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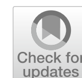

Relative response factors and multiple regression models in liquid chromatography to quantify low-dosed components using alternative standards—proof of concept: total Δ9-THC content in cannabis flowers using CBD as reference

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Abstract

A classical quantitative analysis in liquid chromatography is performed using either a one-point calibration or a calibration line, prepared using a reference standard of the compound(s) of interest. However, in some cases, adequate reference standards may be very expensive, rare to obtain, or have limited shelf-life properties. Also, in herbal matrices, multiple compounds could be necessary to be quantified, needing a whole series of different (related) reference standards. In these cases, the use of relative response (sometimes called relative correction factors) factors (RRFs) towards reference standards, different of the compound to be quantified, gained attraction. This study performed a comparison of the use of RRFs and linear relative response factor models (LRRFM) for the quantification of targeted low-dosed compounds using an alternative standard, since it is known that classical RRFs often fail in lower concentration ranges. For this purpose, the determination of the total Δ9-tetrahydrocannabinol (Δ9-THC + Δ9-THC-A) content in dried cannabis flowers, using UHPLC-DAD, was used as a case study. A chromatographic method was implemented and validated, and the use of classical calibration lines, classical RRF, and the LRRFM was applied and compared, with special focus on the concentration around 0.2% (w/w) total Δ9-THC, the legal limit (in most European countries) in these products. Results showed that the newly presented and validated LRRFM approach outperformed the classical RRFs, especially in the low-concentration ranges and that concentrations obtained with the LRRFM were in accordance with the interpolation results obtained with a calibration line.

Keywords Cannabinoids · Δ9-THC · Relative response factor · Regression · Linear relative response factor models

Introduction

In liquid chromatography (LC), quantification is usually established by using an external reference standard of the compound of interest, for which is worked with one reference standard solution (single-point calibration) or with a calibration curve often obtained by least squares regression between standard solution concentrations and the resulting responses as area under the curves (AUC). In herbal products, often multiple compounds are to be analysed for quality control or legal purposes. However, reference standards of each of the compounds can be very expensive, rare to obtain and often have limited shelf-life properties. The use of relative response (sometimes called relative correction factors) factors (RRFs) towards usually one reference standard for the quantification of multiple compounds therefore gained attraction. RRFs applied in LC with ultraviolet detection (LC-UV) are determined under the same chromatographic and detection conditions. The reference standard should ideally be stable, easily available and inexpensive and have similar UV absorbance and LC retention properties as the compounds to be quantified. Also, regarding the robustness of implementing relative response factors, plotting the relationship of the peak area ratios (reference standard versus analyte of interest) to a UV wavelength range was described

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as a way to find a wavelength with good response ruggedness [1]. A range of RRF-based approaches for LC is described, and applications are already found in the Chinese, US and European pharmacopoeias [2–5].

One of the earliest applications in LC (although not with UV detection) which implemented RRFs describes a high-performance liquid chromatography (HPLC)-refractive index detector (RID) method with benzyl alcohol as standard for the determination of ginkgolides and bilobalide in *Ginkgo biloba* [6]. In the simplest form, a RRF is defined as a ratio between the response factor (RF) of the reference standard and the RF of the analyte, where RF is peak area divided by the concentration [7]. Also, a slope-based method can be used, defining RRFs as the ratio of the slopes of two linear calibration curves of reference standards [8]. Some derived methods, such as "quantitative analysis of multi-components by single marker" (QAMS) [9], "simultaneous determination of multiple components" (SDMC) [10] and "single standard to determine multi-components" (SSDMC) [11], are often applied within the field of quality control of traditional Chinese medicine products. For the determination of RRFs in the absence of authentic compounds with known purity, other techniques were successfully developed such as strategies of combining multiple detectors whether or not in tandem [8, 12].

This study wants to investigate the use of RRFs in the case of low-dosed components, for which expensive reference standards are required. For this purpose, the quantification of Δ9-tetrahydrocannabinol (Δ9-THC) in dried cannabis flowers was chosen as case study.

In recent years, many cannabidiol (CBD) shops appeared in different European countries and sell a large variety of cannabis(-derived) products such as cannabis flowers for smoking purposes [13]. These products claim to contain low levels of Δ9-THC, the cannabinoid responsible for most of the psychoactive effects of cannabis, and therefore would not be subjected to drug laws and therefore are legal in some countries that do not have an explicit ban of these products in their legislation. For many users of CBD-related products, the motivation for use is linked to anticipated health benefits. However, health claims should be authorised under European Commission (EC) Regulation No. 1924/2006, and have not been authorised for cannabis products. The use of product labelling and disclaimers indicates how retailers of low-total-Δ9-THC cannabis products are playing into country-specific legal frameworks that may permit the sale of such products. This development has given rise to concern at the policy level with regard to both the legal status of these products and their potential to cause harm [14].

In most European countries, a maximum total Δ9-THC content of 0.2% (w/w) is authorised [15]. The labelled content, however, could in some cases be linked to dubious certificates of analysis. Different problems are encountered during the analysis of these products, such as matrix complexity and herbal sample inhomogeneity, high costs of cannabinoid reference standards and the low analytical Δ9-THC limit [16]. Those two last parameters made these products an ideal test case for this study on RRFs, where the focus is on the quantification of Δ9-THC and Δ9-tetrahydrocannabinolic acid (Δ9-THC-A) in cannabis flowers with ultra-high-performance liquid chromatography coupled to a diode array detector (UHPLC-DAD), using CBD as reference standard. These compounds where chosen since there is only a legal limit on the total Δ9-THC (Δ9-THC + Δ9-THC-A) content of these products.

Low recovery percentages in standards and spiked blank matrixes, when using common slope-based RRFs, were an issue in early experiments of this study, especially because of the low legal total Δ9-THC limit. In another study [11], it was concluded that conversion factors (such as RRFs) were only applicable in determined concentration ranges in addition to the corresponding linearity ranges. Moreover, they concluded that conversion factors should not be calculated by the slope-based method, especially when the intercept was not neglectable. The proposed approach could be applied for low-concentration compounds as for trace compounds in forensic sciences and is also appropriate for screening purposes, e.g. in the early stages of the investigation of impurities in new pharmaceutical or herbal products. In this study, an improved strategy was developed and validated with potential to extend the applicable concentration ranges, compared to the ranges when implementing ordinary RRFs, and with respect to acceptable trueness and precision. Using a validated total linear relative response factor model (LRRFM) approach, Δ9-THC and Δ9-THC-A were quantified simultaneously in cannabis flowers. This was done by using one reference standard calibration curve, which in this study was set up for CBD, and LRRFMs between CBD and Δ9-THC and its acidic precursor form Δ9-THC-A.

