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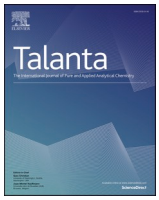

Electrochemical sensors for fast classification of different *Cannabis sativa* L. samples according to total Δ^9-tetrahydrocannabinol content

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ABSTRACT

In this work, we investigated the ability of an electrochemical sensor to recognize *Cannabis sativa* L. samples with different total content of Δ^9-tetrahydrocannabinol (Δ^9-THC), determined by the levels of the psychoactive cannabinoid and of its biosynthetic precursor Δ^9-tetrahydrocannabinolic acid (Δ^9-THCA), using a multivariate approach. The voltammetric responses recorded with screen-printed electrodes modified with carbon black reflected the compositional differences from the different samples, in terms of cannabinoids of the vegetal material. PLS-DA models allowed for the correct classification of most *C. sativa* samples into the classes of legal and illegal samples according to total Δ^9-THC content, based on threshold limits defined by the EU/US (0.3 % w/w) and Italian (0.6 % w/w) regulations. Satisfactory results were achieved in both cases, obtaining classification efficiency values in prediction of the external test set equal to 85 % and 100 % for the EU/US and Italian thresholds, respectively. The obtained results suggest the possibility to consider the proposed method as a starting point for the implementation of an automated device for rapid prescreening of total Δ^9-THC content directly on site.

1. Introduction

Cannabis sativa L., a member of the Cannabaceae family, is one of the world oldest domesticated crops cultivated for recreational, medicinal, and industrial purposes [1,2].

C. sativa can be classified as fibre-type (hemp), drug-type (medicinal cannabis) and recreational-type, based on the phytocannabinoid content and related usage [3]. The term "phytocannabinoids" refers not only to the chemical substances isolated from *C. sativa* exhibiting the typical C21 terpenophenolic skeleton, but also to their derivatives and transformation products. In the *C. sativa* plant, cannabinoids are mostly present in their carboxylated acidic form, mainly including Δ^9-tetrahydrocannabinolic acid (Δ^9-THCA) and cannabidiolic acid (CBDA). By means of a decarboxylation process, which occurs spontaneously under the action of heat and light, cannabinoic acids are converted into their neutral compounds, Δ^9-tetrahydrocannabinol (Δ^9-THC) and cannabidiol (CBD), respectively (Fig. 1). These cannabinoids display remarkable similarities, but they differ in their pharmacological properties. Indeed, only Δ^9-THC shows psychotropic effects, while CBD has anti-inflammatory, neuroprotective and antiepileptic properties.

Recreational-type *C. sativa*, also known as marijuana, is characterised by the presence of a high content of Δ^9-THC, and for this reason it is an illicit drug in several countries. On the other hand, fibre-type *C. sativa*, that was originally used for its stem-fibers, has recently spread in many countries, despite its poorer cannabinoid profile, thanks to the noticeable presence of CBD. Moreover, the continuous improvement of plant genetics and cultivation methods has resulted in the availability of different products with a great variety of compositions and structures, so that legal fibre-type inflorescences must be strictly distinguished from marijuana. Usually, the discrimination between legal

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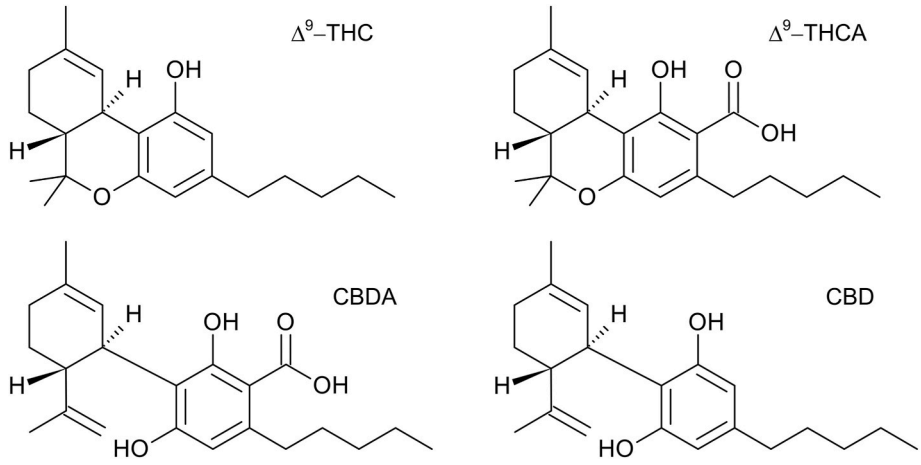

Fig. 1. Chemical structure of the main cannabinoids of *Cannabis sativa* L.

and illegal *C. sativa* samples is based on the total Δ^9-THC content (Δ^9-THC_{TOT}), defined by the formula:

$$\Delta^9 - THC_{TOT}\ (\%\ w/w) = \Delta^9 - THC\% + \left(\Delta^9 - THCA\% \times\ 0.877\right) \quad \text{Eq. (1)}$$

where Δ^9-THC% and Δ^9-THCA% are the concentrations (%, w/w) of Δ^9-THC and Δ^9-THCA determined in the sample on dry weight basis, and 0.877 is the Δ^9-THC/Δ^9-THCA molecular weight conversion factor [4]. Determining Δ^9-THC_{TOT} allows for the quantification of all potential Δ^9-THC present in plant material, since when the plant material is exposed to heat, light, or alkaline conditions, Δ^9-THCA will convert to Δ^9-THC.

According to the Common Agricultural Policy of the European Union (EU), the threshold limit of Δ^9-THC_{TOT} in *C. sativa* has been set equal to 0.3 % w/w [5]. The same limit holds for the federal law of the United States (US) [6]. If one *C. sativa* crop is detected at harvest to contain a higher quantity of Δ^9-THC, the plants could be confiscated and/or destroyed. According to the Italian regulation [7], no responsibility is placed on the farmer until Δ^9-THC_{TOT} does not exceeds the value of 0.6 %.

Therefore, the development of methods for the rapid identification of *C. sativa* inflorescences directly in the field, i.e., before harvest and the further processing of the plant products, is urgently needed. Several methods characterised by portable instrumentation, simple preparation of the samples and low analysis time, have been recently proposed for *in situ* and fast cannabinoid screening in *C. sativa* plants, such as near infrared, Raman and fluorescence spectroscopies [8–12], colorimetric assays [13–15], and biosensors based on various transduction methods [16–18]. Among these, electrochemical methods are particularly promising, since cannabinoids are electroactive species [19–26]. In a recent paper [27], some of us proposed the use of two screen printed electrodes (SPEs) for the fast quantification of Δ^9-THCA in recreational type *C. sativa* extracts. The developed method allowed for the quantification of Δ^9-THCA in samples in which it was the predominant or unique cannabinoid present, as it often occurs to *C. sativa* samples used for recreational purposes. In these samples, the recorded voltammetric signal was clearly attributable to Δ^9-THCA only, so that a rapid quantification of this analyte was possible using a simple univariate calibration curve. The results obtained with the electrochemical method proposed were in excellent accordance with reference data obtained by chromatography, with the advantage of having reduced analysis time and cost. However, one question remained unanswered. In fact, samples characterised simultaneously by a Δ^9-THCA content well above the legal limit and a high CBDA content gave rise to electrochemical signals resulting from the overlap of the oxidation signals of the two cannabinoids, making quantification of Δ^9-THCA not possible. This also means that the identification of illegal samples by direct observation of the voltametric signals and quantification through univariate calibration curves can be affected by high CBDA contents, thus resulting unreliable.

