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An innovative method for the extraction and HPLC analysis of bioactive policosanols from non-psychoactive *Cannabis sativa* L.

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ABSTRACT

Policosanols (PCs) refer to a mixture of long-chain aliphatic alcohols. Sugar cane is the main industrial source of PCs, but others, including beeswax and *Cannabis sativa* L., are also known. In the raw material PCs are bonded to fatty acids to form long-chain esters, known as waxes.

PCs are mainly used as a cholesterol-lowering product, even though their efficacy is controversial. More recently, the pharmacological interest in PCs has increased, as they have been investigated as antioxidant, anti-inflammatory and anti-proliferative agents.

Given their promising biological implications, the development of efficient extraction and analytical methodologies for the determination of PCs is extremely important to identify new potential sources of these compounds and to ensure the reproducibility of biological data. Conventional techniques used for the extraction of PCs involve time-consuming approaches leading to low yields, while analytical methods for their quantification are based on gas-chromatographic (GC) techniques, which require an additional derivatization step during the sample preparation to increase their volatility.

In the light of all the above, this work was aimed at the development of an innovative method for the extraction of PCs from non-psychoactive *C. sativa* (hemp) inflorescences, taking advantage of the microwave-assisted technology. In addition, a new analytical method based on high-performance liquid chromatography (HPLC) coupled with an evaporative light scattering detector (ELSD) was developed for the first time for both the qualitative and quantitative analysis of these compounds in the extracts. The method was validated according to ICH guidelines, and it was applied to the analysis of PCs in hemp inflorescences belonging to different varieties. The results were analyzed using Principal Component Analysis (PCA) and hierarchical clustering analysis to rapidly identify samples with the highest content of PCs, which might find an application as alternative sources of these bioactive compounds in both the pharmaceutical and nutraceutical fields.

1. Introduction

Policosanols (PCs) refer to a mixture of long chain primary aliphatic alcohols (C_{20}OH-C_{36}OH). They are widely used in food supplements because of their beneficial effects on human health. To date, sugar cane represents the main source of commercial PCs [1]. In nature, they are also present in different plant waxes, beeswax and other natural sources, including *Cannabis sativa* L. inflorescences [2].

Previous studies have shown that PCs have a wide array of biological activities, mainly based on a lowering-lipid effect [2–4]. However, this topic is controversial, as very recently a network meta-analysis, that aimed to compare the lipid-lowering activity of various nutraceuticals in adults, has highlighted that PCs have no effect on lipid profile [5]. PCs have been widely applied in the fields of dietary food supplements, human and animal feed additives [2,4]. Nevertheless, the bioactivity investigations on PCs are still poor, especially for their properties against oxidative stress, inflammation and cancer [4]. The antioxidant potential of PCs was mainly assessed *in vitro* [6,7]. More recently, a study from Cho et al. [8] has revealed the ability of a r-HDL-containing Cuban PCs mixture to prevent LDL-oxidation and a significant anti-inflammatory

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activity to protect zebrafish embryo death under the presence of carboxymethyl-lysine [8]. Other studies have suggested a protective effect of PCs from different natural matrices on learning and memory impairment caused by different damages [9,10]. In addition, Cuban PCs have also demonstrated to be effective anti-proliferative agents on human gastric carcinoma SNU-16 cells and in a xenograft mouse model [11]. Lately, the anti-viral potential of PCs was investigated: in particular, the ability of a nano-lipid emulsion of PCs to inhibit the proliferation of SARS-CoV-2 and the related mechanism of action have been elucidated with promising results [12].

Methods for the extraction of PCs from natural sources encompass long processes and return low recoveries of compounds [4]. It should be noted that free PCs in the raw waxy material are poorly present, being them highly bonded to fatty acids to form esters. Therefore, either a saponification or *trans*-esterification step is necessary to free them from the ester form to increase the extraction yield of long-chain alcohols [4]. Other extraction procedures are based either on the supercritical fluid technology with carbon dioxide (CO_2) or on the usage subcritical liquefied dimethyl ether [4,13]. Recently, a microwave-assisted method has been described for PCs from beeswax with a better yield and in a short time in comparison with conventional methods [14]. Beeswax has been found to represent a very promising alternative source of PCs compared to the exploited sugar cane wax, as its concentration is 60 times higher than any other conventional known natural source [15]. PCs from beeswax are richer in long-chain alcohols, including $C_{30}OH$ and $C_{32}OH$, than sugar cane wax [8]. As for PCs from *C. sativa*, the most abundant PCs have been found to range from C_{24} to C_{30} chain lengths [2]. PCs with an odd chain length were identified in extracts from non-psychoactive *C. sativa* (hemp) inflorescences, even if at low abundance [2].