Materials and methods

Reagents, chemicals

The extraction solvent methanol (MeOH) absolute (HPLC grade), also used as diluent for the preparation of the standard solutions, formic acid and acetonitrile (HPLC-S gradient grade) were purchased from Biosolve BV (Valkenswaard, The Netherlands). Ultrapure water was prepared with a MilliQ-Gradient A 10 system (Millipore, Billerica, USA).

Reference standards

Cannabidiol pharmaceutical grade was purchased from Fagron (Nazareth, Belgium). Reference standard solution

Δ9-THC in ethanol (EtOH) was purchased from Lipomed AG (Arlesheim, Switzerland). Reference standard solution Δ9-THC-A in acetonitrile (ACN) was purchased from Dr. Ehrenstorfer-LGC Standards GmbH (Wesel, Germany). CBD and Δ9-THC were stored at 4 °C whereas Δ9-THC-A was stored at − 20 °C.

Preparation of standard solutions

Preparation of the working standard stock solutions

For preparation of the calibration curves, working stock solutions of 0.25, 0.10 and 0.10 mg/ml of, respectively, CBD, Δ9-THC and Δ9-THC-A were prepared by diluting the appropriate amount of certified reference standard solution or dissolving the appropriate amount of pharmaceutical-grade compound in absolute methanol.

For the preparation of working standard stock solutions used to spike with, the same procedure as described above was followed, but for CBD, a 1.0-mg/ml working solution was prepared.

Preparation of the calibration standard series

The calibration standard series were prepared by diluting each of the working standard stock solutions in absolute methanol in order to obtain final concentrations of 6.25, 12.5, 25, 50, 62.5, 75, 125 and 150 µg/ml for CBD and 5, 10, 25, 50, 75 and 100 µg/ml for Δ-9-THC and Δ9-THC-A. All concentrations were made in microliter scale in UHPLC vials (with or without micro-insert), and for each concentration, a maximum of 900 µl was made.

Herbal sample material

Blank matrix material

Fibre-type herbal cannabis material (originating from industrial agriculture and analysed before in the laboratory with a validated gas chromatography-flame ionization detector (GC-FID) method) with negligible amounts of Δ-9-THC, Δ9-THC-A and CBD was used as blank matrix for recovery testing of spiked extracts (‘Preparation of spiked blank matrix samples for recovery testing’). The herbal material, already available in a homogenous dried form, was made from fresh flowers (processed within 48 h after harvest) that were dried at 68 °C for a minimal 24 h followed by a semi-grinding of the material in a mortar and a final sieving through a 1-mm-sieve-width screen. For Δ9-THC-A, the amount of naturally occurring levels was evaluated by using analyses of the ‘blank’ matrices with the UHPLC method described in ‘UHPLC-DAD analysis’. Finally, two suitable blank matrixes were selected, with low (background) levels of the natural present cannabinoids of interest. Blank matrices were used for the preparation of validation samples for Δ9-THC, Δ9-THC-A and CBD. Necessary blank corrections were performed during calculations.

Herbal material for real-sample analyses

Dried herbal samples of cannabis flowers for smoking purpose (CBD cannabis) were voluntarily donated by distributors. The samples were stored at room temperature, in airtight bags, and protected from light. Prior to extraction, for each of the samples, an aliquot of the herbal material was slightly ground to a homogenous semi-fine fraction.

Preparation of herbal extracts

An extraction procedure was developed by monitoring the extraction yield of CBD from a real sample, analysed before with the European GC-FID method [17], implemented and accredited in our laboratory, as a function of different process parameters such as extraction solvent composition and extraction time. The tested extraction solvent compositions were 80 and 90% MeOH/water (v/v), pure methanol, 80% ACN/water (v/v), pure acetonitrile and 96% EtOH/water (v/v). For each of the extraction solvents, the extraction yield of CBD was monitored as a function of extraction time until the same concentration of CBD was obtained as the one measured with GC-FID. The classical solvent hexane, used in the European method, was not taken into account, since its use in liquid chromatography is not recommended. Also, it is the general trend to ban toxic solvents such as hexane and use more environmentally friendly and less toxic solvents. In the optimized extraction procedure, a volume of 10.0 ml methanol absolute was added with a volumetric glass pipette to 100 mg of homogeneous herbal material in a 15-ml polypropylene Falcon® centrifuge tube. The tubes (caps sealed with Parafilm®) with content were protected from light in order to prevent photodegradation of certain cannabinoids and were shaken horizontally for 90 min on an oscillator (Edmund Bühler Swip SM25) set at 200 oscillations per minute. The 90 min was necessary to obtain results for CBD as close as possible to the results obtained with GC-FID. Although an extraction of 90 min is not practical in routine analysis, the focus here was not to optimise an analytical method, but to assure the complete extraction of the cannabinoids of interest to be able to investigate the use of the RRF approaches in this context. After the extraction process, the tubes were centrifuged (Heraeus Multifuge 3S-R) for 5 min at g-force 3000 and the supernatants were filtered through a 0.22-µm PTFE filter. For some experiments described in ‘Preparation of spiked blank matrix samples for recovery testing’ and ‘Real-sample analysis’, other ratios of herbal material to extraction solvent volume were

used, in order to make higher-concentrated extracts. This was taken into account during the validation process of the UHPLC method.

Preparation of solutions for the assessment of the LRRFM and RRF methods at the lower concentration part of their application ranges

This assessment test was set up in order to be able to discriminate between the performance of both response factor methods at cannabinoid concentrations in the lower part of the determined concentration ranges. At each of the three validation days for Δ9-THC and Δ9-THC-A, two independent control solutions were made in methanol at concentrations of about 6 and 10 µg/ml. The solutions were made and mixed in UHPLC vials with micro-insert.