In this paper, we propose a solution to this main issue exploiting a multivariate approach, where we focused our attention on the overall features of the voltammetric signals, rather than on the intensity of specific peaks at defined potential values. After a preliminary exploratory data analysis aimed at evaluating whether and how cannabinoid content affects the whole dataset structure, we focused on the development of classification models to distinguish legal samples from illegal ones based on Δ^9-THC_{TOT} as required by EU/US and Italian legislations, instead of focusing only on Δ^9-THCA and CBDA. The choice of classification models instead of calibration models is driven by the fact that the proposed method is intended for possible future applications as a rapid prescreening: a simple qualitative "yes/no" (i.e., "legal/potentially illegal") indication would be more directly interpretable even by non-expert personnel than a quantitative determination, which in any case should be confirmed by official analytical methods. This represents an innovative procedure in the field of drugs of abuse, that could allow for the development of a device for rapid prescreening of real samples in various scenarios, such as customs. To reach this goal, SPEs modified by carbon black (SPEs-CB) have been used to collect electrochemical signals in extracts of different *C. sativa* samples. The functionalization procedure of SPEs-CB, in fact, is simple and it employs cheap materials, while electrochemical measurements are very easy and fast to be carried out [28]. The voltammetric signals recorded in different extracts were used as a sort of "fingerprint" to recognize *C. sativa* plants and classify them as "illegal" or "legal". Sample classification was accomplished by multivariate classification models capable of detecting small differences in the recorded voltammetric signals, and deriving from the concentration of various cannabinoids present in the extracts. The same approach has been successfully used by some of us in the past with different matrices and for various purposes [28–31].

The responses of the electrode system were first screened via Principal Component Analysis (PCA). Then, classification models were built and validated by Partial Least Squares - Discriminant Analysis (PLS-DA). Two classes were considered in each model, corresponding to illegal and legal samples on the basis of their Δ^9-THC_{TOT} content determined by HPLC, and considering the threshold limits established for Δ^9-THC_{TOT}. Specifically, these limits were set at 0.3 %, in alignment with both EU and US legislation, and at 0.6 %, in accordance with Italian law.

2. Experimental

The entire experimental procedure, including experimental measurements and chemometric strategy, is schematized in Fig. 1S of the

Table 1
Δ^9-THC_{TOT} and CBD_{TOT} values calculated from HPLC data.

# sample	Δ^9-THC_{TOT} (% w/w)	CBD_{TOT} (% w/w)	# sample	Δ^9-THC_{TOT} (% w/w)	CBD_{TOT} (% w/w)
1_A	–	3.67	26_A	0.43	12.33
1_B	–	3.82	26_B	0.38	12.19
2_A	–	1.67	27_A	0.44	11.85
2_B	–	1.66	27_B	0.47	12.17
3_A	–	1.63	28_A	0.46	13.66
3_B	–	1.41	28_B	0.50	13.69
4_A	–	3.78	29_A	0.46	–
4_B	0.18	4.43	29_B	0.74	–
5_A	–	3.21	30_A	0.50	11.54
5_B	–	3.15	30_B	0.55	12.22
6_A	–	3.22	31_A	0.67	15.37
6_B	–	3.13	31_B	0.56	12.33
7_A	–	2.80	32_A	0.73	9.21
7_B	–	2.38	32_B	0.75	9.09
8_A	–	3.16	33_A	1.39	–
8_B	–	3.05	33_B	1.76	–
9_A	–	2.65	34_A	1.53	8.79
9_B	–	2.63	34_B	1.51	6.90
10_A	–	1.07	35_A	3.31	–
10_B	–	1.09	35_B	3.02	–
11_A	–	0.66	36_A	6.62	10.47
11_B	–	0.67	36_B	5.02	7.88
12_A	–	3.90	37_A	6.83	–
12_B	–	3.87	37_B	5.57	0.39
13_A	–	6.61	38_A	7.49	6.12
13_B	–	6.42	38_B	8.44	0.59
14_A	–	5.41	39_A	9.62	–
14_B	–	5.24	39_B	9.97	–
15_A	0.151	5.82	40_A	10.02	–
15_B	0.173	6.26	40_B	10.19	–
16_A	0.157	3.55	41_A	10.62	–
16_B	0.165	3.91	41_B	13.83	–
17_A	0.183	5.87	42_A	11.37	0.33
17_B	0.175	5.80	42_B	11.78	0.32
18_A	0.249	3.84	43_A	17.94	–
18_B	0.186	3.52	43_B	11.39	–
19_A	0.206	7.10	44_A	11.63	–
19_B	0.212	6.79	44_B	19.02	–
20_A	0.214	5.91	45_A	13.14	–
20_B	0.209	5.61	45_B	14.91	–
21_A	0.253	7.74	46_A	14.80	–
21_B	0.265	7.97	46_B	16.57	–
22_A	0.254	3.79	47_A	17.66	–
22_B	0.258	4.04	47_B	15.96	–
23_A	0.295	10.32	48_A	17.20	–
23_B	0.272	9.64	48_B	17.19	–
24_A	0.335	10.71	49_A	18.21	–
24_B	0.332	10.84	49_B	18.18	–
25_A	0.374	11.15	50_A	18.68	–
25_B	0.464	12.87	50_B	21.35	–

- Means below the limit of detection (LOD) or the limit quantification (LOQ).

supplementary material.

2.1. Chemicals, solvents, and plant material

Standard solutions of Δ^9-THCA, Δ^9-THC, CBDA and CBD (1 mg/mL either in acetonitrile or methanol) were purchased from Restek Italia (Cernusco sul Naviglio, Italy) and Merck Life Science (Milan, Italy). HPLC grade acetonitrile (ACN), formic acid (HCOOH) and ethanol (EtOH) were from Sigma-Aldrich (Milan, Italy). Boric acid (H3BO3) 99.8 % pure, sodium acetate (CH3COONa) 99.8 % pure, and HCl 37 % were from Carlo Erba (Cornaredo, Italy). Sodium phosphate monobasic (NaH_2PO_4) 98 % pure was from CalbioChem, sodium hydroxide (NaOH) 98 % was from Fisher Chemical and potassium chloride (KCl) ≥99.0 % pure was from Sigma-Aldrich. Dimethylformamide (DMF) 99.8 % pure was from Scharlau (Scharlab Italia, Lodi, Italy). Water was purified using a Milli-Q Plus185 system from Millipore (Milford, MA, USA) to a final resistivity of 18 MΩ×cm.