As for the analysis of PCs, most of the methods previously described in the literature are based on the application of gas chromatography (GC) [14,16–18]. However, one of the main drawbacks of this technique is the requirement of a derivatization step during sample preparation to increase the volatility as well as the thermal stability of these compounds [14,16]. High-performance liquid-chromatography (HPLC) under reversed-phase (RP) conditions, coupled with an evaporative light scattering detector (ELSD), has been described for the analysis of triacontanol ($C_{30}OH$) in liquid samples [19]. A significant advantage of HPLC-ELSD, over GC methods, is the direct analysis of the purified mixture without derivatization and related accuracy issues. Ultra-high-performance liquid chromatography coupled with high resolution mass spectrometry (UHPLC-HRMS) has been recently applied to the analysis of underivatized PCs in hemp inflorescence with ionization assistance by lithium ion adduct formation [2]. In this way, PCs could be readily separated by RP conditions and detected by MS.

In the light of all the above, this work was focused on the development of an innovative method for the extraction and purification of PCs from hemp inflorescences, by combining a *trans*-esterification and a hydrolysis reaction in one single step, taking advantage of the microwave-assisted technology. A new analytical method was set up and fully validated for both the qualitative and quantitative analysis of the target compounds using HPLC-ELSD. The HPLC-ELSD method developed in this work is the first that allows for the separation of five PCs in the enriched extracts from hemp. The method developed was applied to the analysis of PCs in hemp inflorescences belonging to different varieties. The results were analyzed by using Principal Component Analysis (PCA) to underly patterns and relationships among samples based on PC content. A hierarchical clustering analysis was also carried out to rapidly identify samples having the highest content of PCs, that could be exploited as alternative sources for their extraction and application in both the pharmaceutical and nutraceutical fields.

2. Material and methods

2.1. Chemicals and solvents

n-Hexane, ethanol (EtOH), chloroform ($CHCl_3$), acetonitrile (ACN) and methanol (MeOH), all HPLC purity grade ($\geq$ 99.8%), and potassium hydroxide (KOH) were purchased from Sigma-Aldrich (Milan, Italy). Reference PCs, including tetracosanol ($C_{24}OH$; $\geq$ 99%), hexacosanol ($C_{26}OH$; $\geq$ 97%), ocatacosanol ($C_{28}OH$; $\geq$ 99%), $C_{30}OH$ ($\geq$ 96%) were also from Sigma-Aldrich (Milan, Italy). HPLC purity grade methyl-tert-butyl ether (MTBE) was from Carlo Erba (Milan, Italy). Water (H_2O) was purified by using a 185 Millipore Milli-Q Plus System (Milford, MA, USA).

2.2. Plant material

Fibre-type *C. sativa* female inflorescences, belonging to Bernabeo (C1), Carma (C2), Carmagnola (C3), Carmaleonte (C4), China (C5), Codimono (C6), Carmagnola Selezionata (C7), Eletta Campana (C8), Fibranova (C9), Fibrante (C10), Futura (C11) and Santhica (C13) varieties were kindly provided by Dr. Gianpaolo Grassi of the research center CREA-CIN (Rovigo, Italy). The Kompolti variety (C12) was purchased from Materia Labs (Siena, Italy) and it was selected for the optimization of the PC extraction method. All hemp inflorescences used in this study are approved for commercial use by the European Union and had a certified Δ^9-THC content below the legal limit of 0.2%. Samples were collected during the blooming time (i.e. July-August) in 2018. For each sample, hemp inflorescences were manually separated from twigs and seeds prior the extraction procedure.

