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Analytical quality by design: Development and control strategy for a LC method to evaluate the cannabinoids content in cannabis olive oil extracts

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ABSTRACT

Cannabidiol (CBD) and Δ^9-tetrahydrocannabinol (Δ^9-THC) are considered as the most interesting cannabinoids in *Cannabis sativa L.* for the clinical practice. Since 2013, the Italian law allows pharmacists to prepare and dispense cannabis extracts to patients under medical prescription, and requires the evaluation of CBD and Δ^9-THC content in cannabis extracts before sale. Cannabis olive oil extracts are prepared from dried female cannabis inflorescences, but a standard protocol is still missing. In this study, a fast RP-HPLC/UV method has been developed to quantify CBD and Δ^9-THC in cannabis olive oil extracts. The analytical quality by design strategy has been applied to the method development, setting critical resolution and total analysis time as critical method attributes (CMAs), and selecting column temperature, buffer pH and flow rate as critical method parameters. Information from Doehlert Design in response surface methodology combined to Monte-Carlo simulations led to draw the risk of failure maps and to identify the method operable design region. The method was validated according to the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines and then implemented in routine analysis. A control strategy based on system control charts was planned to monitor the developed method performances. Evaluation data were recorded over a period of one year of routine use, and both the CMAs showed values within the specifications in every analysis performed. Hence, a new risk evaluation for the future performances of the method was achieved by using a Bayesian approach based on the routine use data, computing the future distribution of the two CMAs. Finally, a study focusing on the monitoring of CBD and Δ^9-THC concentrations in cannabis olive oil extracts was carried out. The developed method was applied to 459 extracts. The statistical analysis of the obtained results highlighted a wide variability in terms of concentrations among different samples from the same starting typology of cannabis, underlining the compelling need of a standardised procedure to harmonise the preparation of the extracts.

1. Introduction

Cannabis sativa L., belonging to the Cannabaceae family, is able to biosynthesize a wide variety of secondary metabolites: 565 chemical compounds have been identified to date, among which about 120 cannabinoids (CNBs) [1]. CNBs are a unique class of terpenophenolic compounds found only in *Cannabis sativa* L. They are biosynthesized and accumulated in glandular trichomes, which are densely expressed in reproductive structures of female plants. These compounds are synthesized as their corresponding

Abbreviations: ACN, acetonitrile; AQbD, analytical quality by design; ATP, analytical target profile; CBD, cannabidiol; CBD-A, cannabidiolic acid; CBN, cannabinol; CNBs, cannabinoids; CMA, critical method attribute; CMP, critical method parameter; DoE, Design of Experiments; ICH, International Council for Harmonisation; MeOH, methanol; MODR, method operable design region; QbD, quality by design; RSM, response surface methodology; Δ^9-THC, Δ^9-tetrahydrocannabinol; THF, tetrahydrofuran.

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carboxylic acid forms and are decarboxylated into their neutral forms, during exposure to light, heat or as a result of prolonged storage [2]. The main cannabinoid acids found in fresh plant material are cannabigerolic acid, Δ^9-tetrahydrocannabinolic acid A, cannabidiolic acid (CBD-A) and cannabichromenic acid [3]. The main neutral CNBs found in dry cannabis inflorescence are Δ^9-tetrahydrocannabinol (Δ^9-THC), cannabidiol (CBD), cannabigerol, as well as cannabichromene and cannabinol (CBN) [4]. Δ^9-THC and CBD (Supplementary Fig. S1) are recognized as the compounds with a pharmaceutical importance and represent a therapeutic option for many pathologic conditions, as in analgesia for neuropathic pain, in chemotherapy-related nausea and vomiting and in other conditions such as HIV-associated anorexia [5,6].

There are several medical cannabis products and magistral preparations with different Δ^9-THC and CBD ratios on the market; the most common formulations are cannabis decoction filter bags, unit doses formulations for inhalation and cannabis extracts, mainly in olive oil or ethanol [7]. Cannabis olive oil extracts are prepared by pharmacists from dried cannabis inflorescences and present several advantages: first, the positive effects of phytoextracts, where there is a mixture of many CNBs, are strengthened with respect of those of the single CNBs [8]. Furthermore, pharmacists do not have the need of highly specialized equipments to prepare the formulation, which is ready to be taken by patients without other manipulations [9]. The most common method adopted to prepare cannabis olive oil extracts consists in adding dried cannabis inflorescences (10 g) to pharmaceutical grade olive oil (100 mL). Then, the oily solution is put in a water bath at 100 °C in order to perform the decarboxylation of acidic CNBs, and finally a filtration step concludes the manufacturing procedure [9]. However, this procedure is not a standardised protocol, and each pharmacist is allowed to make variations, leading to differences in both the extraction and the decarboxylation efficiencies and thus to formulations with a high variability in CNBs concentrations among different pharmacies and batches [10].

Several chromatographic methods have been described for the determination of the major CNBs [6]. GC offers short analysis time and high resolution in the analysis of the major CNBs present in cannabis plants and in biological samples, however it does not allow to distinguish between the acidic and neutral CNBs due to the heat applied, inducing the decarboxylation of acidic compounds [9]. Additionally, the thermal conversion of the acidic CNBs may not be total, and the drastic conditions could lead to the opening of the Δ^9-THC ring or to its oxidation [11]. LC techniques offer the possibility to operate at room temperature and allow to distinguish between the acidic and neutral CNBs, and have been applied to evaluate the original CNBs composition in cannabis samples [12–19]. RP-HPLC-UV is suited for the analysis of plant materials with a high content of CNBs; it has lower sensitivity and selectivity than RP-HPLC-MS, but it is less expensive and widely present in most laboratories.

According to the Italian legislation, "the titration of active ingredient/s must be performed for each magistral preparation with sensible and specific methodologies" [20]. As it is not well specified what active ingredients it refers, the analysis of cannabis extracts is currently performed evaluating only Δ^9-THC and CBD concentrations [10]. Therefore, the aim of this study was the development of a simple RP-HPLC-DAD method, which could match the Italian law requirements and could easily be applied to monitor the magistral preparations in terms of Δ^9-THC and CBD content. The method was set up to perform the routine analyses in a lower analysis time with respect to previous methods. These mostly present long run times because usually they are aimed to isolate as many compounds as possible, for example to determine the original composition of plant cannabinoids [12] or to characterize different cultivars [13]. The method was optimized by analytical quality by design (AQbD) [21–24], and within this approach another novelty of the study was the planning of a comprehensive control strategy for achieving a complete risk evaluation, based on control charts and on a Bayesian approach.

