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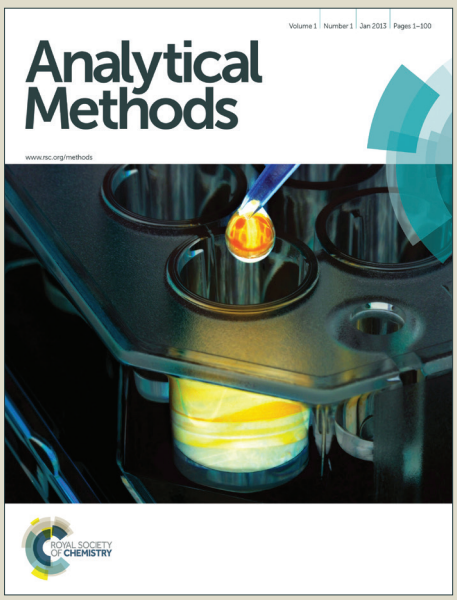

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Rapid capillary electrophoresis method with LED-induced native fluorescence detection for the analysis of cannabinoids in oral fluid

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Abstract

In the present study, a capillary electrophoresis method with native fluorescence detection for the quantification of the two main marijuana cannabinoids, tetrahydrocannabinol (THC) and cannabidiol (CBD), in oral fluid is described. The reported CE method makes it possible to assess illegal cannabis use in approximately 10 min, including saliva sample collection, pre-

treatment procedures and capillary electrophoresis (CE) analysis. The proof of the principle, demonstrated on a home-made lab scale instrument, has a potential to be easily translated onto a truly portable instrument for on-site measurements. The saliva sample collection/preparation/pre-concentration procedure was combined into one step using the Salivette® sampling device. No separate precipitation of proteins and/or derivatisation was required. The baseline CE separation of the two cannabinoids was achieved in less than 7 min by applying non-aqueous background electrolyte (BGE), composed of a 2.5 mM sodium hydroxide in a methanol-acetonitile mixture (1:1). Cannabinoids were detected at their second $\lambda_{ex}/\lambda_{em}$ maximum (280/307 nm) with LODs values of 0.19 and 0.17 µg/mL for THC and CBD, respectively. The recovery of the cannabinoids from the collection pad was greater than 80% for both cannabinoids tested at 2.5 µg/mL, and the inter-day precision was less than 6% for all analytes. The procedure was applied to oral fluid specimens after the controlled *al libitum* smoking of one cannabis cigarette.

Introduction

Cannabis (marijuana) is the most widely used illegal substance in the world[1, 2]. Humans smoke or ingest cannabis for its psychotropic effects. Tetrahydrocannabinol (THC) is the primary psychoactive compound in cannabis and its confirmation is an important element in assessing drug exposure in the workplace, while driving or during drug treatment. Cannabidiol (CBD), the other natural cannabinoid found in relatively high concentration in cannabis, contributes little to cannabis's psychotropic activity; quite the contrary: it may have antipsychotic properties[3]. Its concentration relative to THC can be useful in distinguishing the smoking of cannabis from the administration of cannabinoid-containing pharmacotherapies[4].

The widespread use of illegal cannabis is generally the reason that cannabinoids are intensively studied and that the search for possible new methods for determining usage is still very active[5, 6]. The quest for the development of an analytical method is targeted at the ability to identify the narcotics used by drivers/criminals as quickly as possible, so that appropriate preventive measures can be taken. For such a method to be useful, several factors have to be considered, including non-invasive sampling and sample preparation time, the portability of instruments, instrument start-up time, and actual analysis time.

The use of oral fluid (OF) as an alternative biological matrix for drug abuse testing has received increased attention in forensic and clinical chemistry. Saliva sample collection, compared to blood sampling, is easy, non-invasive and does not require any special training. In addition, for drug abuse control, saliva is available at any time and a sample can be collected in public view to prevent adulteration or sample substitution. An OF clean matrix contains approximately 98% water, with electrolytes, mucus, proteins and small molecules. Oral fluid contains predominately the parent drug rather than drug metabolites, and therefore is a good indicator of intoxication states[7]. During cannabis smoking, oral mucosa is immediately contaminated with cannabinoids contained in cannabis vapour and THC concentrations in saliva often exceed 1000 µg/L for a short time after smoking[8, 9].