2.3. Extraction of PCs from hemp inflorescences

A portion of 10 g of previously sieved inflorescences was weighed and extracted twice with 120 mL of *n*-hexane in an ultrasonic bath (Sonorex RK-100 H, Bandelin, Berlin, Germany). After filtration, the solvent was evaporated using a rotary evaporator (Laborota 4000 Heidolph) at room temperature. The oily residue obtained was dissolved with 30 mL of warm EtOH (75 °C) and the sample was placed to winterize for 24 h at –20 °C. The waxy precipitate was centrifuged at 8000 rpm for 30 min at a temperature of 2 °C and then filtered under vacuum, washed with 5 mL of EtOH and dried overnight in a desiccator. The obtained waxy material was placed in a closed glass vial with 5 mL of EtOH and 100 mg of KOH. The vial was put in the microwave apparatus (FlexiWAVE, Milestone, Sorisole (BG), Italy) for the one-step *trans*-esterification and hydrolysis reaction by setting the temperature and holding time at 80 °C and 30 min, respectively, while a medium speed magnetic stirring was selected. The resulting grayish suspension was filtered under vacuum, washed with 10 mL of water, and dried in a desiccator overnight. The final product was a grayish-white powder. The extraction procedure was performed in duplicate for each sample.

A portion of 7 mg of the obtained residue was dissolved in 1 mL of $CHCl_3$ and filtered through syringe 0.22 μm PTFE filter prior the HPLC-ELSD analysis for the quantification of PCs.

2.4. HPLC-ELSD analysis of PCs

The analyses of PCs in extracts from hemp inflorescences were performed on an Agilent Technologies (Waldbronn, Germany) modular model 1260 Infinity II system, consisting of a vacuum degasser, a quaternary pump, a thermostated column compartment and an evaporative light scattering detector (ELSD). Separation of target compounds was carried out on an Atlantis™ dC_{18} column (150 × 3.0 mm, 3 μm, Waters, Milford, MA, USA). The mobile phase consisted of ACN (solvent A) and a mixture MTBE-MeOH 90:10 (*v/v*) (solvent B), eluted under the following gradient: 0–1 min isocratic elution at 20% B; 1–16 min linear gradient from 20% to 45% B, which was held constant for 4 min; the column was

finally brought to 20% B in 5 min. The flow-rate was set at 1.5 mL/min and the injection volume was 10 μL. The ELSD evaporator temperature was set at 35 °C, while nebulizer temperature was 30 °C. Nitrogen flow rate was set at 1.50 SLM.

All the hemp extracts analyzed in this study were injected in triplicate.

2.5. HPLC-ELSD method validation

In this study, the HPLC-ELSD method developed for the analysis of PCs was validated in agreement with the International Guidelines for Analytical Techniques for the Quality Control of Pharmaceuticals (ICH guidelines) [20].

As regards linearity, the stock standard solution of PCs (C_{24}OH, C_{26}OH, C_{28}OH and C_{30}OH) was prepared by weighing an accurate amount of reference compound (2.5 mg for C_{24}OH, C_{26}OH, C_{28}OH and 20 mg for C_{30}OH) into a 5 mL volumetric flask. Then, $CHCl_3$ was added until the solution was adjusted to volume. The calibration curves were built at five concentration levels covering the following concentration ranges: 52 – 520 μg/mL for C_{24}OH; 49 – 492 μg/mL for C_{26}OH; 60 – 602 μg/mL for C_{28}OH and 97 – 780 μg/mL for C_{30}OH. Injections were performed in triplicate for each concentration level. The calibration curve was obtained by plotting the peak area of the compound at each level versus the concentration of the sample, fitting by least square regression model. A quadratic calibration curve was obtained as the ELSD response is not linear, but it follows a quadratic relationship [19].

The limit of detection (LOD) and the limit of quantification (LOQ) of the reference compounds were experimentally determined by HPLC analysis of serial dilutions of a standard solution to reach a signal-to-noise (S/N) ratio of 3 and 10, respectively.

Intra-day and inter-day precision of the chromatographic system was evaluated by performing multiple injections of the stock standard solution of PCs (consisting of C_{24}OH, C_{26}OH and C_{28}OH) within the same day for three consecutive days. Then, the %RSD of retention times and peak areas was evaluated.

The precision of the extraction technique for hemp inflorescences was evaluated by repeating the extraction procedure on the inflorescence of the same hemp sample (C12) within the same day for three consecutive days. Each extract was then injected into the HPLC-ELSD system for the quantitative analysis of target analytes.

Accuracy of the extraction procedure was assessed by means of the recovery test. It involved the addition of a known quantity of reference compound (C_{24}OH, C_{26}OH, C_{28}OH and C_{30}OH) to half the sample weight of raw material used for the extraction procedure to reach 100% test concentration. The spiked samples were then extracted and analyzed with the proposed analytical method. Recovery test was performed in duplicate and was calculated as the percentage of the response in the extracted samples compared to the control samples.