AQbD principles directly derive from quality by design (QbD), a quality paradigm introduced in the pharmaceutical field by the US Food and Drug Administration and reported in International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) Q8(R2) guideline [25]. QbD leads to obtain the design space by using statistical tools and methods such as Design of Experiments (DoE) [26,27]. The design space consists in a multidimensional region where the quality is guaranteed in a specific selected percentage [21,25]. The quality of analytical separation methods can be controlled and improved by using the AQbD approach [21,22,28], described in an increasing number of papers involving different techniques such as HPLC [12,29], UHPLC [30], SFC [31,32] and CE [33,34]. The main advantages of this approach are the wide knowledge about the method acquired during its development, and the possibility to define a method operable design region (MODR). Each point within the MODR can be chosen as working point and then validated for the quantitative performances. The use of Design of Experiments (DoE) allows to gain a proper knowledge about how critical method parameters (CMPs) influence the critical method attributes (CMAs). This is crucial to better understand and subsequently control the risk associated to the method performances. The control strategy plays a key-role in the risk management [24] and is defined by ICH guideline Q10 as "*a planned set of controls, derived from current product and process understanding, that assures process performance and product quality*" [35]. This part of the AQbD approach is generally overlooked in many studies. In the present study, an efficient control strategy was implemented as a part of the AQbD approach followed for the developed RP-HPLC method, giving an analysis of the method performance over the first year of its routine use. Control charts and estimation of failure risk in routine were implemented as control strategy tools. During this period, 459 cannabis olive oil extracts prepared by pharmacists of the Florentine area were analysed to evaluate their Δ^9-THC and/or CBD content. The collected data were statistically treated, pointing out a wide variability in Δ^9-THC and CBD content also from the same starting typology of cannabis, and thus highlighting the necessity to standardise the procedures used by pharmacists for the preparation of the extracts.

2. Materials and methods

2.1. Chemicals and reagents

All reagents used to prepare solutions and to perform the chromatographic elution were HPLC grade. Milli-Q water used to prepare buffer solutions was obtained by a Millipore® purification system (Billerica, MA, USA). K_2HPO_4 and HCl were purchased from Carlo Erba (Milan, Italy), acetonitrile (ACN) and tetrahydrofuran (THF) from Sigma-Aldrich (St. Louis, MO, USA), while methanol (MeOH) from Honeywell (Seelze, Germany). Reference standards for Δ^9-THC and CBD were purchased from o2si (Charleston, SC, USA) and reference standards for CBD-A and CBN were obtained from Sigma-Aldrich. The analysed formulations were prepared from Bedrocan®, Bediol® and Bedrolite® (Bedrocan BV, Veendam, The Netherlands) with standardized Δ^9-THC and CBD concentrations: 22%, 6.3%, <1% of Δ^9-THC and <1%, 8%, 9% of CBD, respectively. Other formulations were prepared from FM2®, available only within the Italian territory and produced by the Military Pharmaceutical Chemical Works of Florence (Florence, Italy), with 5–8% labelled content of Δ^9-THC and 7.5–12% of CBD.

2.2. Solutions, buffers and cannabis samples

The standard stock solutions of Δ^9-THC (0.4 mg mL^{-1}) and CBD (0.4 mg mL^{-1}) were prepared in MeOH. Phosphate buffers were prepared by mixing a proper volume of 50 mM K_2HPO_4 with 1 M HCl to the desired pH and then with ultrapure water to the desired volume to obtain a final concentration of 5 mM. As for cannabis samples, the starting sample called "solution A" was a pool of real cannabis olive oil extracts samples coming from different galenic laboratories of pharmacies. The "solution A", entirely made up of FM2® olive oil extracts samples, was kept far from heat and light sources during storage. 40 μL of "solution A" were added to 960 μL of THF, obtaining "solution B". Injected samples were obtained by adding 950 μL of MeOH to 50 μL of "solution B" (a total dilution of 500 times of the starting sample). The analysed formulations were prepared in two different ratios for each cannabis variety. Bedrocan® 100 mg mL^{-1} and Bedrocan® 70 mg mL^{-1} were obtained by adding 1 mL of pharmaceutical grade olive oil for each 100 mg or 70 mg of cannabis raw material, respectively, and the same ratios were prepared for the other cannabis varieties (Bediol®, Bedrolite® and FM2®).

2.3. HPLC equipment and conditions

All chromatographic runs were carried out using a Thermo-Fisher Surveyor Plus™ HPLC system (Thermo Fisher Scientific, Waltham, MA, USA) consisting of a quaternary solvent pump, an autosampler and a DAD (UV–vis). Chromatographic separations were achieved using an Agilent Poroshell® 120 SB-C18 analytical column (2.7 μm, 150 mm × 2.1 mm i.d.) (Agilent Technologies, Santa Clara, CA, USA), protected by an Agilent Poroshell® SB-C18 guard column (2.7 μm, 5 mm × 2.1 mm i.d.). Detection wavelength was 222 nm and injection volume was 10 μL. Equipment control, data acquisition and integration were performed with ChromQuest™, 4.2.34 version (Thermo Fisher Scientific, Waltham, MA, USA). The final selected working conditions (with related range defining the MODR) were the following: the mobile phase consisted of a mixture of ACN/water containing 5 mM of K_2HPO_4 adjusted to pH 3.45 (range 3.11–3.50) in 75/25 v/v ratio. The flow rate was set to 0.38 mL min^{-1} (0.31–0.39 mL min^{-1}) and the column temperature was 53 °C (44–55 °C).

2.4. Validation

Selectivity was evaluated by performing four injections of the sample in the optimized conditions. System repeatability was assessed performing six replicated injections of the sample on three consecutive days and calculating the within-day and between-day RSD values of CBD and Δ^9-THC areas and analysis time. For evaluating linearity, two samples for each of six different concentration values of both the analytes were analyzed in the range 0.001–0.040 mg mL^{-1} in order to cover the expected range for concentration values obtained from the dilution of the real samples. Accuracy and precision as degree of repeatability were evaluated by performing three replicated injections at three concentration levels covering the linearity range.