To date, the assessment of illicit cannabis use, based on oral fluid analysis, employs on-site screening tests and, in the case of positive results, follow-up confirmatory analysis in the lab. For on-site testing, such immunoassay devices as DrugWipe®, Cozart® RapiScan, Rapid STAT®, Dräger DrugTest® 5000, Oratect XP saliva kits etc. are available commercially. The main drawbacks of the immunoassay tests are cross-reactivity and poor analyte recovery from the device, which leads to low diagnostic sensitivity (about 60%) and often to inadequate

performance[10, 11]. In the next step of the assessment of narcotic intoxication, chromatographic separation techniques play a dominant role. Usually, GC and HPLC are coupled to a mass spectrometer (MS)[12-15], because these detection techniques provide very reliable identification of separated compounds. Each of these techniques has advantages and drawbacks for on-site analysis. For instance, GC can be made portable and the separation times are relatively short[16]. However, the analysis typically requires derivatization steps because cannabinoids are insufficiently volatile. HPLC, on the other hand, cannot readily be made portable and thus requires the sample to be delivered to an analytical laboratory. In addition, despite the lower amounts of proteins found in OF with respect to blood, extensive sample preparation is needed in order to avoid relevant matrix effects[17]. This is generally carried out using solid phase[15, 18] or liquid-liquid extraction modes[19]. The recovery values of these techniques often decrease up to 50% and the accuracy of the methods suffers.

Capillary electrophoresis (CE) and related techniques are increasingly being employed in forensic analysis, as documented in several recent reviews[20-22]. The exceptional power of separation and resolution, short analysis time, economical use of reagents, and minimum sample requirements make CE an attractive methodology for forensic laboratories. CE can easily be miniaturized, unlike GC or HPLC[23]. CE start-up time is significantly shorter than that of HPLC, because there is no need for lengthy equilibration of the separation column with an eluent. In addition, CE analysis times are unquestionably the shortest of the available separation techniques. In contrast to these advantages, the primary disadvantage limiting its use is its poor concentration sensitivity, particularly when applied with on-column ultraviolet (UV) absorbance detection, due to low sample-injection volume and short optical path length. More adequate detection limits for abused drugs in the context of miniaturized analytical techniques can be obtained by light-emitting diode (LED) induced fluorescence detection[24, 25].

The combination of extremely high stability, long lifetime, small size, low cost and commercial availability at wavelengths ranging from deep-UV to near-IR regions make LEDs an attractive light source[26-29].

In this study, we propose the hypothesis that the capillary electrophoresis method with a native LED-induced fluorescence detector (LED-IF) is an alternative analytical technology to laboratory GC/LC-MS and roadside drug tests. The proof of the principle was demonstrated on a home-made lab scale instrument, with the goal of transforming it further to a portable format for on-site measurements. The LED-fluorescence detector used in this work operated at a deep UV wavelength, providing the detection of the two main marijuana cannabinoids, THC and CBD, at their excitation/emission maximum without the need for derivatization. A saliva sample collection/preparation/pre-concentration procedure was combined into one step using the Salivette® sampling device and was carried out in several minutes. A rapid and effective clean-up was carried out with good recovery values. The electrophoretic conditions obtained with a non-aqueous background electrolyte allowed for good separation of analytes in only 6 min. The developed method was thoroughly validated and used for the analysis of real oral fluid specimens after controlled marijuana smoking.