In this work, 50 samples of *C. sativa* female inflorescences were analyzed. The fibre-type samples were provided by Assocanapa S.r.l. (Carmagnola, Turin, Italy), Materia Medica Processing S.r.l. (Siena, Italy) and Ipergrow S.r.l. (Pomezia, Rome, Italy). The recreational samples were available at the Toxicology Laboratory of the Forensic Institute of the Department of Biomedical, Metabolic and Neural Sciences of the University of Modena and Reggio Emilia. The plant material was stored at +4.0 °C in the dark, away from humidity, to prevent both degradation and decarboxylation of acidic compounds during storage.

2.2. Sample preparation

C. sativa inflorescences (0.25 g), previously deprived of seeds and twigs and properly ground, were weighted and added with 10 mL of EtOH. Cannabinoids were extracted using dynamic maceration at room temperature for 15 min. The extract was then paper-filtered and the residue was subjected to two additional extractions using 10 and 5 mL of EtOH, respectively, following the same procedure. The filtrates were then combined and brought to the final volume of 25 mL with the extraction solvent. The extracts from recreational *C. sativa* were prepared at the Toxicology Laboratory of the Forensic Institute of the Department of Biomedical, Metabolic and Neural Sciences of the University of Modena and Reggio Emilia.

The extraction procedure was carried out in duplicate for each of the 50 samples of *C. sativa* and related extracts are indicated by letters A and B. Therefore 100 extracts, indicated as 1_A, 1_B, 2_A, 2_B, etc., were submitted to electrochemical and HPLC analysis.

2.3. HPLC analysis

HPLC analysis of the extracts was performed following the experimental conditions described in ref. 27. An Agilent Technologies (Waldbronn, Germany) modular model 1260 Infinity II system, consisting of a vacuum degasser, a quaternary pump, a manual injector and a diode array detector was employed. The chromatograms were recorded using an Agilent OpenLab CDS ChemStation Edition (Rev. C.01.10). The separation of cannabinoids was achieved by using an Ascentis Express C_{18} column (150 × 3.0 mm I.D., 2.7 μm, Supelco, Bellefonte, PA, USA). The mobile phase was composed of 0.1 % HCOOH (v/v) in both (A) water and (B) ACN under gradient conditions, as follows: 0–13 min isocratic elution at 60 % B, 13–17 min from 60 to 80 % B, 17–22 min from 80 to 90 % B, which was kept for 8 min. The post-running time and the flow rate were set at 10 min and 0.4 mL/min, respectively. Chromatograms were acquired at the wavelength of 210 nm for the analysis of neutral cannabinoids (Δ^9-THC, CBD), while 220 nm was chosen for the acidic ones (Δ^9-THCA, CBDA).

The results expressed in terms of Δ^9-THC_{TOT} and of total CBD content (CBD_{TOT}) are shown in Table 1.

CBD_{TOT} was assesses according to the formula:

$$CBD_{TOT}\ (\%\ w/w) = CBD\% + (CBDA\% \times 0.877) \quad \text{Eq. (2)}$$

where CBD% and CBDA% are the concentration (%, w/w) of CBD and CBDA determined in the sample on dry weight basis, respectively, and 0.877 is the CBD/CBDA molecular weight conversion factor.

2.4. Electroanalytical tests

All electrochemical measurements were performed using an Autolab PGSTAT-30 (Eco Chemie, Utrecht, The Netherlands) potentiostat/galvanostat, under control of GPES software. Voltammetric measurements were performed with SPE-CB. They were prepared starting from a commercial SPE purchased from Sens4Med (Rome, Italy), consisting of a graphite working and counter electrodes and a silver pseudo-reference electrode. CB N220 was deposited on the working electrode surface of the SPE by drop-casting, following the procedure reported in Ref. [27].

All the electrochemical tests were carried out in a 7:3 (v/v) mixture

of Britton-Robinson buffer (BRB, pH = 7.0) and EtOH, 0.1 M KCl, freshly prepared on a daily basis. 0.1 M BRB was obtained by mixing appropriate amounts of H3BO3, CH3COONa, and NaH2PO4 in deionized water, adjusting the pH by adding either NaOH or HCl. The *C. sativa* extracts were diluted 10 times; 10 μL of extracts were mixed to 20 μL of EtOH and 70 μL of BRB to keep the 7:3 H_2O–EtOH volume proportion.

Differential Pulse Voltammetry (DPV) was used to perform electrochemical analyses. The following parameters were applied: 50 mV pulse potential, 6 mV step potential, 0.1 s pulse time, and 0.4 s interval time (15 mV/s potential scan rate). Before each measurement, the newly fabricated SPE-CB underwent 10 DPV scans in a 7:3 v/v mixture of BRB (pH = 7.0) and EtOH, 0.1 M KCl to stabilize the background signal. After that, the voltammograms recorded in the solution containing the extracts consisted of a single DPV scan in the potential range between (0.0 and + 1.1) V. The signal recorded for each extract was considered without any blank subtraction, and each solution was analyzed with a new SPE-CB.

2.5. Multivariate analysis of DPV signals

The dataset was composed of 100 DPV signals (50 *C. sativa* samples × 2 extracts for each plant sample). Each DPV consisted of 180 current values collected in the potential range from 0.0 V to +1.1 V. The dataset was first subjected to exploratory data analysis by means of PCA using standard normal variate and mean centering (SNV + MC) as data preprocessing methods.

Various classification models were then calculated using PLS-DA, taking into account both different types of signal preprocessing and different threshold limits to distinguish between illegal and legal samples. As for the preprocessing methods, four combinations were considered: standard normal variate and mean centering (SNV + MC), first-order derivative and mean centering (D1 + MC), second-order derivative and mean centering (D2 + MC) and quadratic detrend and mean centering (QD + MC). Regarding the threshold limits, the models were built by setting the threshold that identifies legal samples both as those with Δ^9-THC_{TOT} <0.3 % (EU and US), and as those with Δ^9- THC_{TOT} <0.6 % (IT). In the first case, the dataset consisted of 46 signals identified as legal samples and 54 as illegal samples; in the second case, the dataset consisted of 60 signals identified as legal samples and 40 as illegal samples.