2.5. Software

Modde® 10 software package [36] was purchased from S-IN (Vincenza, Italy) and was employed to generate the Doehlert Design used for response surface methodology (RSM), to perform data analyses and to find the MODR by means of risk failure maps calculated using Monte-Carlo simulations. In Monte-Carlo simulations, different possible original set-points were calculated, expanding the distribution of each factor symmetrically around the set-point until one or more response limits are exceeded according to the specified defects per millions opportunities outside specifications (DPMO) target. The DPMO was used a stop criteria in MODR estimation and was set as 50,000 (5% risk of failure).

Nemrod-W software package [37] was used to generate the Plackett-Burman designs used to select the testing points at the edges of the MODR and to test robustness, and to statistically treat the related responses. A user-written R code [38] was used to analyse the routine use data, especially to compute a probability or risk of failure to meet the specifications in routine.

3. Results and discussion

This section consists of three parts: method optimisation and development, control strategy and monitoring study. The first part concerns all the practical aspects about the AQbD development strategy adopted, while for further theoretical concepts the reader can refer to recent publications [24,39]. In the second part, the attention is focused on the implementation of the analytical method in routine use. More specifically, after one year of method life, all the collected data have been analysed to evaluate if the method performances have remained within the specifications defined during the development. For this purpose, a Bayesian regression model [40] has been used to predict the future behavior of the method based on the routine data and to compute a risk or probability of failure to meet the defined specifications. Lastly, in the third section, the results of a monitoring study concerning the CNBs concentrations found in magistral preparations are presented.

3.1. Method optimisation and development

The development of the analytical method according to the AQbD strategy involved the following steps: (i) definition of analytical target profile (ATP) and scouting phase; (ii) definition of CMAs and CMPs for quality risk assessment; (iii) use of DoE for method optimisation; (iv) definition of MODR; (v) working point definition and robustness assessment.

3.2. Analytical target profile and scouting phase

The first step was to define the ATP, that is the intended purpose of the analytical method [22,23,28]. In this study the ATP was defined by the selective determination of both CBD and Δ^9-THC in the original cannabis olive oil extracts within a maximal analysis time (t) of 6 min. Hence, all the tests carried out during the optimisation/development process were performed directly on a sample of cannabis olive oil extract after a 500 times dilution. For the selective determination of CBD, the baseline separation of CBD peak and the adjacent peak CBD-A was required. The desired resolution (Rs) value was defined as $Rs \geq 0.85$. This target value corresponds to the baseline separation of two peaks of different size [41], such as in the present case, where CBD-A peak is much lower than CBD peak.

In the scouting phase, essential information on the experimental conditions useful for approaching the ATP were obtained by means of preliminary runs and an Agilent Poroshell® 120 SB-C18 Column (2.7 μm, 150 mm × 2.1 mm i.d.) was chosen. A C18 stationary phase is the ideal for hydrophobic analytes such as CNBs in reversed phase conditions. Moreover, it is a superficially porous particles column packing whose characteristics result in high peak resolutions in short retention times. A low pressure is obtained using this type of columns on "classical" HPLC systems, with some UHPLC advantages like the high throughput. This advantage is crucial in the context of the intended routine use of the method. Two different solvents, MeOH and ACN, were tested as constituents of the

future mobile phase. The mobile phase combinations tested on the sample were the following: MeOH/5 mM buffer phosphate (75:25 v/v) and ACN/5 mM buffer phosphate (75:25 v/v). The concentration of phosphate buffer and the percentage of organic solvent were selected on the basis of preliminary experiments, taking into account the resulting selectivity and analysis time. Finally, ACN was selected as organic modifier due to a large reduction of the total run time with respect to MeOH, and an isocratic elution was considered.

3.3. Critical method attributes and critical method parameters for risk assessment

The CMAs are key response variables that should be within an appropriate range to ensure the desired quality of the analytical procedure [21,22]. In this study the CMAs were defined as the total analysis time (t), corresponding to the retention time of the last peak in the chromatogram, and the critical resolution value (Rs) between CBD peak and CBD-A peak.

The CMPs are the parameters whose variations impact one or more CMA(s) and therefore should be monitored or controlled to ensure that the method meets the desired quality. The quality of a chromatographic method could be affected by several parameters. The Ishikawa diagram reported in Supplementary Fig. S2 represents a quality risk assessment tool that classifies the various parameters into groups and helps to visualize and select the most influential ones to be further studied [24,30,42]. Based on this diagram, the identified potential risks have been classified into five different groups of parameters: column, mobile phase, injection, detection, and sample. Column temperature, buffer pH and flow rate were selected as CMPs to be deeply investigated in this study by means of DoE.

3.4. Design of Experiments and method optimisation: response surface methodology

In RSM a full quadratic model (Eq. 1) was chosen and fitted to obtain a predictive model for each CMA. The model is written as follows:

$$y = \beta_0+\beta_1x_1+\beta_2x_2+\beta_3x_3+\beta_{11}{x_1}^2+\beta_{22}{x_2}^2+\beta_{33}{x_3}^2+\beta_{12}x_1x_2 +\beta_{13}x_1x_3+\beta_{23}x_2x_3+\varepsilon \quad (1)$$

where y represents the modelled CMA, x_i one of the three CMPs, β_0 the intercept, β_i the true coefficients and ε the experimental error. The experimental domain for the three CMPs was defined as follows: pH, 2.00–3.50; temperature, 35–55 °C; flow rate, 0.30-0.40 mL min^{-1}. The domain for pH was selected keeping into account the buffering pH range for phosphate (pk_{a1} = 2.15).

A Doehlert Design for three factors was selected to estimate the parameters of the model relating each CMA to the CMPs. Geometrically the Doehlert Designs are polyhedrons based on hyper-triangles (simplexes), with a hexagon in the simplest two-factor case. They have uniform space-filling properties with an equally spaced distribution of points lying on concentric spherical shells, and the number of levels is not the same for all variables: the first factor is investigated at three levels, the second at five levels and all the others at seven levels. Doehlert Designs require a number of experiments equal to k^2+k+n, where k is the number of variables and n the number of repetitions in the centre of the experimental domain. This type of design is well suited for the RSM objective with up to 5 or 6 factors and can be effectively used for optimization and detailed understanding [43].