Preparation of spiked blank matrix samples for recovery testing

Spiking was done in already-prepared blank extracts while taking into account the original herbal extraction ratio ('Preparation of herbal extracts'). Due to the fact that cannabinoids were spiked afterwards, a dilution of the blank extracts took place. To tackle this issue, a double-concentrated blank extract was made according to the extraction method described in 'Preparation of herbal extracts', with 800 mg dry herb material and 40.0 ml absolute methanol in a 50-ml polypropylene Falcon® centrifuge tube as changed parameters. In order to make the validation samples, a part of the double-concentrated filtered extract was spiked with the working standard solutions for spiking purpose and the rest-volume was adjusted with MeOH up to the original herbal extraction ratio as described above. This strategy made it possible to downscale the sample volumes to microliter scale and therefore reduced the consumption of the reference standards.

The blank extracts and the reference standard working solutions to spike with, as well as the calibration curves for the quantification analysis, were prepared daily. The validation samples were prepared in triplicate at three nominal concentration levels in a range around the Belgian legal threshold of 0.2% (w/w) total Δ9-THC. For Δ9-THC and Δ9-THC-A, the levels were 0.1, 0.2 and 0.3% (w/w) with corresponding concentrations in the extracts of, respectively, 10, 20 and 30 µg/ml, while for CBD, the levels 0.5, 2.0 and 5.0% (w/w) were chosen with corresponding concentrations in the extracts of, respectively, 50, 200 and 500 µg/ml. The validation samples of the second and third level of CBD were diluted, respectively, 1:2 and 1:5 with methanol prior to analysis in order to obtain a response within the range of the CBD calibration curve. The spiking process and analysis was repeated for 3 days for Δ9-THC and Δ9-THC-A and for 4 days for CBD.

UHPLC-DAD analysis

An adapted version of a Waters® method [18] was used in this study. Analyses were performed on an ACQUITY UPLC system combined with an ACQUITY UPLC PDA detector from Waters® (Milford, MA, USA). The injection volume was 5 µl on a CORTECS UPLC Shield RP18 column from Waters® with 1.6 µm particle size (2.1 × 100 mm) thermostated at 35 °C. An isocratic elution at 0.7 ml/min was applied, with mobile phase consisting of 48% (v/v) water acidified with 0.1% (v/v) formic acid and 52% (v/v) ACN. The runtime was adapted to 18 min due to the interference of small matrix peaks with the signals of the cannabinoids of interest. Data acquisition and processing were performed with the Empower™ 3 Chromatographic Data software from Waters®. For all cannabinoids, a detection wavelength of 228 nm with 4.8 nm resolution was used. The total Δ9-THC content was determined by adding the Δ9-THC-A content to that of Δ9-THC after equivalent molar mass conversion of the carboxylated form to its corresponding decarboxylated cannabinoid form.

Although the method is used for the analysis of only three compounds, the runtime took 18 min in order to assure all components were eluted from the column in comparison to the runtime of 10.5 min of the original Waters® method. This was due to the fact that the water content in the mobile phase was increased in order to solve interference of some matrix peaks observed during the implementation of the method. The long runtime ensures complete separation and absence of matrix interference in real and spiked samples. This was necessary to ensure correct implementation and evaluation of the applied models. An example of a chromatogram obtained from a real-sample analysis is given in Fig. 1.

Method validation

In order to ensure the reliability of the UHPLC-DAD method, used to obtain the measurements for the evaluation of the RRF approaches, the method was validated according to ISO-17025, applying accuracy profiles, which are based upon the total error approach. This approach, well described in literature [19, 20], estimates the total error by combining the trueness (systematic error) and the random error (intermediate precision), in order to know the difference between the observed result and the true value. For each validation day, blank matrix was spiked three times at three levels with either CBD, Δ9-THC or Δ9-THC-A. The assay results were normalized prior to calculation of the accuracy profiles. This takes into account the assay result relative to the real concentration that was spiked (% recovery) followed by a relative

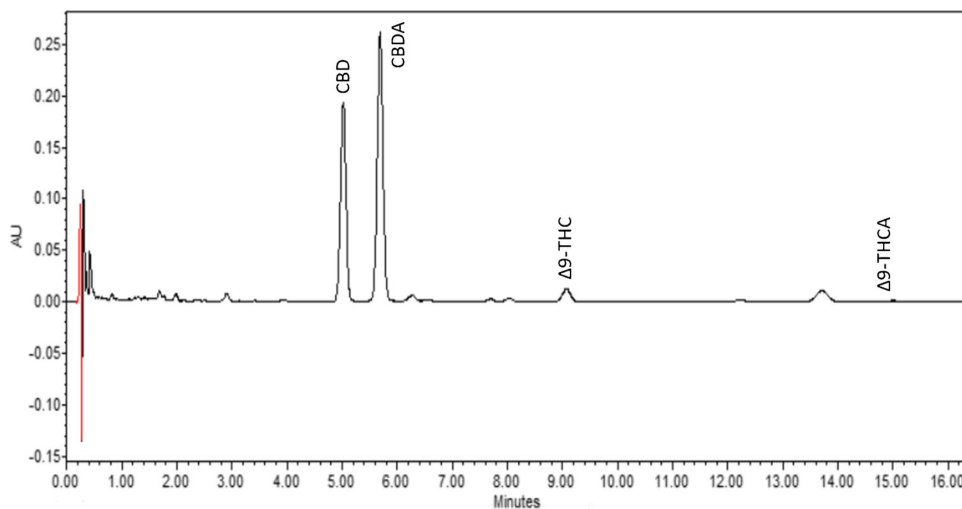

Fig. 1 Chromatogram of a real sample showing the peaks of cannabinoids of interest

levelling towards the nominal spiked concentration. Trueness, precision and accuracy were determined for each concentration level. The accuracy profiles were built by plotting the relative bias (%) of the normalized assay results against the concentration levels, including the acceptance limits and the upper and lower tolerance limits. Also, the total LRRFM approach was validated after applying the LRRFMs on the respective responses from the UHPLC-DAD validation data set ('Method validation of the total LRRFM approach').

Limits of detection and quantification

The limit of detection (LOD) and limit of quantification (LOQ) were estimated by examining the signal-to-noise ratios of the chromatographic cannabinoid responses from diluted cannabis extracts. The extracts were made from herbal material with known cannabinoid content of interest and were prepared with the same extraction ratio and extraction method as described in 'Preparation of herbal extracts' and then further diluted in MeOH. The LOD was determined as the minimum concentration (% (w/w)) with a signal-to-noise (S/N) ratio of at least 3.1. In a similar manner, the LOQ was determined but with a S/N ratio of at least 10:1.