2.6. Statistical analysis

To have an overview of quantitative results obtained by HPLC-ELSD analyses of PCs extracts obtained from hemp inflorescences, all samples were submitted to multivariate statistical analysis by means of PCA [21] and hierarchical clustering analysis, using MATLAB computing platform. In the former analysis, default distance was measured as Euclidean distance and data were classified into a cluster heat map. PCA was carried out by using PLS_Toolbox 8.9.2 software (Eigenvector Research Inc., Manson, WA, USA).

3. Results and discussion

3.1. Extraction of PCs from hemp inflorescences

The procedure for the extraction of PCs from hemp inflorescences was developed in this work starting from preliminary findings described in the literature [2,14]. The first step of the extraction method involved an ultrasound-assisted extraction (UAE) with *n*-hexane, aimed at the recovery of all lipophilic components from the plant material, including waxes, among others. To favor the precipitation of the waxy material, *n*-hexane was evaporated, and the residue re-dissolved in EtOH and placed at the temperature of – 20 °C (winterization process). The waxy precipitate was then recovered by centrifugation at 2 °C and vacuum filtration. In this way, other lipophilic compounds that were co-extracted with sonication, such as cannabinoids, were easily removed with the solvent. To obtain free PCs from the ester form, Venturelli et al. have applied a microwave-assisted technique to perform a first *trans*--esterification reaction and a subsequent hydrolysis on beeswax [14]. In the present paper focused on PCs from hemp inflorescences, the method was significantly modified and improved to combine both the microwave-assisted *trans*-esterification and hydrolysis reactions in one single step. In particular, the procedure was carried out by using EtOH as the reaction solvent and KOH as the strong basis at 80 °C for 30 min. Free PCs were then recovered from the reaction mixture after filtration and washing with H_2O [14].

Several non-psychoactive varieties of hemp inflorescences were studied in this work, and they exhibited different yields. The overall yield of the whole extraction process, calculated as the quantity of the final residue obtained with respect to the starting raw plant material, was between 0.14% and 0.58%. These data provided indications on the content of waxy material in the hemp inflorescences investigated. Among them, samples C3 and C8 proved to be the richest varieties in waxy material, as they had the highest yield in final product (0.58% and 0.52%, respectively), in contrast to samples C2 and C7, which presented the lowest yield (0.14% and 0.17%, respectively).

3.2. HPLC-ELSD method development for the analysis of PCs

As already mentioned, PCs are generally analyzed by means of GC, prior a derivatization process to make them more volatile [14,16–18]. In the literature, few studies are based on HPLC as the analytical technique of choice for the determination of PCs [2,19]. In this context, common detectors normally employed for the analysis of natural compounds are not suitable for PCs. Indeed, since they do not have significant absorption maxima in the UV-Vis range, they are not suitable for the very widely diffused HPLC-UV technique. HPLC-ESI-MS/MS does not provide the optimal solution for PCs analysis either, since PCs are not prone to ionize, thus requiring appropriate mobile phase modifiers [2]. Conversely, HPLC-ELSD represents a good alternative to the above-mentioned detectors, as it offers a faster and simpler sample preparation without a derivatization step [19]. Indeed, Sut et al. have used HPLC-ELSD for the separation and quantification of C_{30}OH in liquid samples, but they did not apply it to other PCs [19].

The present work is the first one based on the development of an efficient HPLC-ELSD method for the analysis of several PCs in natural matrices, such as those derived from hemp. The achievement of a high resolution of the target analytes in the minimum time of analysis was the driving force for the method development. Given the high lipophilicity of the target analytes, the selection of a suitable mobile phase was a crucial point. Indeed, ACN was not enough strong for the elution of PCs with a longer chain. For this reason, a mobile phase composed of both ACN and a mixture of MTBE-MeOH 90:10 (*v/v*) was selected. The gradient was optimized to allow for the separation of all PCs present in the samples analyzed in a total time of analysis of 25 min. The flow rate was kept quite high (i.e. 1.5 mL/min) to improve the peak shape and, thus, the resolution of chromatographic peaks in an acceptable analysis time. As to the detection parameters, the method was optimized to maximize sensitivity, by focusing on evaporation and nebulizer temperature, together with the gas flow rate. Given the volatile nature of the solvents employed, the evaporation and nebulizer temperature of the ELSD detector were set at 35 and 30 °C, respectively, while the gas flow was set at 1.5 SLM, leading to a reduction of the baseline noise and an

improvement of method sensitivity.