The experimental plan with the measured responses is reported in Table 1; each experimental run was duplicated, for a total of 26 runs. The statistical data treatment was performed using MODDE® software. During the analysis, the time response t (min) was transformed into its inverse t^{-1} to obtain a valid model. Subsequently, the models were simplified by dropping insignificant interactions terms to improve the goodness of fit and prediction criteria (coefficient of determination, R^2, and prediction coefficient of determination, Q^2). The ANOVA of the regression model of each CMA also indicated that both models were valid and significant with the following goodness of fit and prediction values, showing the adequacy of the models [43]: *Rs*, Q^2 = 0.526, R^2 = 0.799; *t*, Q^2 = 0.995, R^2 = 0.998. The values of reproducibility, describing the variation of the response under the same conditions compared to the total variation of the response, were satisfactory [43]: *Rs*, reproducibility = 0.7153; *t*, reproducibility = 0.9978.

Table 1
Doehlert Design for response surface methodology.

Exp. no.	pH	Temperature (°C)	Flow rate (mL min^{-1})	Rs	t (min)
1	3.50	45	0.35	0.96	5.83
2	3.12	55	0.35	0.84	5.13
3	3.12	48	0.40	0.85	5.08
4	2.00	45	0.35	0.76	5.94
5	2.38	35	0.35	0.76	6.89
6	2.38	42	0.30	0.79	7.26
7	3.12	35	0.35	0.85	6.93
8	3.12	42	0.30	0.85	7.26
9	2.75	52	0.30	0.86	6.23
10	2.38	55	0.35	0.75	5.10
11	2.38	48	0.40	0.74	4.99
12	2.75	38	0.40	0.77	5.84
13	2.75	45	0.35	0.84	5.97
14	3.50	45	0.35	0.99	5.84
15	3.12	55	0.35	0.88	5.06
16	3.12	48	0.40	0.93	5.00
17	2.00	45	0.35	0.84	5.93
18	2.38	35	0.35	0.80	6.91
19	2.38	42	0.30	0.79	7.21
20	3.12	35	0.35	0.82	6.95
21	3.12	42	0.30	0.82	7.25
22	2.75	52	0.30	0.83	6.19
23	2.38	55	0.35	0.82	5.04
24	2.38	48	0.40	0.78	4.96
25	2.75	38	0.40	0.72	5.85
26	2.75	45	0.35	0.79	5.91

Rs, resolution; *t*, analysis time.

The obtained models were interpreted and graphically represented using iso-response surfaces (contour plots), reported in Fig. 1. Contour plots for the *Rs* response (Fig. 1a) showed that the maximum resolution was obtained with high pH, flow rate, and column temperature values. Contour plots for *t* (Fig. 1b) highlighted that minimum analysis time was obtained with high values of temperature and flow rate. Graphic analysis of effects is reported in Supplementary Fig. S3, pointing out a quadratic effect of pH on *Rs* and a significant interaction effect between flow rate and temperature on *t*.

In order to satisfy the ATP and to arrive at an optimum point to be used for routine analysis, the required values for the CMAs were $Rs \geq 0.85$ and $t \leq 6$ min. The search of the global optimum was performed by the sweet spot plots represented in Supplementary Fig. S4. These plots are useful to highlight the combinations of CMPs leading to the desired values for one CMA (blue zone) and those leading to the desired values for both the CMAs (green zone), also called sweet spot. It was evidenced that passing from lower to higher values of flow rate the green area becomes much wider. However, these plots cannot be directly used to define the MODR. In fact, for this aim the uncertainty of the prediction of individual values of CMAs must be considered, and it is very often a smaller zone than that suggested by sweet spot plots [21–24].