Calculation of relative response factors for the quantification of cannabinoids

Determination of RRFs based on the slope ratio method

The RRFs were determined as the ratio of the slopes of the regression lines for each of the cannabinoids of interest to that of the slope of the regression line of the reference cannabinoid CBD. For a same mass concentration, the response of CBD as reference cannabinoid is expected to be the response of the cannabinoid of interest divided by its RRF. It enables the conversion of a cannabinoid response into a corresponding imaginary CBD response, and the quantification of the cannabinoid (as CBD) can then be done by interpolating the simulated CBD response into the CBD calibration curve. The applicable concentration range for using RRFs of cannabinoids was estimated after converting the range of responses from each cannabinoid calibration curve with the RRF and extracting the shared linear concentration range with the reference calibration curve ('Overview of modelling results').

Determination of LRRFM based on linear regression of real and imputed responses of the cannabinoid of interest and the reference cannabinoid CBD

The real responses (area under the curves, AUCs) of the CBD calibration standards, as reference cannabinoid, and those of the cannabinoid calibration standards of interest (Δ9-THC or Δ9-THC-A) were combined and subjected to a two-step least square regression analysis. The first step involved the calculation of the missing responses for the concentrations of the complementary cannabinoid standards, either CBD as reference cannabinoid or one of the cannabinoids of interest. This was done by interpolating the complementary concentrations in the corresponding calibration curves. After imputation of the missing responses with the fitted values, the response-couples for each known concentration were then subjected to a second linear regression. The outcome of that resulted in a linear equation that describes the relation of the responses of the cannabinoid of interest with the selected reference cannabinoid CBD. To assure quality of the models, obtained from data including imputed values with by definition lack of random error properties, quality parameters (regression statistics to test for significance of a quadratic model, quality coefficient (QC) and coefficient of determination (R^2)) as well as relative recovery results of low-concentration standards and spiked blank matrixes were checked.

Results and discussion

Method validation

Spiked blank matrix samples were analysed in triplicate for each of the three concentration levels during three validation days for Δ9-THC and Δ9-THC-A and four validation days for CBD. A background correction was done by subtracting the integrated peak areas of CBD and Δ9-THC, present in the blank extract, from the appropriate integrated peaks of the validation samples. The natural present background levels were far below the lowest validation level, and no Δ9-THC-A was found in the selected blank matrix. The final concentrations were back-calculated using the calibration curves and were then normalized (% recovery · nominal concentration / 100). Due to the natural origin of the sample material, the β expectation level and the acceptability accuracy limits were set at, respectively, 90 and 15%. The accuracy profiles for CBD, Δ9-THC and Δ9-THC-A are respectively represented graphically in supplementary material Fig. S1 (a), (b), (c).

Linearity of the calibration curves

Each calibration standard was injected three times, and this equally spread over the total runtime of analyses. Linear calibration curves of all cannabinoids were calculated using ordinary least square regression analysis of the peak areas (AUC) correlated with the theoretical cannabinoid concentrations (µg/ml). The linearity was evaluated using quality parameters. The QC was used as a tool to evaluate the average percentage of deviation of the measurements towards each regression model. The limit for the QC was set to be less than 5% [21] and all calculated QCs were compliant to this limit . A Cochran's test at 5% significance level was performed in order to check for homoscedasticity. Homoscedasticity was accepted to be present in all calibration curves. The R^2 values were calculated as part of additional information to support proof of linearity of the calibration curves. All R^2 values were greater than or equal to 0.999. Also, regression statistics were used to test for significance of a quadratic model. When the value zero is within the 95% confidence interval of the coefficient of the quadratic variable x^2, then the quadratic model can be rejected. In all cases, the linearity of the calibration curves was accepted. The results of the linearity data and quality parameters of the calibration curves are summarized in supplementary material Table S2.

Method linearity

The linearity of the method was proven with R^2 values above 0.997 (Table 1), obtained after plotting the obtained and the theoretical results of the spiked samples.

Table 1 Validation results of the trueness, precision, accuracy and uncertainty of the UHPLC-DAD method for the quantification of the investigated cannabinoids

Cannabinoid	Method linearity	Trueness			Precision						Accuracy			Uncertainty		
	R^2	Relative bias (%)			Repeatability (%RSD)			Intermediate precision (%RSD)			β-Expectance tolerance interval (%)			Relative expanded uncertainty (%)		
Concentration level		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
CBD	0.99819	−1.02	3.28	2.20	2.45	0.76	1.17	4.61	3.40	2.72	[−11.70; 9.66]	[−5.91; 12.48]	[−5.03; 9.43]	10.02	7.82	6.15
Δ9-THC	0.99671	−0.57	2.21	−0.10	2.68	1.50	1.07	3.63	2.01	2.02	[−9.04; 7.91]	[−2.60; 7.03]	[−6.75; 6.55]	7.95	4.51	4.56
Δ9-THC-A	0.99761	2.93	2.27	0.12	1.23	1.23	2.03	1.23	1.37	2.03	[0.41; 5.45]	[−0.64; 5.19]	[−3.94; 4.17]	2.66	3.00	4.28

RSD relative standard deviation

Trueness

The trueness estimates the systematic error of an analytical method and is expressed as a relative bias at each concentration level. As shown in Table 1, the relative bias for all the components was below 5% with a maximum relative bias of 3.28% for the second level of CBD. Consequently, the validation requirements were fulfilled.

Precision

The precision estimates the random error of the method and is expressed using a relative standard deviation (RSD). For each concentration level, the repeatability was obtained from the variability of the triplicate measurements. The intermediate precision was investigated for the time-dependent variability of the method. The results are displayed in Table 1. The highest value was seen for CBD with an intermediate precision of 4.61% for the lowest concentration level, which was below 5% and was considered acceptable.

Accuracy

Based on the obtained trueness and precision of the method, the β-expectation tolerance intervals, representing the accuracy of the method, were calculated. Accuracy takes the total error associated with each measurement into account. The accuracy profiles are presented in S1 (a), (b), (c), and the β-expectation tolerance intervals are given in Table 1. The highest value was seen for CBD with an upper β-expectation tolerance limit of 12.48% for the second concentration level. The accuracy profiles show that the β-expectation tolerance intervals do not exceed the acceptance limits of ± 15%, which means that the β-percent (90%) of the future measurements of unknown samples will be included within the tolerance limits. Therefore, this method is considered suitable for the intended purpose.