3.3. Validation of the HPLC-ELSD method

The HPLC-ELSD method optimized in this study was validated according to the international ICH guidelines [20]. In particular, the method was validated for its linearity, sensitivity, precision and accuracy.

For what concerns linearity, the HPLC-ESLD methods showed good linearity for the reference standards selected in this study over the concentration range tested (Table S1, SI). Calibration curves followed a quadratic trend, as the ELSD response was not linear. The values of the limit of detection (LOD) and limit of quantification (LOQ) obtained for each standard are listed in Table S1, indicating a satisfactory sensitivity.

The low intra- and inter-day relative standard deviation (%RSD) for retention times ($\leq$ 3.3%) and peak areas ($\leq$ 1.9%) relative to the target compounds (Table S2, SI) indicate the high precision of the chromatographic system. The extraction precision was also satisfactory, as shown by the SD values reported in Table S3 of the SI.

Accuracy of the analytical method was assessed by means of the recovery test and the % recovery data for PCs were satisfactory, ranging from 86% to 104%.

3.4. Analysis of PCs in hemp inflorescences

Different varieties of hemp inflorescences were taken into consideration in this study to identify the most promising ones as a new source of PCs. All the extracts obtained from the hemp samples were analyzed by means of the HPLC-ELSD to obtain both the qualitative and quantitative PC composition of hemp inflorescences. Peak assignment was confirmed by analyzing the same samples spiked with the compounds of interest (C_{24}OH, C_{26}OH, C_{28}OH and C_{30}OH). Extraction from each hemp variety was performed in duplicate and each sample was analyzed in triplicate.

Fig. 1 shows a representative HPLC-ELSD chromatogram of the final product obtained from hemp (sample C5, i.e. China variety), while Table 1 shows quantitative data of PCs in hemp inflorescences belonging to 13 hemp varieties. All compounds of interest were quantified by means of external calibration with their respective reference compound, with exception of C_{32}OH which was quantified by using C_{30}OH calibration curve.

From a first glance at quantitative data, it is possible to observe a great variability in the content of PCs among hemp varieties (Table 1). In general, higher molecular weight PCs, including C_{28}OH, C_{30}OH and C_{32}OH, are those present in greater amounts, though the same cannot be stated for sample C8, where the major PCs were found to be C_{24}OH and C_{26}OH (Table 1).

To simplify the visualization and understanding of the data obtained from the different varieties of hemp inflorescences considered in the present study, a multivariate analysis was performed. Data were organized in a two-dimensional matrix (60 samples x 5 compounds, considering the replicates measurements), autoscaled and analyzed by PCA (model built with two principal components, LVs, explaining 93% of total variance) for a first exploratory investigation. The analysis of the scores of the first vs. second principal components, LV1 vs. LV2 (Fig. 2A) highlights the presence of various trends among the investigated samples. Firstly, the different replicates (same symbols) of the samples are grouped to each other, showing a good reproducibility of the developed analytical method. As far as samples are concerned, a peculiar behavior of sample C8 can be observed, with high positive values for both LV1 and LV2. There is also a similarity between samples C3 and C13 (with positive values for LV1 and negative ones for LV2) and among samples C2, C4, C6, C7 and C12, that lie to the left side of the plot. Although C10 presents a negative score value for LV1, it seems to be different from the latter samples for a higher value of LV2. Finally, C11, C9 and C1 are located approximately in an intermediate position of the scores plot.