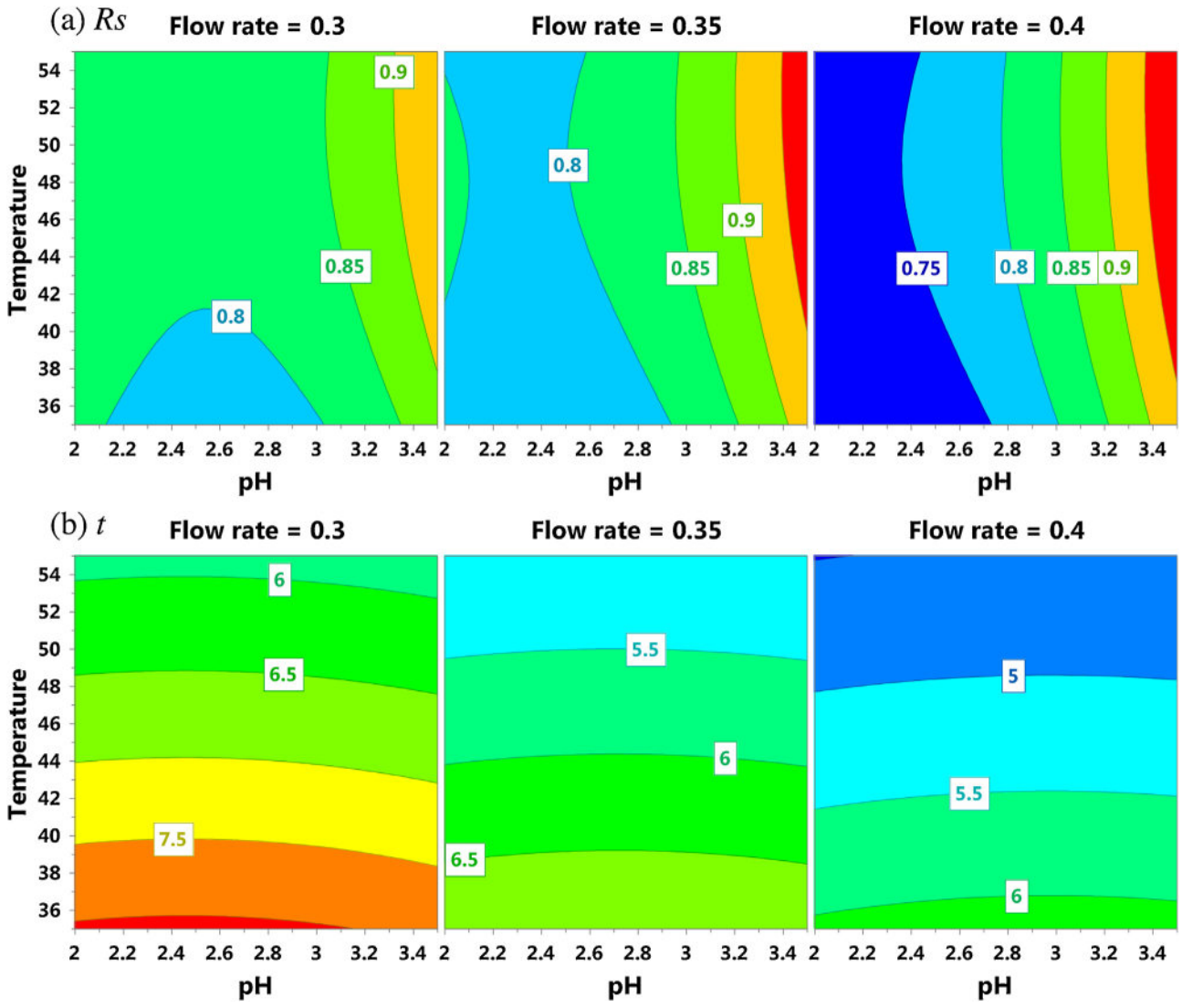

Fig. 1. Contour plots for (a) *Rs* and for (b) *t* obtained by plotting temperature *vs.* pH at three different values of flow rate (0.30, 0.35, 0.40 mL min^{-1}).

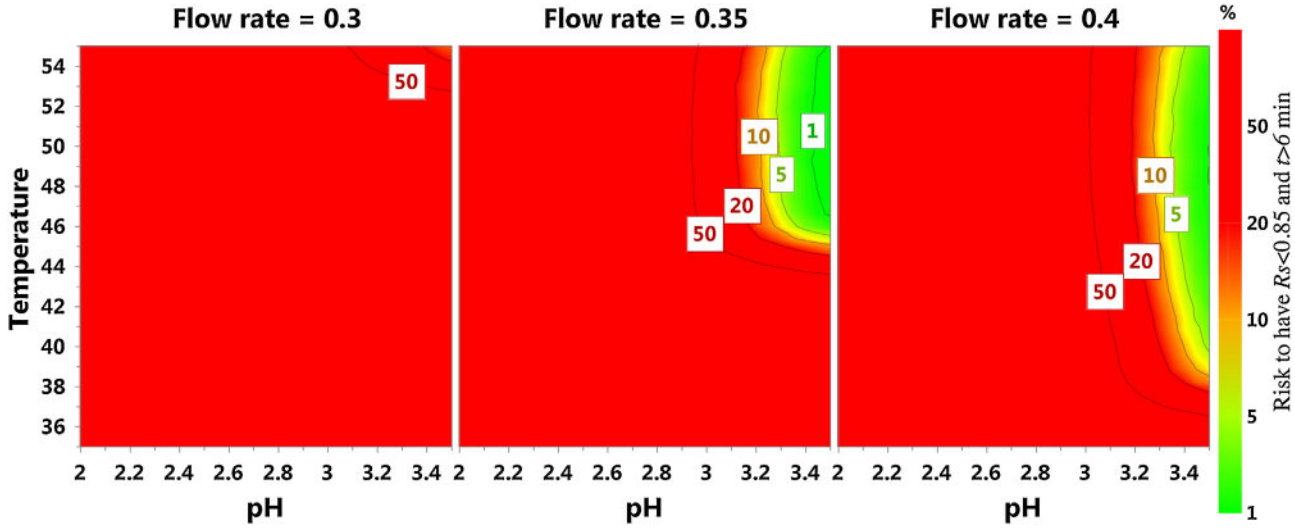

Fig. 2. Probability maps highlighting the MODR, colored in green and included in the line corresponding to 5% risk of failure. The maps are obtained by plotting temperature *vs.* pH at different values of flow rate (0.30, 0.35, 0.40 mL min^{-1}). Requirements for the CMAs: $Rs \geq 0.85$, $t \leq 6$ min. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

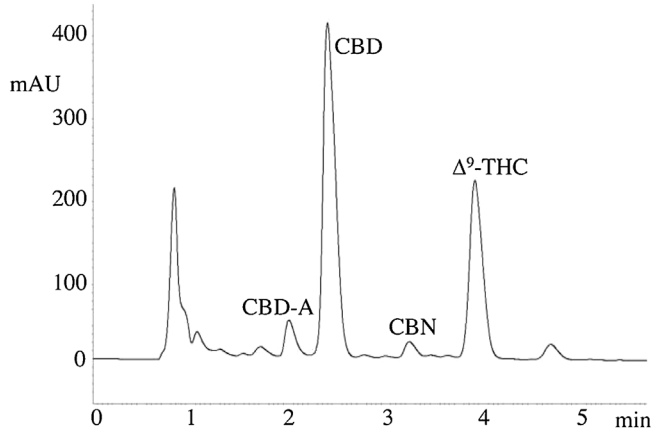

Fig. 3. Chromatogram referring to optimised conditions. Stationary phase, Agilent Poroshell® 120 SB-C18 analytical column. Mobile phase, mixture of ACN/5 mM K_2HPO_4 pH 3.45. Flow rate, 0.38 mL min^{-1}, temperature 53 °C. CBD-A, cannabidiolic acid; CBD, cannabidiol; CBN, cannabinol; Δ^9-THC, Δ^9-tetrahydrocannabinol.

3.5. Method operable design region definition

The MODR is a set of conditions where the CMAs simultaneously satisfy the specifications with a determined probability [24]. Determination of MODR was performed by using Monte-Carlo simulations, implemented in MODDE® 10 software. Monte-Carlo simulations proceeded performing a search by expanding the possible factor ranges from an optimum set-point to the largest possible range where all response predictions are simultaneously still within the specifications [30]. The starting set-point was the following: buffer pH 3.44, column temperature 50 °C and flow rate 0.35 mL min^{-1}. Monte-Carlo simulations were carried out by propagating the errors of the fitted model to each mean predicted CMA to obtain a distribution of the CMAs that accounts for the model error. The MODR was then defined as the multidimensional combination of CMPs where the CMAs simultaneously satisfied the specifications with an assurance (probability) level $\pi \geq 95\%$. Risk of failure maps are represented in Fig. 2 and the MODR (green area) can be identified as the region included within the line defining a risk of failure equal to 5%. The MODR was defined by the following ranges of parameters: buffer pH 3.11–3.50, column temperature 44–55 °C, flow rate 0.31-0.39 mL min^{-1}.