To calculate the PLS-DA models, the dataset was randomly divided into a calibration set (training set) containing 70 signals, used to build the models, and an external validation set (test set) containing the remaining 30 signals, used to validate the models. The signals obtained from the two extracts of the same plant were always kept in the same set. In this way, the test set included 12 legal and 18 illegal samples when the threshold limit was set at Δ^9-THC_{TOT} = 0.3 % and 16 legal and 14 illegal samples when the threshold limit was set at Δ^9-THC_{TOT} = 0.6 %.

The optimal number of latent variables (LVs) was chosen by minimizing the Classification Error, that was estimated using a venetian blinds cross-validation with 10 deletion groups, each including both extracts from the same plant sample. The performance of the classification models was quantified in terms of sensitivity (SENS), specificity (SPEC) and efficiency (EFF) estimated in calibration (CAL), cross-validation (CV), and prediction of the test set (PRED).

Considering the relatively low number of available samples, to further verify the statistical significance of the two best PLS-DA models (one for each threshold limit), a permutation test was also implemented, which consisted in repeatedly and randomly reassigning the samples of the training and of the test set to the two classes (illegal and legal) in a way that they did not match anymore the true groupings. The number of samples assigned to each class was the same as in the original (correct) class assignment, and the two replicates of each sample were always assigned to the same class. For each one of the 100 permutations considered in the present work, a PLS-DA model was calculated on the training set considering the same number of LVs of the correct model and the same crossvalidation procedure, and then it was applied to the test set. For each permutation, the EFF values were calculated in CAL, CV and PRED, and finally their distributions were compared with the corresponding values of the correct model using a one-tailed *t*-test (P = 95 %).

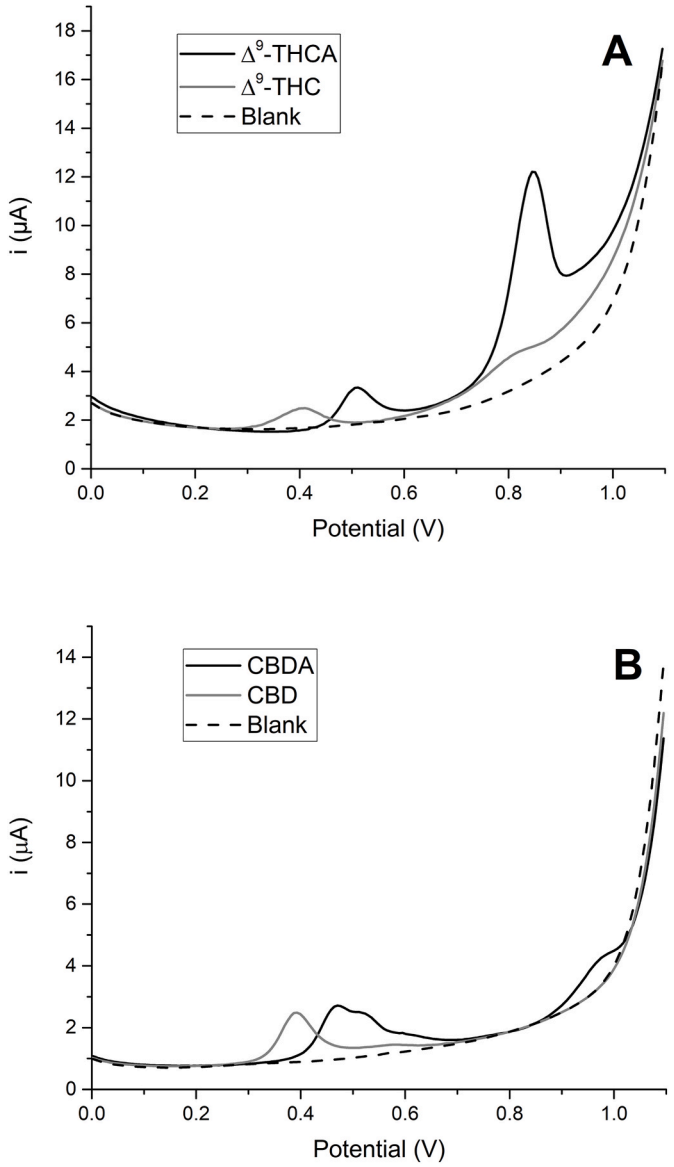

Fig. 2. Voltammetric signals recorded using SPE-CB in 7:3 v/v mixture of BRB (pH = 7.0) and EtOH, 0.1 M KCl in the absence (dotted line) and in the presence of A) 50 μM Δ^9-THCA (black line) and of 50 μM Δ^9-THC (grey line); B) 50 μM CBDA (black line) and of 50 μM CBD (grey line).

The PCA and PLS-DA models were calculated using the PLS Toolbox (ver. 8.8.1, Eigenvector Research Inc., USA) running into the Matlab 9.3 (R2019b) environment (The Mathworks Inc., Natick, MA, USA). The permutation test was calculated using a Matlab function written *ad hoc*.

3. Results and discussion

3.1. Electrochemical analysis of C. sativa samples

The main cannabinoids present in *C. sativa* inflorescences are CBDA and Δ^9-THCA, depending on the chemotypes. The neutral forms, namely CBD and Δ^9-THC, may also be present. All these compounds are electroactive, and they give rise to voltammetric signals characterised by