Uncertainty

The expanded uncertainty represents an interval around the results where the unknown true value can be observed with a confidence level of 95%. The uncertainties are calculated taking into account the intraday and interday variances and the bias obtained during the calculation of the accuracy profile, with a coverage factor k equal to 2 (± 95% confidence). The relative expanded uncertainties (%) are obtained by dividing the corresponding expanded uncertainties by the corresponding concentrations. These results are also shown in Table 1. A maximum relative expanded uncertainty of 10% was found for the lowest level of CBD. All other values were below 8%, and overall, the method was considered to have acceptable relative expanded uncertainties for all tested cannabinoids.

LOD and LOQ

The limits of detection and quantification were determined in matrix and were, respectively, 0.02% (w/w) and 0.05% (w/w) for CBD, 0.03% (w/w) and 0.06% (w/w) for Δ9-THC and 0.01% (w/w) and 0.03% (w/w) for Δ9-THC-A. The presented limits in percent (w/w) are only valid when the mother extract was prepared according to the extraction method ('Preparation of herbal extracts') that was developed for this study.

Calculation of RRFs for the quantification of cannabinoids

Determination of RRFs based on the slope ratio method

The RRFs were determined as the slope ratios of standard calibration curves, as described in 'Determination of RRFs based on the slope ratio method' under 'Materials and methods'. Finally, average RRFs were calculated from the data set of three validation days, and are shown in Table 2.

For each of the three validation data sets, the estimated concentration ranges for applying the RRFs were obtained by first converting the average cannabinoid (Δ9-THC or Δ9-THC-A) responses of the calibration series into CBD responses with the determined average RRFs, followed by interpolation of these responses into the appropriate CBD curves. Then, from these concentration ranges, the average concentration range from three data sets was calculated. A small mismatch was visible at the low border in comparison with the ranges of the corresponding calibration series. As a consequence, the limits of the estimated applicable concentration ranges were then set to match with the ranges of the

Table 2 RRFs calculated with the slope-ratio approach and the applicable estimated concentration ranges

Cannabinoid	Average RRF ($n = 3$)	Average concentration range after conversion with RRF (µg/ml)	Applicable concentration range after matching with the real concentration range (µg/ml)
CBD reference	1	Not applicable	Not applicable
Δ9-THC	1.842	[4.7–99.9]	[5.0–99.9]
Δ9-THC-A	1.470	[4.5–98.7]	[5.0–98.7]

corresponding (Δ9-THC or Δ9-THC-A) calibration curves. The range was further evaluated in the comparison of the assay results of all methods.

Determination of LRRFM based on linear regression of real and imputed responses of the cannabinoid of interest and the reference cannabinoid CBD

Building the LRRFM for Δ9-THC For a same concentration within each data set from three independent calibration series, one response of either CBD or Δ9-THC was known from the analyses. The complementary missing responses were fitted by using linear regression on the corresponding individual calibration series of either CBD or Δ9-THC. These fitted responses were imputed in supplementary material Table S3.

Then, the relation between the two series of responses including known and imputed responses was determined by least squares linear regression (Δ9-THC responses as y values and responses as CBD as x values) with output the LRRFM: $y \approx 1.8414x - 6038.2$. A graphical representation of the LRRFM for Δ9-THC is given in Fig. 2.

Note that the slope coefficient has a similar value as the RRF that was determined with the slope ratio–based approach. The estimated applicable Δ9-THC range of the LRRFM is discussed in 'Overview of modelling results', and the range was further evaluated in the comparison of recovery results of all methods. An overview of the quality parameters for the linearity of the model is given in Table 3. The QC was 2.24% and was far below the critical level of 5%, R^2 was 0.9993 and the test for quadratic model was not significant (the test method is described in 'Linearity of the calibration curves').

For an unknown Δ9-THC concentration with a known Δ9-THC response and a newly determined CBD calibration curve, it is possible to quantify Δ9-THC by firstly calculating the corresponding response as CBD by using the LRRFM (Eq. 1) followed by quantification of the unknown concentration Δ9-THC with the use of the CBD calibration curve (Eq. 2).

$$Response\Delta 9THC = a'_{LRRFM_\Delta 9THC} \bullet Response_{asCBD} + b'_{LRRFM_\Delta 9THC} \quad (1)$$

with $a'_{\text{LRRFM_Δ9-THC}} \approx 1.8414$ and $b'_{\text{LRRFM_Δ9-THC}} \approx -6038.2$

$$[\Delta 9THC]_{\mu g/ml} = \frac{\left(Response_{\text{asCBD}} - b_{\text{CBD_cal_curve}}\right)}{a_{\text{CBD_cal_curve}}} \quad (2)$$

with $a_{\text{CBD_cal_curve}}$ as the slope and $b_{\text{CBD_cal_curve}}$ as the intercept of the CBD calibration curve.

Building the LRRFM for Δ9-THC-A In the same way as the LRRFM for Δ9-THC was built, the LRRFM between Δ9-THC-A and CBD was built (Δ9-THC-A responses as y values and responses as CBD as x values) with output: $y \approx 1.4863x - 14{,}404$. A graphical representation of the LRRFM for Δ9-THC-A is given in Fig. 3. The linearity parameters are summarized in Table 3. The QC of the model was 2.96%, R^2 was 0.9988 and the test for quadratic model was not significant so that the linearity of the model was accepted. An unknown Δ9-THC-A concentration with a known Δ9-THC-A response and a newly determined CBD calibration curve can be quantified in a similar way as described in 'Building the LRRFM for Δ9-THC'. The estimated applicable Δ9-THC-A range of the LRRFM is discussed in 'Overview of modelling results'.

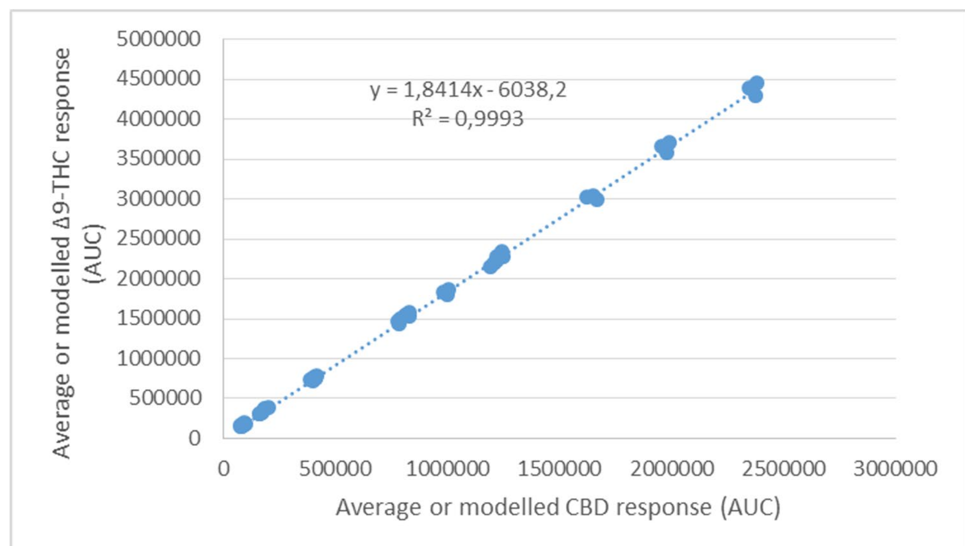

Fig. 2 The LRRFM for Δ9-THC

Table 3 Overview of modelling results of the LRRFM approach, quality parameters and the applicable estimated concentration ranges