3.6. Working point and robustness

Verification points were selected at the boundary of the MODR and tested by using a Plackett-Burman design matrix for validating the MODR, and this step also confirmed the accuracy of prediction of the optimized models. In this matrix, the +1 and -1 levels for each CMP corresponded respectively to the lowest and the highest limits of the MODR range. For choosing the working point within the MODR, the value of temperature and flow rate were increased with respect to the original set-point (50 °C, 0.35 mL min^{-1}), with the aim of further reducing analysis time. Hence, the selected working point was the following: buffer pH, 3.45; column temperature, 53 °C; flow rate, 0.38 mL min^{-1}. The chromatogram obtained with these conditions is reported in Fig. 3.

Robustness is defined as the capacity of the method to remain unaffected by small but deliberate variations applied to the optimized conditions [44]. It was evaluated by using a Plackett-Burman matrix and a linear relationship was hypothesized between the CMAs and the CMPs because factor interactions can be assumed to be negligible for the smallness of the experimental domain under study [45]. The experimental plan with the considered responses is reported in Supplementary Table S1. ANOVA showed that the calculated model for *Rs* was not significant, with $R^2 = 0.700$, while the model for *t* was significant, with $R^2 = 0.998$. In particular, the graphic analysis of effects, reported in Supplementary Fig. S5, showed that significant effects on *t* response were exerted by column temperature and flow rate. In any case, the defined requirements for both CMAs were satisfied in each experiment within the plan. Hence, the method was declared robust with a precautionary statement about the control of instrumental factors.

3.7. Validation and application in routine use

Selectivity, accuracy, precision, linearity and range of the method were evaluated following the ICH guideline Q2(R1) in order to validate the method [44]. As regards selectivity, the resolution value between CBD and CBD-A was $Rs = 1.00 \pm 0.05$ ($n = 4$, $\alpha/2 = 0.025$). The chromatogram of the blank was free of any peaks and no interference with the peaks of target compounds CBD and Δ^9-THC was found. The other validation data are reported in Supplementary Information Tables S2, S3 and S4, and on the basis of the obtained results the method was declared suitable for its purpose and implemented for routine use.

3.8. Control strategy

The aim of the control strategy is to verify that the developed method maintains the designed quality performance throughout its application in routine use. For this analytical method, two control strategies were implemented. First, control charts were used to monitor the compliance of the method through time. Secondly, the risk of the method failure in routine was re-evaluated using routine data, and then compared to the operational risk defined during the method development and validation phases. The control charts enable to monitor the method performances through time and detect if the chosen quality parameters deviate from specifications with time. The CMAs selected for method optimization were also chosen as criteria to monitor the behaviour of the method performance. Data to implement both strategies were collected over a period of one year of routine use (from June 2017 to June 2018). In this range of time, a total number of 459 cannabis olive oil samples (from 22 to 66 samples per month) were analysed in the laboratory. The sample population was highly heterogeneous in terms of typology of cannabis used by pharmacists during the manufacturing process. In fact, 193 cannabis olive oil extracts (42.0%) were prepared from Bedrocan®, 133 (29.0%) from FM2®, 74 (16.1%) from Bediol®, 59 (12.9%) from Bedrolite®. Among these samples, only 136 presented both the CBD-A and CBD peaks in the chromatograms allowing to monitor the critical resolutions (R_s) among these two peaks. For the other samples, a monitoring of the resolution was not allowed because of the absence of the acidic form CBD-A (depending on the manufacturing protocol used or on natural variations in the composition) or both CBD-A and CBD (depending on the chemotype of the cannabis plants) in the case of Bedrocan® cannabis. Regarding the second monitoring criterion, the analysis time (*t*), this parameter was extrapolated from the totality of the sample population.

Fig. 4 shows the control charts for (a) *Rs* and (b) *t* constructed using the data from one year of application in routine. The control limits used are the specifications limits defined during the optimisation phase: 0.85 for *Rs* and 6 min for *t*. As it can be seen, all the analysis times in routine remained under the tolerance limit. Similarly, the resolution values were generally above the limit defined, except one value.

Besides control charts, the risk of failure associated to the use of the method was re-evaluated by computing a probability that the

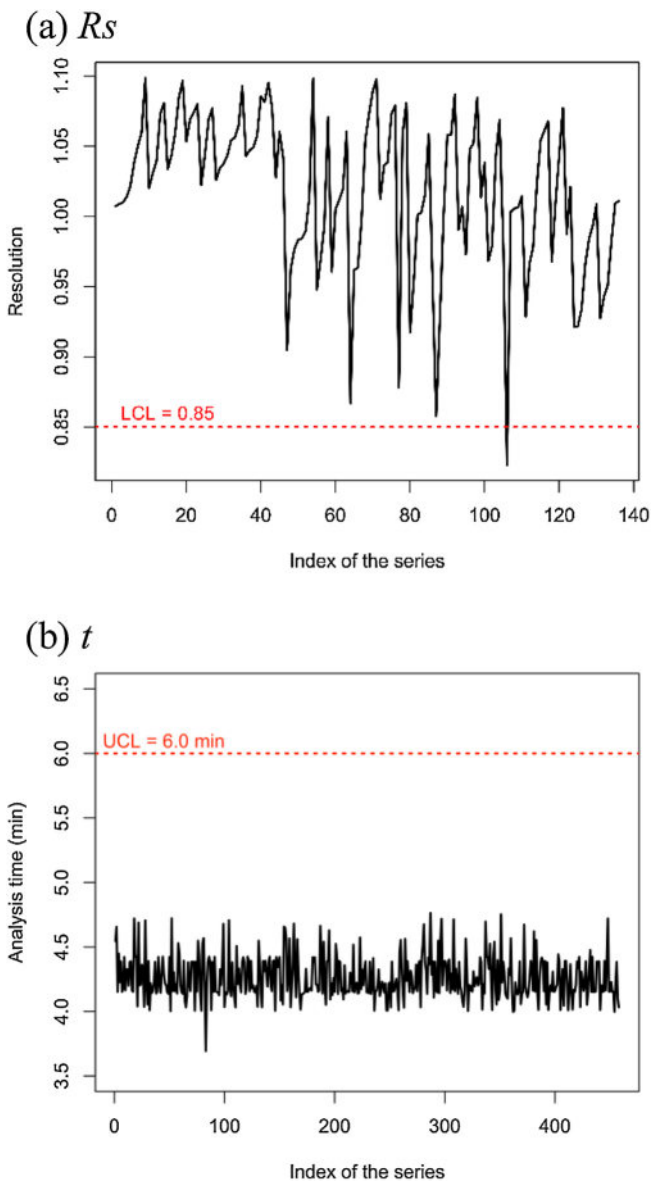

Fig. 4. Control charts for (a) *Rs* and (b) *t* used for monitoring the behavior of these two CMAs during the routine use of the method. LCL = lower control limit and UCL = upper control limit derived from the specifications used for the definition of the MODR.