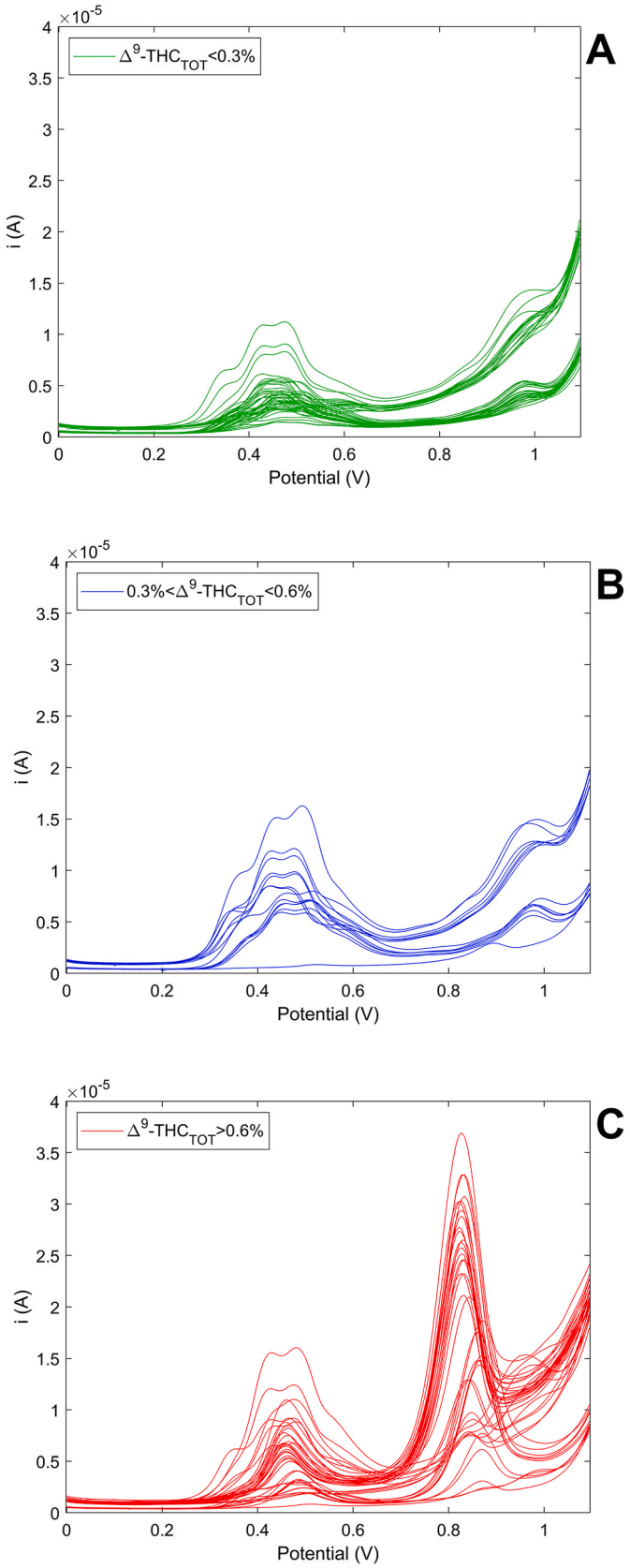

Fig. 3. Comparison of the DPV signals recorded in various *C. sativa* extracts diluted in 7:3 v/v mixture of BRB (pH = 7.0) and EtOH, 0.1 M KCl using SPEs-CB. A) DPV signals of samples with Δ^9-THC_{TOT} <0.3 %; B) DPV signals of samples with 0.3 %< Δ^9-THC_{TOT} <0.6 % and C) DPV signals of samples with Δ^9-THC_{TOT} >0.6 %.

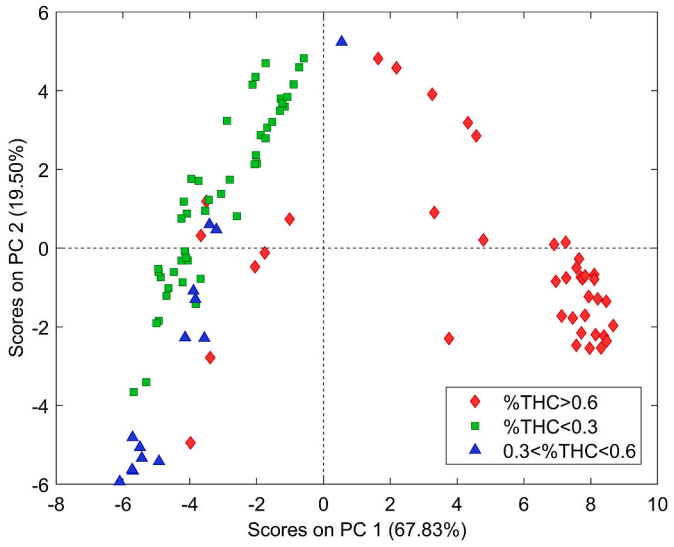

Fig. 4. PC1vsPC2 score plot of the DPV signals obtained by SPEs-CB.

peculiar features. This is well evident from Fig. 2A and B, showing the DPV signals recorded at SPEs-CB in standard solutions of Δ^9-THC and Δ^9-THCA and of CBD and CBDA, respectively. The signals of Δ^9-THCA and Δ^9-THC are significantly different from each other: two well defined oxidation peaks at +0.50 V and at +0.84 V are present in the DPV signal of Δ^9-THCA, while Δ^9-THC is characterised by a single peak at +0.41 V and of a shoulder at +0.80 V. The DPV response of CBDA shows a first oxidation process centered at ca. +0.50 V, characterised by the overlap of several peaks, and a second oxidation process at +0.97 V. CBD is characterised by the presence of a well-defined oxidation peaks located at +0.39 V and of a shoulder at +0.57 V.

Voltammetric signals with features similar to those shown in Fig. 2A and B were obtained from the analysis of the different extracts. Fig. 3 shows the voltammograms recorded in the extracts of *C. sativa* samples having different percentage of Δ^9-THC_{TOT}, according to the quantification deriving from chromatographic analysis. In particular, Fig. 3A shows the DPV signals recorded in all the samples having Δ^9-THC_{TOT} <0.3 %, whereas Fig. 3B shows the DPV signals recorded in all the samples having 0.3 %< Δ^9-THC_{TOT} <0.6 % and Fig. 3C shows the DPV signals recorded in all the samples having Δ^9-THC_{TOT} >0.6 %.

As a general consideration, all the recorded voltammograms show a system of peaks located at ca. 0.45 V. All the investigated cannabinoids have characteristic peaks in the potential range comprised between 0.3 and 0.6 V, as shown in Fig. 2A and B. Therefore, what effectively characterizes the responses recorded from extracts possessing different amounts of Δ^9-THC_{TOT}, grouped in Fig. 3A, B and 3C, are the peaks centered at higher potential values. In particular *C. sativa* samples having Δ^9-THC_{TOT} <0.6 % (Fig. 3A and B) give rise to voltammograms characterized by a peak or a shoulder at ca. +0.95 V, typical of CBDA whereas the voltammograms deriving from *C. sativa* samples having Δ^9-THC_{TOT} >0.6 % (Fig. 3C) show a well-defined peak at +0.8 V, typical of Δ^9-THCA. These pieces of evidence suggest the possibility of discriminating samples on the basis of their respective voltammetric signals.

3.2. PCA on the DPV signals of C. sativa extracts

A PCA model was calculated on the electrochemical signals recorded at the SPE-CB electrode for exploratory data analysis purposes, with the aim of evidencing meaningful patterns within the dataset, without making any a priori assumption.