Cannabinoid	LRRFM towards response CBD	Confidence interval, 95%	Applicable range after conversion with LRRFM model (µg/ml)	Applicable range after matching with corresponding calibration curve (µg/ml)	QC (%)	R^2
Δ9-THC	$Response_{\Delta 9\text{-THC}} = (1.8414 * Response_{asCBD}) - 6038.2$ To calculate $[\Delta 9\text{-THC}]_{\mu g/ml}$: $(Response_{asCBD} - b_{CBD\ cal_curve}) / a_{CBD_cal_curve}$	Intercept: [−22,901; 10,824] Slope: [1.8262; 1.8566]	[4.9–100.2]	[5.0–100.2]	2.24	0.9993
Δ9-THC-A	$Response_{\Delta 9\text{-THC-A}} = (1.4863 * Response_{asCBD}) - 14{,}403.9$ To calculate $[\Delta 9\text{-THC-A}]_{\mu g/ml}$: $(Response_{asCBD} - b_{CBD\ cal_curve}) / a_{CBD_cal_curve}$	Intercept: [−31,330; 2522.6] Slope: [1.4708; 1.5019]	[5.1–98.2]	[5.1–98.2]	2.96	0.9988

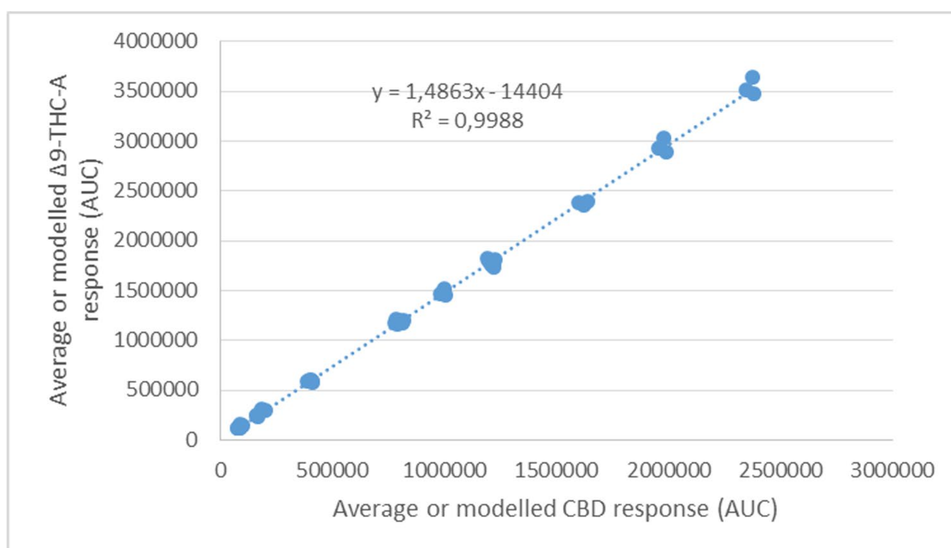

Fig. 3 The LRRFM for Δ9-THC-A

Overview of modelling results The different LRRFMs for Δ9-THC and Δ9-THC-A, together with the linearity quality parameters and concentration ranges, are summarized in Table 3. In a similar manner as described in 'Determination of RRFs based on the slope ratio method' for the slope-based RRFs, also, estimated applicable concentration ranges for the LRRFMs were determined. In contrast with the phenomena of the ranges obtained with the slope-based RRFs ('Determination of RRFs based on the slope ratio method'), the estimated lower-border concentrations (converted with LRRFM) were a closer match with those of the corresponding Δ9-THC and Δ9-THC-A calibration curves with great potential for further extending the range if the validity of the response model and the linearity of the calibration curves can be proved.

Comparison of recovery test results of cannabinoid control solutions and spiked blank matrixes after applying the different RRF approaches

For the comparison of the different RRF approaches, within the same analysis run, all RRF methods were performed on the same integrated chromatographic peaks as used for back-calculated assay results from the corresponding calibration curves.

Assessment of the LRRFM and RRF at the lower concentration part of their application ranges

This assessment test was set up to discriminate between the performance of each of the response factor methods at low concentrations. An overview of the average relative

recoveries towards the back-calculated assay results from the calibration curves is shown in Table 4. The average relative recoveries obtained with the slope-based RRF method were always lower than those obtained with the LRRFM method. Also, with the slope-based RRF method, the relative recoveries decreased further with decreasing cannabinoid concentration. For Δ9-THC, for both methods, the relative recoveries were all between 95 and 105%. For Δ9-THC-A, half of the relative recoveries obtained with the slope-based RRF were below 95%, which indicated an unacceptable performance of the method at the tested concentrations, in contrast to the LRRFM method.