Materials and methods

1.1 Chemicals and reagents

Tetrahydrocannabinol and cannabidiol standard solutions 10 mg/mL and 1 mg/mL and powders were purchased from Lipomed AG (Switzerland). Internal standard (IS) bicalutamide (BCT) and potential interferents, including ethanol, acetaldehyde, nicotine, cotinine, caffeine and ibuprofen were obtained from Sigma-Aldrich (USA). Other drugs, including the analgetics (acetylsalicylic acid, paracetamol, ibuprofen, metamizole, diclofenac, meloxicam, flupirtine,

tramadol, codeine), the antibiotics (doxycycline, amoxicillin, clindamycin, azithromycin, metronidazole, nitrofurantoin, sulfamethoxazole, trimethoprim), the histamine antagonists (loratadine, cetirizine, levocetirizine), the psychoactive drugs (phenobarbital, alprazolam, citalopram, escitalopram, venlafaxine, carbamazepine, olanzapine), and others (doxazocin, finasteride, ambroxol, metoprolol, omeprazole, dydrogesterone, loperamide, warfarin, enalapril, glucosamine, sildenafil, levothyroxine) were purchased from a pharmacy. Non-aqueous background electrolyte compounds, including sodium hydroxide, methanol and acetonitrile (ACN), were obtained from Sigma-Aldrich (USA). All of the chemicals were ACS grade and the organic solvents were HPLC grade.

Cannabis cigarettes with concentrations of THC and CBD of 25 and 20 mg, respectively, were made from the medical marijuana Bediol (Bedrocan, Switzerland) and LM Blue cigarettes.

1.2 Calibrator and quality control solutions

THC 10 mg/mL in ethanol and CBD 1 mg/mL in methanol standard solutions were diluted with acetonitrile to prepare 100 µg/mL stock solutions, which were stored at -20 ºC. A stock solution 100 µg/mL of bicalutamide used as IS was prepared in acetonitrile. Fresh OF samples for the preparation of the working and quality controls (QC) were donated by staff personnel, and collected with the Salivette® device (Sarstedt, Numbrecht, Germany). Calibration solutions 0.5, 1.0, 2.0, 5.0 and 10.0 µg/mL were prepared by the addition of the appropriate amount of the cannabinoid stock solutions and IS (0.5 µg/mL) to blank oral fluid samples, with the following sample preparation. QC solutions were prepared in the same way from different cannabinoid standard lots than those used for the calibrators.

1.3 Oral fluid collection and sample preparation

Fresh OF samples were collected with the Salivette® device and centrifuged at 10000 rpm for 1 minute. The centrifugate was discarded and 1000 µL of acetonitrile was added to the pad. The Salivette tube was centrifuged again under the same conditions and the obtained centrifugate was injected for CE analysis. The collected samples were stored at -20 ºC.

1.4 CE apparatus and analysis

The LED-fluorescence detector for CE used for the determination of cannabinoids was designed and constructed by Laser Diagnostic Instruments AS (LDI), Estonia. It was described in detail in our previous study[25]. A UV-LED (Roithner Lasertechnik, Austria) was used as the fluorescence excitation source (λ = 280 nm). An interference filter of 307 nm (Andover Corporation, USA) was used to block reflected UV radiation and select the required spectral region for fluorescence signal registration.

The CE apparatus was constructed in-house. Uncoated, fused-silica capillaries, i.d. 75 µm and o.d. 360 µm (Polymicro Technologies, Phoenix, AZ, USA) were used for the analyses. The total capillary length was 60 cm with the detection zone placed at 15 cm from the capillary end. Prior to injection, the capillary was rinsed sequentially with 0.1 M NaOH and background electrolyte for 2 min each. Samples were injected into the capillary by hydrodynamic flow at a height differential of 20 cm for 30 seconds. Separations were performed at +17 kV. Before the measurements, new capillaries were conditioned by rinsing them sequentially with 1 M sodium hydroxide and Milli-Q water. Between analyses, the capillaries were rinsed with electrolyte solution for 2 min.

1.5 Method validation

The developed CE method was evaluated for linearity, limits of detection and quantification (LOD and LOQ), selectivity, inter-day precision, accuracy, carry-over, extraction recovery and matrix effect (ME).

Calibration was performed by the method of standard additions. For this, blank OF samples were spiked at five different concentration levels and each concentration level was injected five times. Analyte responses were normalized to an internal standard and quantified by linear least-squares regression. Bicalutamide (BCT) was utilized as the IS. Linearity was checked from 0.5 to 10 µg/mL for both cannabinoids, THC and CBD. The correlation coefficient (R^2) was required to be at least 0.99, and residuals <20% at the LOD and <15% at the other concentrations.