The score plot of the first two PCs obtained by the PCA model (accounting for 87.33 % of total data variance) is shown in Fig. 4. A clearly visible clustering occurs along PC1 (67.83 % of explained variance):

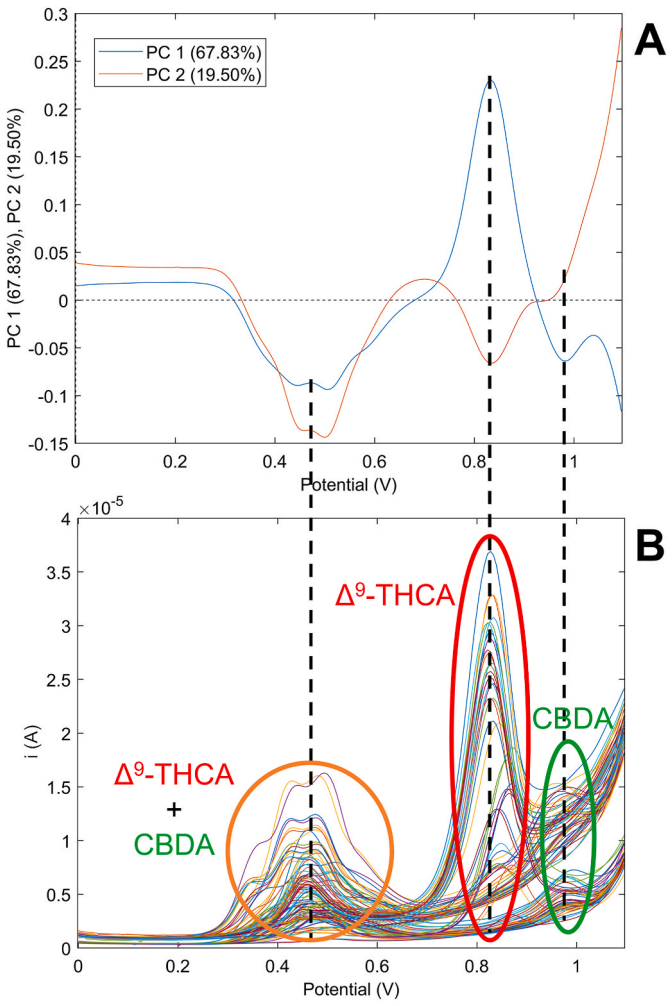

Fig. 5. A) loadings plots for PC1 and PC2 obtained by PCA on the DPV signals; B) original DPV signals. The vertical dashed lines connect the maximum/minimum values of the loading plots to relevant features of the original signals.

most of the samples with Δ^9-THC_{TOT} >0.6 % have positive PC1 values, while samples with Δ^9-THC_{TOT} <0.6 % have generally negative PC1 values. Notably, the samples with Δ^9-THC_{TOT} >0.6 % located at negative PC1 values are those simultaneously containing CBDA at a very high concentration value. A partial separation between samples having 0.3 %< Δ^9-THC_{TOT} <0.6 % and Δ^9-THC_{TOT} <0.3 % occurs along PC2 (19.50 % of explained variance); indeed, most of the samples with 0.3 %< Δ^9-THC_{TOT} <0.6 % have PC2 negative values. Overall, the first two principal components of the model suggest the possibility to differentiate the samples based on the total Δ^9-THC content in the sample.

The PC1 and PC2 loading plots shown in Fig. 5 provide an interpretation of what observed in the corresponding score plot, indicating the contributions of different regions of the electrochemical signal to the directions of the principal components. For a clearer interpretation of the loading vectors, Fig. 5 compares the loading plots (Fig. 5A) and the original signals (Fig. 5B), in order to easily identify the most useful regions for distinguishing samples with high Δ^9-THC_{TOT}. Fig. 5A shows that the highest positive loading values of PC1 are located in the potential region corresponding to the second peak of Δ^9-THCA. This is consistent with the position in the score plot in Fig. 4 of the samples colored in red, which, having Δ^9-THC_{TOT} >0.6 % and a very intense second peak of Δ^9-THCA, are almost all located at positive PC1 values. The loadings of PC1 also show the contributions related to the first and second CBDA peaks, but with negative values. This is also consistent with the score plot and with the different types of original signals in Fig. 3, as samples with Δ^9-THC_{TOT} >0.6 % generally exhibit less intense peaks at ca. +0.95 V for CBDA, attributable to lower content of this cannabinoid compared to other samples. On the other hand, the main contribution to the loadings of PC2 is given by the system of peaks at lower potential values in the voltammograms, primarily due to CBDA, and by the second peak of Δ^9-THCA. Both contributions have negative values. Consistently, the blue-colored samples in Fig. 4, ie., those with 0.3 %< Δ^9-THC_{TOT} <0.6 %, are almost all grouped in the quadrant with negative values of both PC1 and PC2, since almost all these samples show the highest values of CBDA concentration and, at the same time, a Δ^9-$THCA_{TOT}$ content above the EU and US legal limits.

3.3. Classification of C. sativa samples on DPV signals with PLS-DA

Based on the encouraging results obtained from the exploratory data analysis, the electrochemical signals were then used to build

Table 2
Classification results of the PLS-DA models obtained using different preprocessing methods. The values of SENS and SPEC are referred to the class of illegal samples; note that, for a given couple of SENS and SPEC values, SENS(legal) = SPEC(illegal) and SPEC(legal) = SENS(illegal). The higher the SENS/SPEC/EFF value, the greener the colour of the corresponding cell.

	Threshold 0.3%					Threshold 0.6%				
Preprocessing	**#LV**	**SET**	**SENS**	**SPEC**	**EFF**	**#LV**	**SET**	**SENS**	**SPEC**	**EFF**
SNV+MC	2	CAL	0.806	0.912	0.857	6	CAL	0.923	0.977	0.950
		CV	0.778	0.912	0.842		CV	0.885	1.000	0.941
		PRED	0.722	1.000	0.850		PRED	1.000	1.000	1.000
D1+MC	2	CAL	0.750	0.941	0.840	4	CAL	0.885	0.977	0.930
		CV	0.722	0.912	0.811		CV	0.846	0.977	0.909
		PRED	0.722	1.000	0.850		PRED	0.857	1.000	0.926
D2+MC	2	CAL	0.778	0.912	0.842	4	CAL	0.885	0.977	0.930
		CV	0.750	0.912	0.827		CV	0.846	0.977	0.909
		PRED	0.833	1.000	0.913		PRED	0.857	1.000	0.926
QD+MC	3	CAL	0.778	0.941	0.856	5	CAL	0.885	0.977	0.930
		CV	0.694	0.941	0.808		CV	0.846	0.955	0.899
		PRED	0.722	1.000	0.850		PRED	0.857	1.000	0.926

