the cuticular waxes extracted by SC-CO_2 are C_{27}, C_{29} and C_{31} for all the vegetable species studied. These results are in agreement with the previous literature related to the same vegetable matter [32–39]. However, data in the literature are referred only to straight paraffins. Analysis performed in this work demonstrates, instead, the presence of some high-molecular-weight paraffinic alcohols (namely, C_{24}, C_{26}, C_{28} and C_{30}). The largest percentages of these compounds are found in marjoram (16.22%), tobacco (5.94%) and lavender (5.48%). Additionally, aldehydes and traces of a paraffinic acid, namely octacosanoic acid, are detected in jasmine and tobacco. More specifically, C_{28} and C_{30} aldehydes are the most widespread compounds and the largest percentage of C_{28} aldehyde is found in tobacco (6.57%) and marjoram (6.40%).

The prevalence of long-chain alkanes is confirmed, and they range from C_{23} to C_{33}, with a prevalence of odd paraffins, as C_{27}, C_{29} and C_{31}, that largely confirm as the major components, though their relative proportions vary from one vegetable species to another [43,44]. Some small quantities of paraffinic alcohols, aldehydes and fatty acids are also identified, as expected. They all show the same carbon atoms' skeleton of the identified paraffins, with the further presence of a functional group: i.e., alcoholic, aldehydic or acid group.

Table 1. Percentage (area %) of the compounds identified by GC−MS of cuticular waxes extracted by SC−CO_2 plus fractional separation from different vegetable matter, studied in this work.

Compound Identified	Chamomile	Basil	Ginger	Jasmine	Lavender	Tobacco	Marjoram	Tangerine	Cannabis	Rosemary	Clove Buds
Tricosane, $C_{23}H_{48}$	10.69	0.10	-	1.25	-	-	0.05	-	0.04	0.07	-
Tetracosane, $C_{24}H_{50}$	0.99	0.15	-	0.18	-	0.08	0.12	-	0.51	0.07	-
Pentacosane, $C_{25}H_{52}$	15.12	5.38	-	6.88	2.32	3.97	3.53	2.17	12.04	3.41	5.80
1-Tetracosanol, $C_{24}H_{50}O$	0.65	-	-	-	0.69	-	2.81	-	2.36	0.09	-
Hexacosane, $C_{26}H_{54}$	1.26	1.76	-	1.75	-	0.39	1.41	0.73	3.08	1.45	1.50
Methylhexacosane, $C_{27}H_{56}$	-	-	-	-	-	-	1.38	-	0.44	0.62	-
Heptacosane, $C_{27}H_{56}$	20.94	22.52	8.33	28.70	20.40	3.69	12.93	28.45	61.85	23.01	65.80
1-Hexacosanol, $C_{26}H_{54}O$	0.65	-	-	2.52	2.32	1.69	9.78	0.41	4.06	1.41	-
Octacosane, $C_{28}H_{58}$	1.63	3.33	1.07	3.19	2.74	-	1.85	3.56	2.88	1.87	3.90
Octacosanal, $C_{28}H_{56}O$	-	1.51	0.38	1.42	-	6.57	6.40	0.23	0.14	1.74	-
Nonacosane, $C_{29}H_{60}$	16.47	21.53	19.01	33.24	31.60	18.34	11.54	41.09	15.73	22.25	22.30
Methylhexacosanoate, $C_{17}H_{34}O_2$	0.78	-	0.73	-	-	-	-	-	-	-	-
1-Octacosanol, $C_{28}H_{58}O$	0.33	3.46	-	2.94	5.48	5.94	16.22	1.65	-	2.46	-
Triacontane, $C_{30}H_{62}$	1.36	3.34	2.09	1.61	2.54	3.11	1.40	2.93	0.35	1.67	-
Octacosanoic acid, $C_{28}H_{56}O_2$	-	-	-	0.86	-	0.09	-	-	-	-	-
Methylheptacosanoate, $C_{29}H_{58}O_2$	0.69	2.40	1.74	0.51	-	4.02	2.36	0.34	-	2.14	-
Triacontanal, $C_{30}H_{60}O$	-	0.57	-	0.19	-	1.14	2.84	0.12	-	0.32	-
Hentriacontane, $C_{31}H_{64}$	9.68	16.69	19.27	7.12	13.45	27.84	8.57	11.41	0.26	13.20	-
1-Triacontanol, $C_{30}H_{62}O$	1.03	4.14	2.85	2.12	3.92	1.89	4.24	2.34	-	2.22	-

Table 1. *Cont.*

Compound Identified	Chamomile	Basil	Ginger	Jasmine	Lavender	Tobacco	Marjoram	Tangerine	Cannabis	Rosemary	Clove Buds
Dotriacontane, $C_{32}H_{66}$	0.61	0.96	0.76	0.17	0.52	0.89	0.30	0.16	0.07	0.38	-
Tritriacontane, $C_{33}H_{68}$	1.13	1.23	1.06	0.52	0.84	1.36	0.54	0.49	0.56	0.62	-
Methyldotriacontane, $C_{33}H_{68}$	-	0.50	0.16	-	1.41	0.34	0.30	0.82	-	0.13	-

4. Conclusions

In the present work, a detailed study on the composition of cuticular waxes extracted and fractionated by SFE is reported. GC-MS analysis confirmed that the separation from the other extractable materials was accurate, and these products can be considered a sort of fingerprint of the specific vegetable matter. C_{27}, C_{29} and C_{31} were the most abundant compounds found in the investigated vegetable materials, in line with the previous findings reported in the literature. Moreover, the specific selectivity of SC-CO_2 extraction towards non-polar or slightly polar compounds makes these cuticular waxes suitable for higher added-value applications, such as in the medical and pharmaceutical field, in which purity and biocompatibility are key features that justify the selection of a more complex extraction process with respect to the traditional ones.

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