LOD and LOQ were determined by measuring a series of decreasing concentrations of fortified saliva samples. LOD was determined as the lowest analyte concentration with an S/N ratio of at least 3 for both cannabinoids, with acceptable peak shapes. LOQ was the lowest concentration that could be quantified with acceptable precision (%CV<20%) of the target concentration.

Selectivity was defined as the ability to identify and quantify an analyte in the presence of potential endogenous or exogenous interferences. Potential endogenous interferences were assessed by the analysis of six OF samples from volunteers fortified with IS. Exogenous interferences were assessed by the analysis of OF samples fortified with 45 common drugs and alcoholic beverages listed above. Moreover, exogenous interference caused by the smoking of common tobacco cigarettes was also evaluated.

Precision and accuracy were assessed at low (0.5 µg/mL), medium (2.5 µg/ml) and high (10 µg/mL) QC concentrations in an oral fluid matrix. Precision and accuracy were studied by analysing three replicates on ten different days (n=30). The experimental precision was expressed as the relative standard deviation. Accuracy was calculated as the difference between mean and target concentrations (Ct) (A%=mean/Ct×100).

Fortified OF samples exceeding the linear range for THC and CBD (100 µg/mL) were extracted and analysed to evaluate carry-over. Blank samples containing IS were injected after each carry-over challenge to quantify potential carry-over from the previous injection.

Extraction recovery (R%) was calculated at two concentration levels (2.5 and 10 µg/mL) by comparing mean peak areas in blank samples fortified with the analytes and IS before (A) and after (B) extraction with the Salivette® device (n=5). Accordingly, R%=A/B×100. The interference of the matrix with the S/N ratio of each analyte was calculated as the slopes ratios of the calibration curves obtained in solvent (ACN) and in OF.

Results and discussion

1.6 Method development

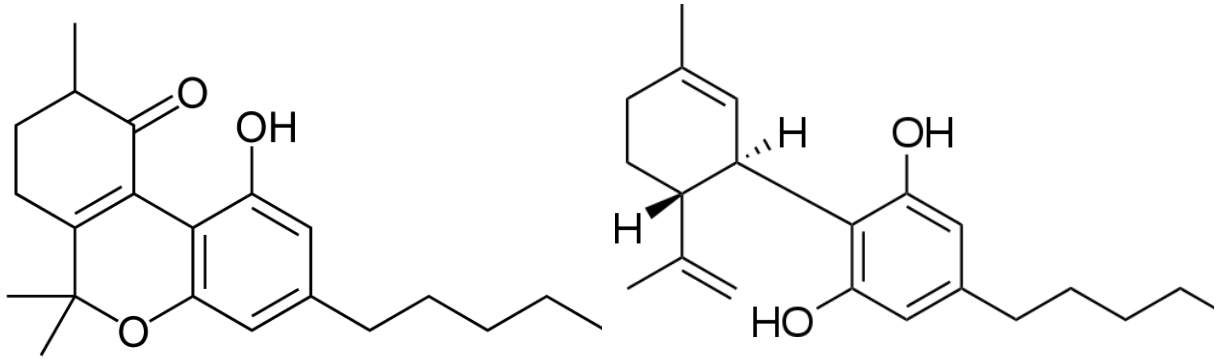
1.6.1 Fluorescence emission spectra of cannabinoids

The fluorescence emission spectra of THC and CBD were first investigated to estimate the feasibility of this method. It was found that both cannabinoids emitted fluorescence wavelengths around 307 nm when excited at 230 and 280 nm. Therefore, it was possible to use a 280 nm light-emitting diode with an interference filter 307 nm to detect them in CE without the need for fluorescent derivatization. The use of the first $\lambda_{ex}/\lambda_{em}$ maximum of cannabinoids (230/307 nm) would provide a higher quantum yield (Figure 1S in supplemental

material) and, therefore, detection sensitivity; however, the absence of the commercially available 230 nm LEDs restricted the possibility of their use for this application.