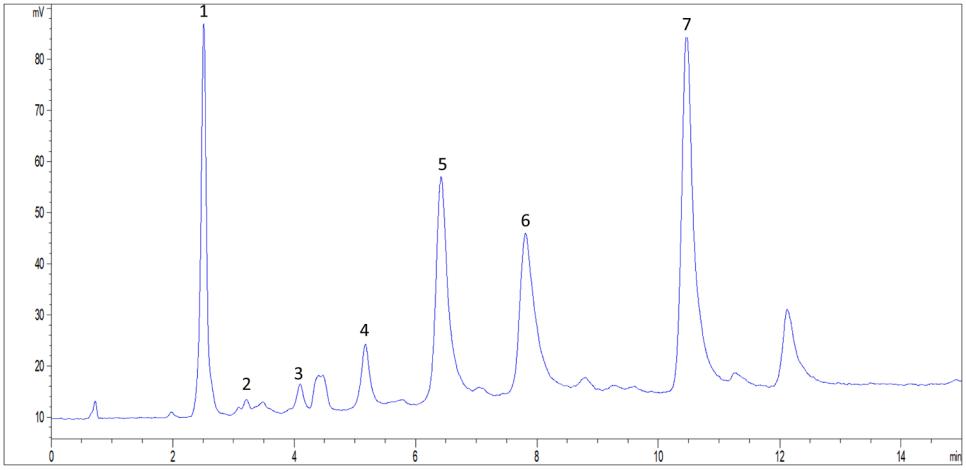

Fig. 1. Representative HPLC-ELSD chromatogram of PCs extracted from hemp inflorescences (sample C5). For peak identification: 1. fatty acids, 2. C_{24}OH, 3. C_{26}OH, 4. C_{28}OH, 5. C_{30}OH, 6. C_{32}OH, 7. hydrocarbons.

Table 1
Content of PCs in hemp inflorescences. Data are expressed as μg/g ± SD ($n = 6$).

Compound	t_R (min)	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12[a]	C13
$C_{24}OH$	3.2	23.7 ± 3.1	5.6 ± 1.9	36.5 ± 2.1	19.2 ± 0.9	12.3 ± 1.3	9.4 ± 0.2	10.9 ± 1.1	155.4 ± 0.3	20.8 ± 3.4	69.1 ± 5.0	31.8 ± 3.0	13.5 ± 1.0	63.9 ± 5.9
$C_{26}OH$	4.1	46.3 ± 4.1	6.5 ± 1.8	64.8 ± 6.1	22.9 ± 1.7	9.4 ± 1.1	12.4 ± 0.4	25.6 ± 2.4	158.4 ± 5.8	53.3 ± 4.2	44.4 ± 8.1	46.3 ± 2.7	13.7 ± 1.3	69.2 ± 6.7
$C_{28}OH$	5.2	79.7 ± 4.2	14.7 ± 4.0	112.2 ± 8.6	38.5 ± 1.7	19.9 ± 1.0	24.7 ± 0.7	43.5 ± 3.1	98.8 ± 1.7	71.8 ± 9.0	39.4 ± 8.2	61.2 ± 2.0	24.4 ± 2.0	92.0 ± 10.0
$C_{30}OH$	6.4	66.3 ± 0.3	29.3 ± 5.3	105.5 ± 5.0	46.3 ± 2.5	38.4 ± 0.1	37.1 ± 3.4	21.6 ± 1.9	99.9 ± 1.1	80.0 ± 7.9	47.4 ± 6.5	47.6 ± 1.1	35.6 ± 3.2	141.2 ± 9.1
$C_{32}OH$	7.8	57.1 ± 1.0	23.1 ± 5.0	78.6 ± 8.2	43.9 ± 3.1	36.3 ± 0.2	27.2 ± 1.4	51.5 ± 5.7	96.2 ± 0.5	50.8 ± 7.2	36.0 ± 5.8	68.8 ± 1.6	31.2 ± 2.9	76.4 ± 21.4

Experimental conditions as in Section 2.3.
[a] $n = 18$.

From LV1 vs. LV2 loadings plot (Fig. 2B), it is possible to point out that, having all the loadings a positive contribution for the first component, the separation between C8 from the other samples is mainly due to its global higher PC amount. On the contrary, samples positioned in left of the Fig. 2A plot are different with respect to C8 sample, with lower PC content. Finally, samples C3 and C13 present a high content of the investigated PCs, but, compared to C8, they have a relatively higher amount of $C_{28}OH$ and $C_{30}OH$ (negative LV2 loading values) and a lower one of $C_{24}OH$ and $C_{26}OH$ (positive LV2 loading values).

Lastly, the amounts of PCs identified in the samples were analyzed to obtain a graphical representation of their distribution and a hierarchical clustering of the different profiles across the hemp varieties. The MATLAB computing platform was used to generate a cluster analysis (Fig. 3). Clusters were formed based on the level of "similarity" between samples according to the content of PCs. In particular, hemp varieties were subdivided using hierarchical clustering with default distance measured as Euclidean distance, i.e. the square root of the sum of the square differences.