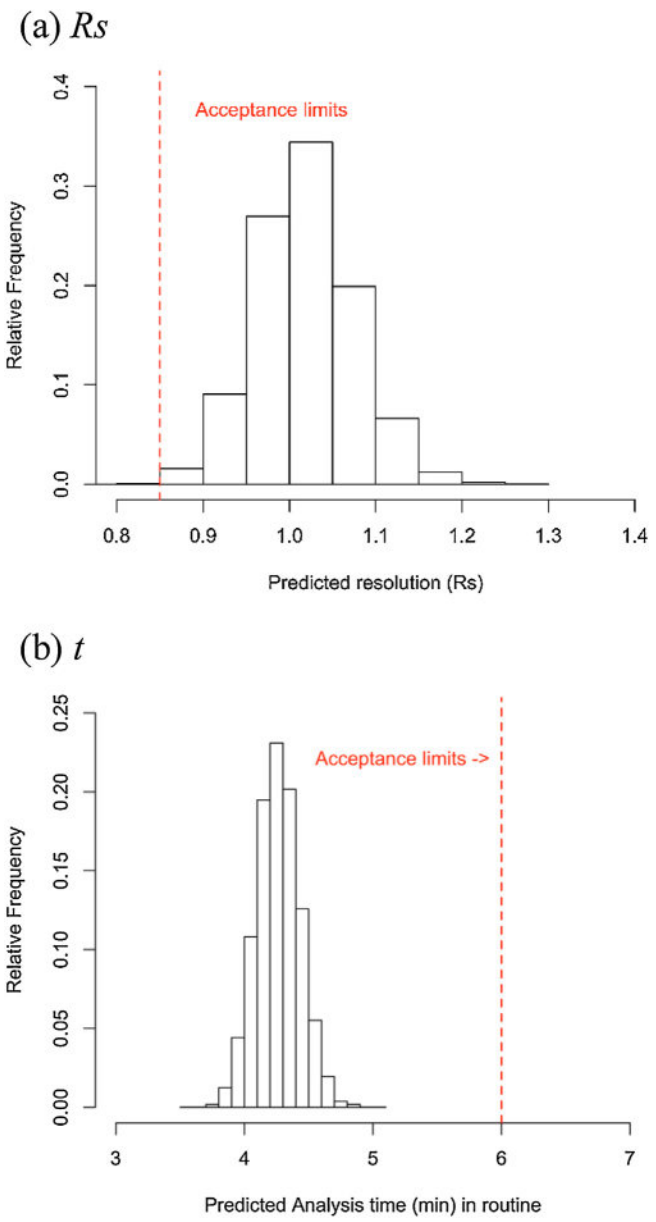

Fig. 5. Histogram of 10,000 predicted (future) (a) *Rs* and (b) *t* values using the data from the use of the method in routine. The predicted values of *Rs* are compared to the defined specification (red dotted vertical line) and the proportion of values below the specification is computed as the updated risk of the method failure in routine and compared to the defined operational risk of 5%. The predicted values of *t* are compared to the defined specification (red dotted vertical line) and the proportion of values above the specification is computed as the updated risk of the method failure in routine and compared to the defined operational risk of 5% (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

CMAs will fall out of the defined specifications on the basis of the routine use data collected. A Bayesian regression model has been used for that purpose as follows [46]. The Bayesian approach to linear regression assumes a prior probability distribution on the regression coefficients and on the noise variance, and a probability distribution on each measured CMA value given the model parameters (also called likelihood). Then, combining these distributions using the Bayes theorem, a joint posterior probability distribution of the coefficients and noise variance given the data is derived. Hence, contrary to classical least square regressions, which focus on point estimates of the coefficients and noise variance, the outcome of the Bayesian regression analysis is the posterior probability distribution of the model parameters. This distribution can be used to extract values of the parameters (coefficients and noise variance) that are more plausible. More importantly, the posterior distribution of the model parameters is used to derive the future distribution of each of the two CMAs for example by propagating uncertainty from the model parameters to the model equation.

The obtained future distribution is the predictive probability distribution of the CMAs given the routine data and accounts both the variability in the routine data and model parameters uncertainty. This risk based approach of routine data is more and more recommended for any risk assessment problem. The reader is referred to Ref [46] for further information on how to compute the predictive distribution of one or several CMAs. A sample of size 10,000 of the predictive distribution has been derived for each CMA using Markov Chain Monte Carlo (MCMC) methods [46]. Histograms of these distributions are reported in Fig. 5 for each CMA. Based on this predictive distributions of the CMAs, the updated risks of not meeting the defined method specifications in routine was computed as

Table 2
Statistical analysis of cannabis olive oil samples. Mean, RSD, median, minimum and maximum, 1st and 3rd quartiles values of Δ^9-THC and CBD concentrations in cannabis olive oil extracts from Bedrocan®, FM2®, Bediol® and Bedrolite®.

Typology of Cannabis	Number of samples	Mean (mg mL^{-1})	RSD (%)	Median (mg mL^{-1})	Minimum (mg mL^{-1})	Maximum (mg mL^{-1})	1st Q (mg mL^{-1})	3rd Q (mg mL^{-1})
Bedrocan® 100 mg mL^{-1}	95							
Δ^9-THC		16.600	10.71	16.490	10.900	22.500	15.640	17.480
CBD		ND	ND	ND	ND	ND	ND	ND
Bedrocan® 70 mg mL^{-1}	98							
Δ^9-THC		12.700	14.07	13.090	5.200	16.010	11.650	13.740
CBD		ND	ND	ND	ND	ND	ND	ND
FM2® 100 mg mL^{-1}	51							
Δ^9-THC		6.258	12.43	6.160	3.800	9.000	5.975	6.685
CBD		12.630	12.04	12.700	6.900	16.200	12.20	13.280
FM2® 70 mg mL^{-1}	82							
Δ^9-THC		4.849	14.42	4.955	2.300	6.500	4.513	5.235
CBD		9.206	14.81	9.375	4.700	14.060	8.715	9.852
Bediol® 100 mg mL^{-1}	17							
Δ^9-THC		5.538	13.98	5.561	3.600	6.900	5.379	5.900
CBD		8.640	14.60	8.912	6.490	10.500	7.800	9.600
Bediol® 70 mg mL^{-1}	57							
Δ^9-THC		3.977	14.58	4.000	2.580	5.100	3.610	4.500
CBD		6.546	18.04	6.400	4.220	12.550	6.000	6.900
Bedrolite® 100 mg mL^{-1}	12							
Δ^9-THC		ND	ND	ND	ND	ND	ND	ND
CBD		8.916	10.02	9.100	6.965	10.232	8.540	9.419
Bedrolite® 70 mg mL^{-1}	47							
Δ^9-THC		ND	ND	ND	ND	ND	ND	ND
CBD		6.652	18.81	6.600	5.000	13.818	6.163	7.000