1.6.2 CE conditions

The cannabinoids studied in the present work represent weakly acidic molecules with hydroxyl groups attached to unsaturated aromatic hydrocarbon rings, and they exhibit pKa values above 9.5 and 10.5 for CBD and THC, respectively (see Figure 1). Thus, in very basic electrolytes, cannabinoids are deprotonated at the oxygen atom and migrate as anions towards the cathode. THC and CBD are almost insoluble in water and the use of organic solvents for separation media has many advantages compared to aqueous CE. Besides the improved solubility for water-insoluble compounds, a non-aqueous medium in CE provides increased selectivity and reduced currents, which allow for the application of high field strengths to achieve rapid separation[30]. The application of a very basic electrolyte system consisting of sodium hydroxide dissolved in methanol-acetonitrile for the analysis of acidic species was first proposed by K. Altria et.al.[30] and confirmed by U. Backofen[31] et.al. for the determination of cannabinoids in hair. In the present study, both cannabinoids and IS (bicalutamide) were separated in 2.5 mM NaOH dissolved in a methanol-acetonitrile (1:1) mixture (Figure 2). It is interesting to note that CBD, which has two phenol moieties and the same mass, migrated faster than THC. This can be explained by the fact that in strongly basic media CBD is oxidized to a quinone, and becomes less charged than THC[32].



Tetrahydrocannabinol, Mw 314.5, pKa 10.5 Cannabidiol, Mw 314.5, pKa 9.7

Figure 1. The chemical formulae, molecular masses and pKa of studied cannabinoids. pKa values can change depending on the environment.