In parallel to the clustering analysis, a heat map was also generated by applying a colorimetric scale representing the normalized concentrations of PCs in the samples. The colorimetric scale goes from –2.5 (deep blue) for low concentration values, 0 (white) for average values, to 2.5 (red) for high values. In this way, it is possible to get information about PC concentrations in relation to other samples. In Fig. 3 it is possible to visualize how both hemp varieties and PCs were ordered by similarity. Indeed, two macro-families can be identified: on the right-hand side of the graph all the varieties that were characterized by a higher content of PCs are present, namely samples C3, C8, and C13; on the left-hand side of the graph, a big cluster, including all hemp varieties having a lower average amount of PCs, is visible. Refining the analysis, within the first cluster, sample C8 is the one having the higher PC content, with particular regard to $C_{24}OH$ and $C_{26}OH$, which represent the most abundant compounds of this class. Samples C3 and C13 formed another cluster, being them characterized by a very similar quantitative PC profile. On the other hand, the macro-group on the left-hand side of the graph can be divided into four main clusters. In particular, samples C1, C9 and C11 showed an average amount of PCs, within which the most represented were $C_{28}OH$, $C_{30}OH$, and $C_{32}OH$. Samples C4 and C7 were characterized by a slightly lower total PC amount, with the most abundant one being $C_{32}OH$. Samples C2, C5, C6 and C12 were grouped into a quite homogeneous cluster, as they had the lowest total PC amount. In particular, sample C2 was the one characterized by the lowest PC content within this group. Sample C10 alone represented a separate group, as it was characterized by the lowest content of almost all PCs, with the only exception of $C_{24}OH$, which represented the major long-chain fatty alcohol.

The results of the cluster heat map analysis highlighted that samples C8, C13 and C3 are those with the highest content of total PCs. Therefore, these hemp varieties could be of potential interest, especially in a circular economy perspective, where both residual hemp inflorescences deriving from industrial processes and waste material may be exploited to obtain products of both pharmaceutical and nutraceutical interest.

4. Conclusions

In this work, a new method for both the extraction and the HPLC analysis of PCs from hemp inflorescences belonging to different varieties was developed for the first time. As to the sample preparation, extraction of fatty alcohols was achieved by an initial UAE with *n*-hexane of the waxy material, followed by a one-step microwave-assisted reaction for their *trans*-esterification and hydrolysis to free the fatty alcohols from their esters. To determine PC profile in hemp inflorescences a new method based on HPLC coupled with ELSD was developed that allowed for the separation of the main five long-chain alcohols in the extracts.