Comparison of LRRFM and RRF method results from spiked validation samples versus results obtained with linear regression on calibration curves

The normalized (% recovery · nominal concentration / 100) assay results obtained with the LRRFM and RRF method were statistically compared with the normalized assay results that were obtained with linear regression on the appropriate calibration curves. This was done by regression analysis with the reference method in the x-axis and the LRRFM or RRF method in the y-axis. All the normalized results from the spiked validation samples were used (3 samples per level, 3 levels, 3 days). The blank matrices that were

Table 4 Assessment of relative recoveries in a comparison between the LRRFM method and the slope-based RRF method at low concentration levels

Cannabinoid	Validation day	Concentration (µg/ml)	Average ($n = 2$) relative recoveries (%) towards assay results obtained with classical calibration curves	
			Method LRRFM (result and 95% CI)	Method RRF
Δ9-THC	1	6.12	98.79 ± 0.110	95.89 ± 0.233
		10.09	99.71 ± 0.001	97.83 ± 0.002
	2	6.12	100.42 ± 0.111	97.39 ± 0.015
		10.09	99.40 ± 0.030	97.53 ± 0.004
	3	6.12	99.85 ± 0.011	96.60 ± 0.041
		10.09	100.30 ± 0.013	98.33 ± 0.050
Δ9-THC-A	1	6.01	101.79 ± 0.068	(*) 93.79 ± 0.362
		9.92	101.20 ± 0.040	96.90 ± 0.213
	2	6.01	104.52 ± 0.122	96.88 ± 0.054
		9.92	102.26 ± 0.085	97.89 ± 0.038
	3	6.01	95.02 ± 0.099	(*) 86.93 ± 0.422
		9.92	96.12 ± 0.035	(*) 91.57 ± 0.150

(*) relative recoveries smaller than 95%

spiked with Δ9-THC and Δ9-THC-A were measured in the same run as the Δ9-THC and Δ9-THC-A calibration curves. With a slope value of 1 and an intercept value of 0 within the 95% confidence interval, there is, respectively, no relative or absolute systematic error between the two methods and, as a consequence, both methods are set not to differ from each other significantly. No relative and absolute systematic errors were found between results obtained with linear regression and the LRRFM method, so both methods did not differ from each other significantly. For the comparison between the linear regression method versus the RRF method, an absolute systematic error was found.

Applying the LRRFM method to Δ9-THC and Δ9-THC-A spiked blank matrix samples resulted for all tested levels in relative recovery percentages between 95 and 105% (Table 5). Applying the slope-based RRF method resulted in lower relative recovery percentages that decreased towards the lower levels of spiked Δ9-THC and Δ9-THC-A. The relative recovery of the 10 µg/ml Δ9-THC-A spiked matrix of the third validation day was below 95%.

Real-sample analysis

A pre-analysis of three cannabis samples ('Herbal material for real-sample analyses') revealed that the Δ9-THC-A levels in undiluted extract, obtained with the extraction method described in 'Preparation of herbal extracts', were lower than the LOQ. Therefore, the extraction process was repeated with double the amount of herbal material and half the amount of extraction solvent in order to obtain a 4-times-concentrated extract compared to the original extraction method. Then, the undiluted extracts were analysed. A summary of the results is shown in Table 6. As a consequence of the changed extraction ratio that was used for the mother extract, the LODs and LOQs in percent (w/w) presented in 'LOD and LOQ' were not valid for this setup and were estimated to be 4 times lower for these 4-times-concentrated extracts in comparison to the original extraction method described in 'Preparation of herbal extracts'. This is supported with the back-calculated Δ9-THC-A concentration from the calibration curve of ≈ 0.022% (w/w) for sample 1 which was lower than the LOQ of 0.03% (w/w) for an original extract although it corresponded with a Δ9-THC-A concentration of ≈ 8.7 µg/ml in the undiluted concentrated extract which was slightly below the lowest validated concentration level of 10 µg/ml and above the theoretical LOQ of 3 µg/ml both for an original extract. The same is true for sample 2. The total Δ9-THC content in all analysed samples was below the legal Belgian threshold of 0.2% (w/w) total Δ9-THC. Results obtained with both RRF methods were all within 95–105% of the back-calculated concentrations using the calibration curves. The acceptable results from the slope-based RRF method can be explained due to the higher

Table 5 Comparison of relative recovery results obtained after applying both RRF approaches in analyses of spiked blank matrixes

Cannabinoid	Nominal concentration (μg/ml)	Validation day	Relative average ($n=3$) recoveries (%) towards assay results obtained with classical calibration curves with 95% confidence limit	
			Method LRRFM	Method RRF
Δ9-THC	10	1	99.59 ± 0.044	97.58 ± 0.092
		2	99.57 ± 0.075	97.55 ± 0.010
		3	100.27 ± 0.015	98.19 ± 0.055
	20	1	100.48 ± 0.022	99.46 ± 0.048
		2	98.65 ± 0.010	97.68 ± 0.001
		3	100.65 ± 0.002	99.62 ± 0.008
	30	1	100.75 ± 0.006	100.05 ± 0.014
		2	98.38 ± 0.007	97.71 ± 0.001
		3	100.76 ± 0.003	100.04 ± 0.011
Δ9-THC-A	10	1	101.29 ± 0.012	96.45 ± 0.063
		2	102.44 ± 0.059	97.80 ± 0.026
		3	96.07 ± 0.026	(*) 91.37 ± 0.112
	20	1	100.81 ± 0.002	98.97 ± 0.013
		2	100.49 ± 0.047	98.67 ± 0.021
		3	96.96 ± 0.004	95.15 ± 0.017
	30	1	100.67 ± 0.012	99.76 ± 0.066
		2	99.85 ± 0.015	98.96 ± 0.007
		3	97.25 ± 0.004	96.37 ± 0.018

(*) relative recovery smaller than 95%

Table 6 Summary of assay results of real cannabis samples with the comparison of both RRF methods

Sample	Δ9-THC			Δ9-THC-A			Total Δ9-THC		
	Concentration, back-calculated from calibration curve (%w/w)	Relative average ($n=2$; independent extracts 3 × injected) recoveries (%) towards assay results obtained with classical calibration curves with 95% confidence limit		Concentration, back-calculated from calibration curve (%w/w)	Relative average ($n=2$; independent extracts 3 × injected) recoveries (%) towards assay results obtained with classical calibration curves with 95% confidence limit		Concentration, back-calculated from calibration curve (%w/w)	Relative average ($n=2$; independent extracts 3 × injected) recoveries (%) towards assay results obtained with classical calibration curves with 95% confidence limit	
		Method LRRFM	Method RRF		Method LRRFM	Method RRF		Method LRRFM	Method RRF
1	0.125 ± 0.003	100.977 ± 0.007	100.523 ± 0.016	0.022 ± 0.001	101.444 ± 0.052	95.621 ± 0.278	0.144 ± 0.003	101.039 ± 0.001	99.873 ± 0.035
2	0.102 ± 0.003	100.892 ± 0.012	100.341 ± 0.026	0.023 ± 0.001	101.407 ± 0.036	95.818 ± 0.193	0.122 ± 0.004	100.976 ± 0.005	99.601 ± 0.049
3	0.102 ± 0.002	100.890 ± 0.006	100.337 ± 0.014	0.031 ± 0.001	101.134 ± 0.010	97.272 ± 0.052	0.129 ± 0.002	100.941 ± 0.003	99.696 ± 0.023

cannabinoid content in the 4 × more concentrated extract. The low relative recovery results of the slope-based RRF method were at the border of acceptability for Δ9-THC-A. But the low content of Δ9-THC-A had a minor impact on the total Δ9-THC content.