RSD, relative standard deviation; Δ^9-THC, Δ^9-tetrahydrocannabinol; CBD, cannabinol; 1st Q, first quartile; 3rd Q, third quartile; ND, not detected.

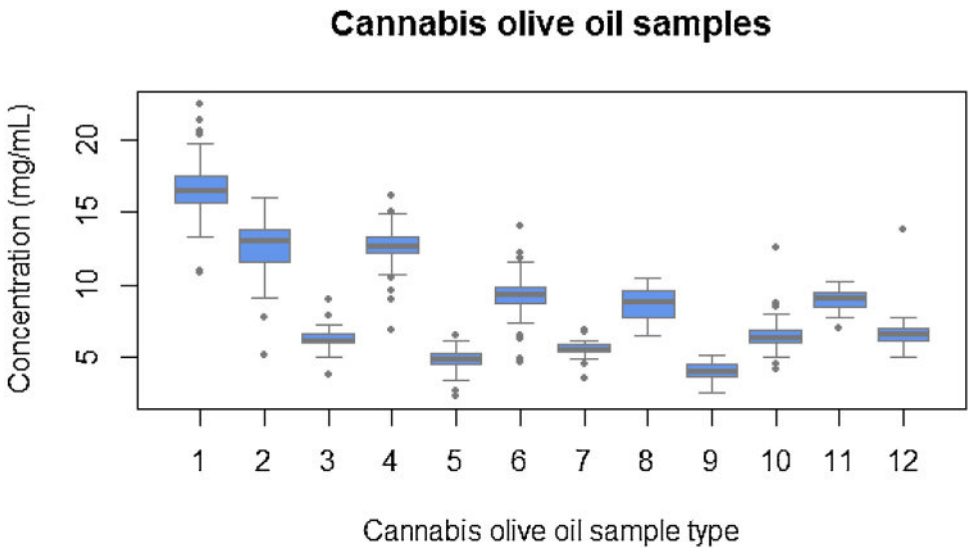

Fig. 6. Distribution of Δ^9-THC and CBD concentrations in cannabis olive oil extracts from Bedrocan®, FM2®, Bediol® and Bedrolite® represented by Box-plots. For each typology of samples, a Box-plot is represented and numbered from 1 to 12 with the following correspondence: 1) Δ^9-THC in Bedrocan® 100 mg mL^{-1} samples; 2) Δ^9-THC in Bedrocan® 70 mg mL^{-1}; 3) Δ^9-THC in FM2® 100 mg mL^{-1}; 4) CBD in FM2® 100 mg mL^{-1}; 5) Δ^9-THC in FM2® 70 mg mL^{-1}; 6) CBD in FM2® 70 mg mL^{-1}; 7) Δ^9-THC in Bediol® 100 mg mL^{-1}; 8) CBD in Bediol® 100 mg mL^{-1}; 9) Δ^9-THC in Bediol® 70 mg mL^{-1}; 10) CBD in Bediol® 70 mg mL^{-1}; 11) CBD in Bedrolite® 100 mg mL^{-1}; 12) CBD in Bedrolite® 70 mg mL^{-1}. On the "y" axis, the cannabinoids concentration (mg mL^{-1}) found in samples is presented.

the proportion of the predicted samples out of the specification for both CMAs. Compared to the reference or operational failure risk defined during the method development (5%), the updated risk was very low, as shown in Fig. 5: less than 0.1%. Thus, the assurance that the method will meet the specifications in routine is about 99.9%, that is greater than the operational assurance defined during the development of the analytical method. Hence, the method has remained in compliance with the ATP defined at the beginning of the development process.

3.9. Monitoring study

The developed method was applied to samples of cannabis olive oil extracts prepared by pharmacists of the Florence area. The data collected were used to monitor the concentration of Δ^9-THC and CBD in these magistral preparations. For this study, the same 459 extracts described above from different varieties were analysed. For each formulation type, the mean and median concentrations of Δ^9-THC and/or CBD, the RSD and the minimum and maximum

values found, together with the first and the third quartile values, are reported in Table 2. The obtained results can be effectively displayed in Fig. 6, reporting the Box-plots showing the distribution of Δ^9-THC or CBD concentrations in the samples analyzed. The values obtained highlighted the widely variable concentrations of the analytes between formulations. It could be reasonably stated that such variability comes from both the application of different protocols adopted by pharmacists during the preparation and the possible natural fluctuation in cannabis inflorescences. This issue highlighted that in order to minimise the first cited effect a standard manufacturing protocol for olive oil extracts is strongly recommended.

4. Conclusions

In this paper the AQbD approach for the optimization of a HPLC method to evaluate Δ^9-THC and CBD content in cannabis olive oil extracts is presented. The AQbD strategy made it possible to achieve a good understanding about the parameters which affect the process and to control them. Experimental design enabled to reduce the number of experiments, to detect the interactions between variables and to obtain a mathematic predictive model, leading to the definition of the MODR. The control strategy made it possible to keep under control the behaviour of the selected CMAs, and a Bayesian regression model has been used to compute their future distribution and the risks of the method not meeting the specifications in routine. This analytical method can be easily applied for the routine analysis on cannabis olive oil extracts. The assessment of the risk linked to method performance after implementation in routine use highlighted the robustness of the quality parameters related to the chromatographic method. Finally, routine analysis has demonstrated the very high variability of Δ^9-THC and CBD in the magistral preparations, indicating the strong need of a dedicated preparation protocol in order to assure the quality of the olive oil extracts.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2019.01.032.

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