The above-described analytical method was fully validated for what

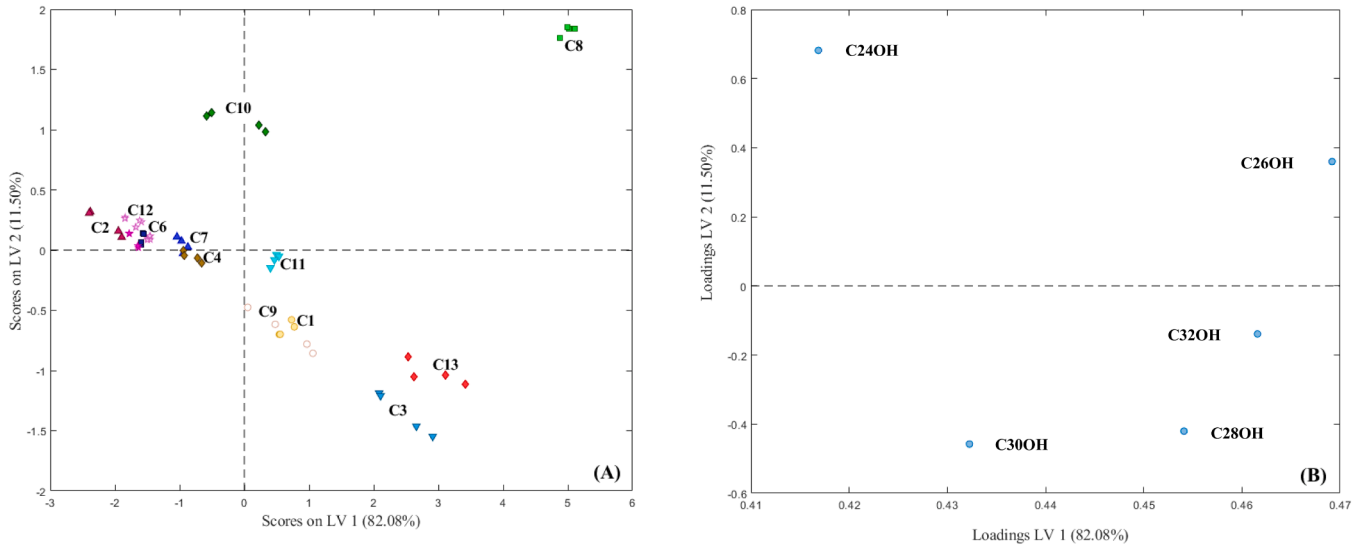

Fig. 2. LV1 vs. LV2 score (Fig. 2A) of hemp inflorescence samples (scores are colored considering the hemp variety) and loading (Fig. 2B) plots.

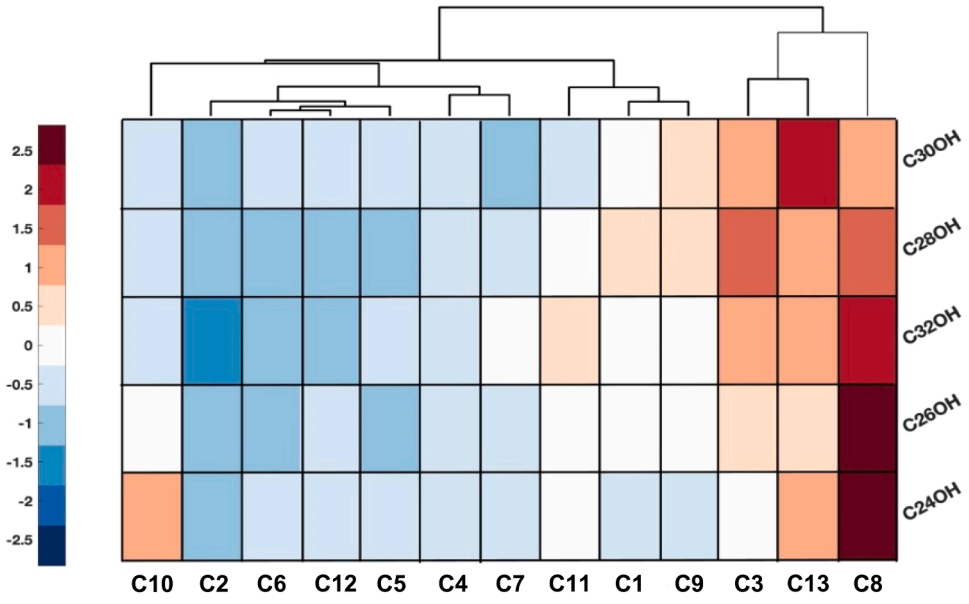

Fig. 3. Cluster heat map representing the relative amounts of PCs detected in the inflorescences of different hemp varieties analyzed by HPLC-ELSD.

concerns linearity, sensitivity, precision and accuracy, according to the international ICH guidelines and it was successfully applied for the quantification of PCs in 13 samples of hemp inflorescences. The quantitative analysis of the hemp samples revealed a heterogeneous distribution of the fatty alcohols among the samples analyzed. As a general observation, alcohols with longer carbon chain seemed to be present in greater amount with respect to the others.

This study identified hemp as a new promising source of PCs. In this view, residual inflorescences deriving from industrial extraction processes and waste materials could be further investigated to obtain PCs for a possible application in both the pharmaceutical and nutraceutical fields in a circular economy perspective.

CRediT authorship contribution statement

Virginia Brighenti: Data curation; Formal analysis; Investigation; Methodology; Software; Validation; Visualization; Writing - original draft. **Alberto Venturelli**: Conceptualization; Writing - review & editing. **Clarissa Caroli**: Data curation; Formal analysis; Investigation; Methodology; Writing - original draft. **Lisa Ancheschi**: Data curation; Formal analysis; Investigation; Methodology; Writing - original draft. **Megi Gjikolaj**: Data curation; Formal analysis; Investigation; Methodology. **Caterina Durante**: Conceptualization; Data curation; Formal analysis; Software; Validation; Visualization; Writing - review & editing. **Federica Pellati**: Conceptualization; Data curation; Formal analysis; Funding acquisition; Project administration; Resources; Supervision; Validation; Visualization; Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2023.115547.

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