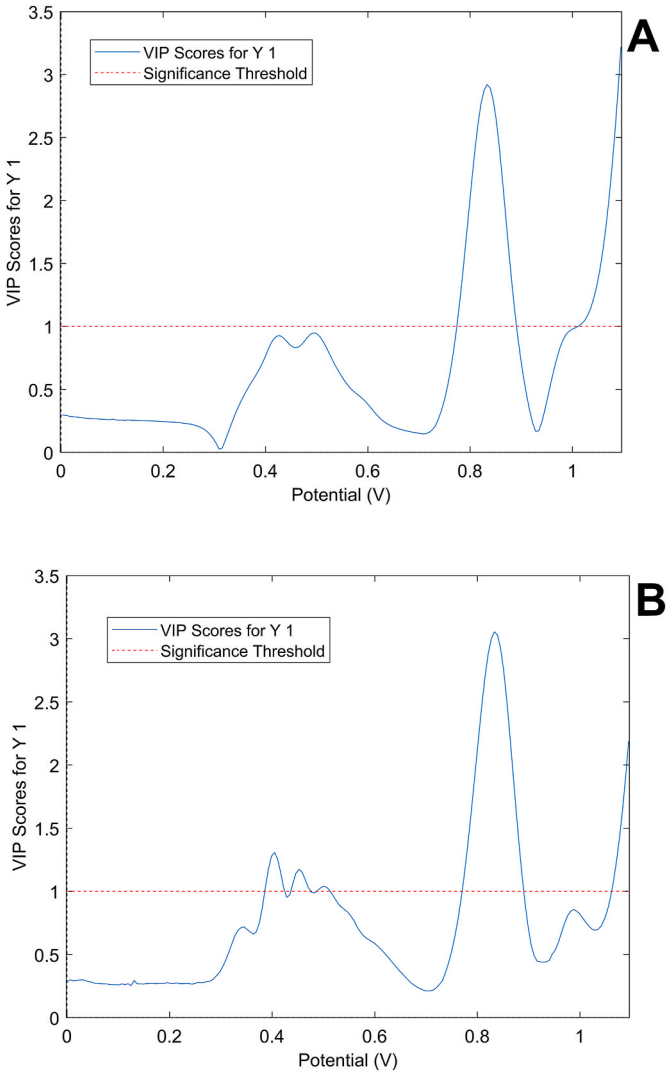

Fig. 6. VIP scores plots of PLS-DA model with Δ^9-THC_{TOT} = 0.3 % threshold limit (A) and with Δ^9-THC_{TOT} = 0.6 % threshold limit (B). The horizontal dashed line indicates the threshold value (=1).

classification models, in order to evaluate the capability to classify *C. sativa* samples based on threshold values of practical interest. To this purpose, both the threshold limits of Δ^9-THC_{TOT} = 0.3 % and Δ^9-THC_{TOT} = 0.6 % were considered to distinguish between legal (below the limit) and illegal (above the limit) samples.

The results in terms of SENS, SPEC and EFF values for calibration (CAL), cross-validation (CV), and prediction (PRED) as a function of the different preprocessing methods used are shown in Table 2. All calculated models showed performance ranging from good to excellent and, in general, the models calculated considering the Δ^9-THC_{TOT} threshold limit of 0.3 % required fewer latent variables but led to lower classification efficiency values, compared to the models calculated considering the Δ^9-THC_{TOT} threshold limit of 0.6 %. Additionally, a consistent observation across all models is that the sensitivity values are higher for the class of legal samples, i.e., these samples are correctly identified as belonging to their respective class more frequently than illegal samples.

The best preprocessing method for both the threshold limits was found to be SNV + MC, as it yielded the highest EFF values in CV. Using this pretreatment, a correct prediction of all the test set samples was reached by the model with threshold limit of Δ^9- THC_{TOT} = 0.6 %. Regarding the model with the threshold limit of Δ^9-THC_{TOT} = 0.3 %, it accurately predicted 100 % of legal samples and 72.2 % of illegal samples. This indicates that 13 out of the 18 illegal samples in the test set were classified correctly.

The Variable Importance in Projection (VIP) scores plots for the model with the threshold limit set at Δ^9-THC_{TOT} = 0.3 % and Δ^9-THC_{TOT} = 0.6 % are shown in Fig. 6A and B, respectively. According to the "greater than one" criterion, the signal variables positioned above the red dashed lines in Fig. 6 – marked at a value of 1 – are recognized by the models as the most informative for distinguishing between legal and illegal samples [31]. Therefore, the most significant contribution in both models for the identification of legal and illegal samples is represented by the second peak due to Δ^9-THCA oxidation, which is located in the potential range around +0.84 V.

This explains why illegal samples with low Δ^9-THCA content, resulting in a second peak of very low intensity in the voltammograms, can be misclassified by the model. This is exemplified by the 5 samples of the test set of the model with threshold limit of Δ^9-THC_{TOT} of 0.3 % that were erroneously classified as legal. In these samples, Δ^9-THC_{TOT} ranges between 0.3 % and 0.6 %, but the signal of Δ^9-THCA is either very low or masked by the signal of CBDA, as shown in Fig. 3B. Actually, these samples represent borderline cases. It is known that some of the products marketed as hemp have a Δ^9-THC_{TOT} content slightly exceeding the legal limit, thus potentially falling under the classification of marijuana [32]. On the other hand, it is possible to clearly distinguish a sample with Δ^9-THC_{TOT} well above the legal limit. Therefore, we can state that the developed SPE-CBs remain suitable for rapid screening, enabling clear discrimination between *C. sativa* samples with high concentrations of Δ^9-THC and Δ^9-THCA and those primarily containing CBD and CBDA.

The statistical significance of the best classification models, i.e., of the PLS-DA models calculated using the SNV + MC signal preprocessing considering both the 0.3 % and the 0.6 % threshold limits of Δ^9-THC_{TOT}, was also checked by means of a permutation test. Fig. 7 shows the results of the permutation test, where the EFF values of the correct model (full squares) and of the permutations (empty circles) are reported as a function of the percentage of matching between the correct class assignation and the randomly shuffled one. The first row shows the results of the model calculated considering the 0.3 % threshold limit, while the second row is referred to the 0.6 % threshold limit; the three columns are referred to the EFF values estimated in calibration of the training set (left), in cross-validation (middle) and in prediction of the test set (right).

To evaluate whether the EFF values obtained for the correct models (i.e., the best PLS-DA models) were statistically significant, they were compared with the distribution of the corresponding EFF values from permutations, using a one-tailed *t*-test (P = 0.05).

Table 3 shows the EFF values calculated using the best PLS-DA models, the mean (m) and standard deviation (s) of the EFF values resulting from the permutation test, and the corresponding results of the one-tailed t-tests. The reported results show that the significance of the EFF values obtained with the best PLS-DA models are much lower than 0.05, confirming that the good performance of the classification models is not due to chance.

4. Conclusions

In this paper, we explored the feasibility of distinguishing legal and illegal *C. sativa* samples through a simple and fast voltammetric method using SPE-CB sensors, coupled with multivariate analysis of the signal dataset.