As a matter of test (data not shown), the performance between the relative response factor methods outside the range of a cannabinoid (Δ9-THC-A) was also compared in 4 × dilutions of the previous concentrated extracts in methanol in order to reach the same extraction ratio from the original extraction method. For these samples, then, most of the assay results for Δ9-THC-A were below the lowest validation level and LOQ. Applying the LRRFM of Δ9-THC-A then resulted in relative recoveries (towards assay results obtained from interpolation on the calibration curves) between 89.6 and 92.0% whereas applying the slope-based RRF resulted in unacceptable relative recoveries between 63.7 and 74.2% for Δ9-THC-A. Taking into account the total Δ9-THC content resulted in relative recoveries between 98.4 and 99.0% with the LRRFM method, and those relative recoveries obtained with the slope-based RRF method were all below 95% (between 92.4 and 94.3%). The lower effect on the total Δ9-THC content, for these samples, was explained by the low Δ9-THC-A content in comparison to the Δ9-THC content.

Method validation of the total LRRFM approach

To validate the total LRRFM approach, the LRRFM between CBD and Δ9-THC/Δ9-THC-A was applied on the integrated peaks that were obtained from the validation setup of the UHPLC-DAD method ('Method validation'). The final concentrations were back-calculated using the appropriate LRRFMs and CBD calibration curves and were then normalized (% recovery · nominal concentration / 100). The β confidence level and the acceptability accuracy limits were also set at, respectively, 90 and 15%. The results are summarized in Table 7, and the corresponding accuracy profiles are presented in supplementary material Fig. S4 (a), (b).

The linearity of the total LRRFM method was considered linear as R^2 values for all components were above 0.994. The relative bias for all the components was below 5% with a maximum relative bias of 2.85% for the first concentration level of Δ9-THC-A. Consequently, the trueness of the total LRRFM approach method was considered acceptable. The repeatability and intermediate precision, expressed as %RSD, for all total LRRFM approaches and for all tested concentration levels, were below 5%. Therefore, the precision was considered acceptable. Regarding the accuracy, the highest upper limit of 13.54% for a β-expectation tolerance interval was seen for the lowest concentration level of Δ9-THC-A. That value was within the 15% acceptance limit, and therefore, the accuracy of the method was considered acceptable. A maximum relative expanded uncertainty of 7.70% was found for the lowest level of Δ9-THC, and overall, the method was considered to have acceptable relative expanded uncertainties for all tested cannabinoids. Consequently, the validation requirements were fulfilled for the total LRRFM approach.

Table 7 Validation results of the trueness, precision, accuracy and uncertainty of the total LRRFM approach (UHPLC-DAD method + LRRFM) for the quantification of the investigated cannabinoids

Cannabinoid	Method linearity	Trueness			Precision						Accuracy			Uncertainty		
	R^2	Relative bias (%)			Repeatability (%RSD)			Intermediate precision (%RSD)			β-Expectance tolerance interval (%)			Relative expanded uncertainty (%)		
Concentration level		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Δ9-THC	0.99479	−0.76	2.15	−0.13	2.66	1.51	1.07	3.53	2.78	2.99	[−8.96; 7.44]	[−5.38; 9.67]	[−10.10; 9.85]	7.70	6.40	6.83
Δ9-THC-A	0.99692	2.85	1.67	−0.64	1.23	1.22	2.06	3.12	1.90	2.11	[−7.84; 13.54]	[−3.41; 6.75]	[−4.84; 3.55]	7.32	4.32	4.43

RSD relative standard deviation

Conclusion

A linear relative response factor model method was developed, validated and proposed as an enhanced approach of using RRFs at low concentrations in a validated UHPLC-DAD method. The LRRFM method was demonstrated for the quantification of Δ9-THC and Δ9-THC-A using one reference standard (CBD). Assay results obtained with this LRRFM method were proven not to be significantly different from results obtained with interpolation on linear calibration curves. The advantage of applying the LRRFM method is that, for low concentrations and within a validated range, the recovery values remained between 95 and 105% whereas recovery results from the slope-based RRF method were often lower than 95%. This effect was demonstrated in control solutions and in spiked validation samples as well as in real-sample analyses. Because of the low legal total Δ9-THC threshold of 0.2% (w/w) in cannabis, this LRRFM method was extremely

useful and superior to the slope-based RRF method in the validated UHPLC-DAD method. Where low concentrations are expected in UHPLC-DAD analyses and where reference material is rare and expensive (often in herbal analyses but not limited to that domain), the LRRFM model can be useful to overcome the unacceptable low recovery issues when applying ordinary slope-based RRFs.

Once a LRRFM is developed for a compound and another certain reference compound and within well-determined chromatographic conditions, its use could reduce the costs of UHPLC-DAD analyses, when expensive reference standards are needed.

In this study, the analysis of the total Δ9-THC content in CBD smoking products was used as a case study for the LRRFM approach, but it could also find applicability in different types of analyses like quality control and risk evaluation of products, pill testing in the context of harm reduction or forensic analysis. In the context of pill testing, these facilities do not always have the resources to have a whole series of reference standards at their disposal, making the use of RRF approaches very interesting and cost saving. If low-dosed compounds should be quantified, the LRRFM approach presented in this paper would be useful. The same is true for the analysis of drugs of abuse in a forensic context. For highly dosed drugs like, e.g. Δ9-THC in cannabis, cocaine, …, the preference should go to classical calibration or to easily applicable RRF approaches as is for example done by several laboratories for the quantification of total Δ9-THC in cannabis using cannabinol as reference standard [22]. LRRF models would be more useful for the quantification of low-dosed compounds like, for example, drugs adulterated in cutting agents or the dosage of new psychotropic substances, like the fentanyl series. In the latter case, one fentanyl derivative could be used to dose different analogues. The model could be applied in the impurities dosage of a new pharmaceutical or herbal product.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00216-022-04208-y.

Declarations

Conflict of interest The authors have no conflicts of interest to declare.

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