Exploratory PCA revealed the clustering of the majority of samples with Δ^9-THC_{TOT} lower or higher than 0.6 %, mainly due to the levels of Δ^9-THCA and CBDA. Afterwards, PLS-DA models were built based on two different threshold limits, corresponding to the maximum values of Δ^9-THC_{TOT} allowed for the cultivation and commercialization of *C. sativa* samples: in both the European Union and United States this limit is set at a Δ^9-THC_{TOT} value of 0.3 %, but according to the Italian regulation contents of Δ^9-THC_{TOT} up to 0.6 % can be tolerated. In the

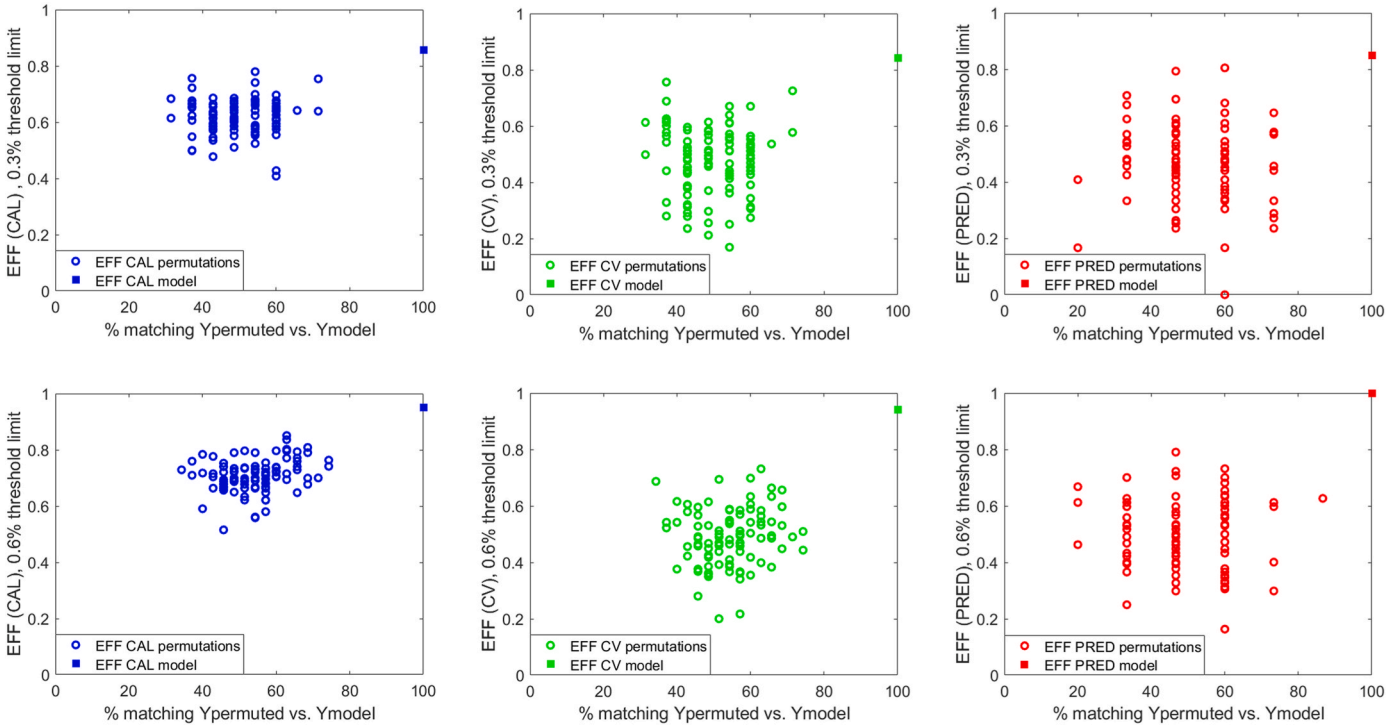

Fig. 7. Comparison of the classification efficiency values obtained for the best PLS-DA models (full squares) with the corresponding values obtained with the permutation test (empty circles).

Table 3
Results of the one-tailed t-tests performed on the EFF values shown in Fig. 7.

Threshold	Parameter	Best model	Permutations		One tailed *t*-test		
			m	s	t_{CRIT}[a]	t_{CALC}	$P(t_{CALC})$
0.3 %	**EFF (CAL)**	0.857	0.616	0.063	1.660	3.835	1.11×10^{-4}
	EFF (CV)	0.842	0.478	0.118	1.660	3.079	1.35×10^{-3}
	EFF (PRED)	0.850	0.457	0.150	1.660	2.623	5.05×10^{-3}
0.6 %	**EFF (CAL)**	0.950	0.708	0.058	1.660	4.184	3.10×10^{-5}
	EFF (CV)	0.941	0.489	0.101	1.660	4.488	9.71×10^{-6}
	EFF (PRED)	1.000	0.495	0.125	1.660	4.052	5.05×10^{-5}

[a] $P = 0.05$; $df = 99$.

latter case, an excellent result was achieved (classification prediction EFF = 100 %), while for the EU/US threshold limit the result was good, although not all illegal samples were recognized as such. Indeed, a misclassification occurred for samples with Δ^9-THC_{TOT} slightly exceeding the legal limit and also containing high concentration of CBDA and CBD. This is the case for many products marketed as hemp, which actually have a borderline composition, as they exhibit a Δ^9-THC_{TOT} content just above the legal limit. These samples should be classified as marijuana according to the actual US regulation [32] and they may pose a particular challenge for forensic laboratories. On the other hand, it must be considered that nowadays *C. sativa* cultivated for illegal market generally possesses a content of the psychoactive cannabinoid even 50 times higher than the legal limits [33], so that we can finally assume that the proposed model ensures 100 % correct classification in these cases.

In conclusion, this study confirms the effectiveness of SPE-CB sensors and of the proposed chemometric procedure for a very fast routine control of *C. sativa* samples. In perspective, the system could find even *in situ* applications. In fact, the next step of this work will be focused on the integration of the sensing/classification system into a portable and easy to use device to achieve a rapid in-situ analysis of *C. sativa* samples, also integrating the automatic extraction procedure [34]. To this aim, the dataset size will be significantly increased by analysing a high number of samples, in order to improve the robustness of the classification model and to better estimate the prediction error.

CRediT authorship contribution statement

Alessandro Monari: Investigation, Validation, Formal analysis, Data Curation, Writing – original draft. **Giorgia Foca:** Methodology, Validation, Data Curation, Writing – review & editing. **Alessandro Ulrici:** Methodology, Validation, Data Curation, Writing – review & editing. **Barbara Zanfrognini:** Writing – review & editing, Visualization. **Virginia Brighenti:** Investigation. **Patrizia Verri:** Investigation, Resources. **Federica Pellati:** Methodology, Resources, Writing – review & editing. **Chiara Zanardi:** Methodology, Funding acquisition, Writing – review & editing. **Laura Pigani:** Conceptualization, Methodology, Resources, Writing – Original Draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2024.126958.

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