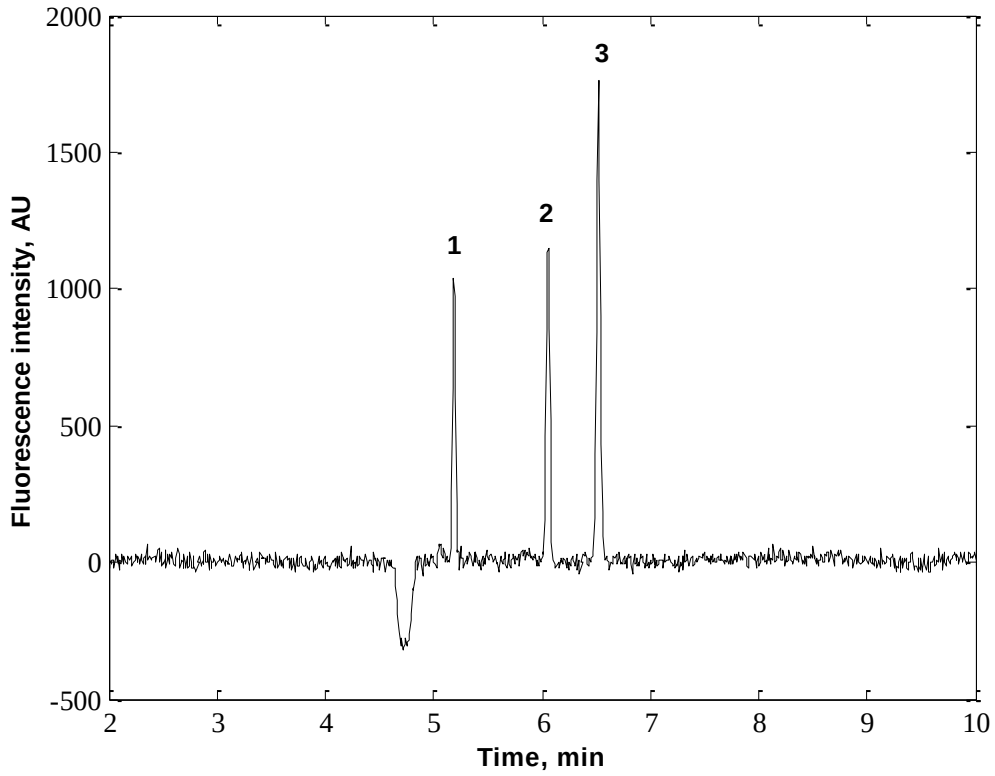


Figure 2. Separation on tetrahydrocannabinol (THC) and cannabidiol (CBD) standards by non-aqueous CE with LED-fluorescence detector. Experimental conditions: capillary I.D. 75 µm; running electrolyte 2.5 mM sodium hydroxide dissolved in methanol-acetonitrile (1:1); separation field 283 V/cm; $\lambda_{ex/em}$=280/305 nm. Peaks: 1-CBD (2 µg/mL), 2-THC (2 µg/mL), 3- IS (1 µg/mL).

1.6.3 Optimization of the extraction procedure

A careful optimization of the extraction step was needed, particularly to achieve the best sensitivity for the detection of cannabinoids in OF. As was mentioned above, during cannabis smoking, oral mucosa is contaminated with cannabinoids contained in cannabis vapour. In the present study, we exploited the strong adsorption of oral mucosa with cannabinoids to Salivette® cotton swabs, which allowed us to use the Salivette® device for sample collection, analyte pre-concentration and extraction. First, a Salivette® tube, containing the collected OF sample was centrifuged and the obtained oral fluid was withdrawn. Then, cannabinoids were extracted by the addition of a certain amount of ACN to the swab, providing denaturation of the mucosa proteins and release of the analytes, followed by centrifugation. The sampling/extraction procedure was examined in terms of the remaining amount of OF in the swab and the loading volume of ACN for maximum extraction efficiency, while the sampling time was kept constant (1 min) according to the Salivette® producer recommendations. For this, blank and fortified (1 µg/mL of each cannabinoid) OF samples from six volunteers were extracted and analysed. The amount of the saliva obtained after the first centifugation varied, depending on the person, from 0.1 to 1.5 mL. However, the remaining amount of oral fluid in the swab after the first centrifugation was constant, 0.33±0.03 mL(n=6), providing normalization of the sampling procedure. The ACN loading volume was varied from 0.5 to 2 mL. The addition of decreased amounts of ACN to the swab produced lower recovery and peak broadening, caused by the weak stacking effect, while an increased amount of ACN led to sample dilution and lower sensitivity. The best extraction recovery values (over 80%) with minimum sample dilution were obtained when 1 mL of ACN was added to the swab (Table 1).

1.7 Method validation

The data of the method validation are summarized in Table 1. The calibration curves were generated using an unweighted least-square regression model. A linear relationship was obtained between the concentration injected and the corrected peak area for both cannabinoids within the range 0.5-10 µg/mL. The calibration curve R^2 always exceeded 0.994. Limits of detection and quantification of 0.19 µg/mL and 0.41 µg/mL for THC, and 0.17 µg/mL and 0.36 µg/mL for CBD were achieved. In spite of that, the GC-MS technique for the analysis of cannabinoids still outperformed the proposed CE protocol in LOD; the real concentrations of cannabinoids in saliva were usually much higher (1-5 µg/mL) for a short time after smoking than the declared cut-offs[9]. Therefore, the proposed CE technique can be successfully applied for confirmation analysis during the intoxication period (0-3 hours after cannabis use[33, 34]).

The selectivity of the method was proven by the evaluation of endogenous and exogenous matrix effects. Endogenous matrix effects were determined in the OF fortified with IS collected from six drug-free volunteers. There were no endogenous signal contributions for any analyte of interest (Figure 3).

Exogenous interferences were assessed by fortifying QC samples with forty-five potential interfering drugs. Under applied CE separation conditions, only tramadol, codeine, citalopram, escitalopram, venlafaxine, metoprolol and omeprazole peaks were observed on the electropherograms. The analysis of the drug-fortified (1 µg/mL) OF samples confirmed that these drugs do not contribute to the measured concentrations of THC and CBD in OF (Figure 4). All drugs, except omeprazole, co-migrated with the EOF peak. The omeprazole migration time was about 14 min (electropherogram (c), peak not shown).

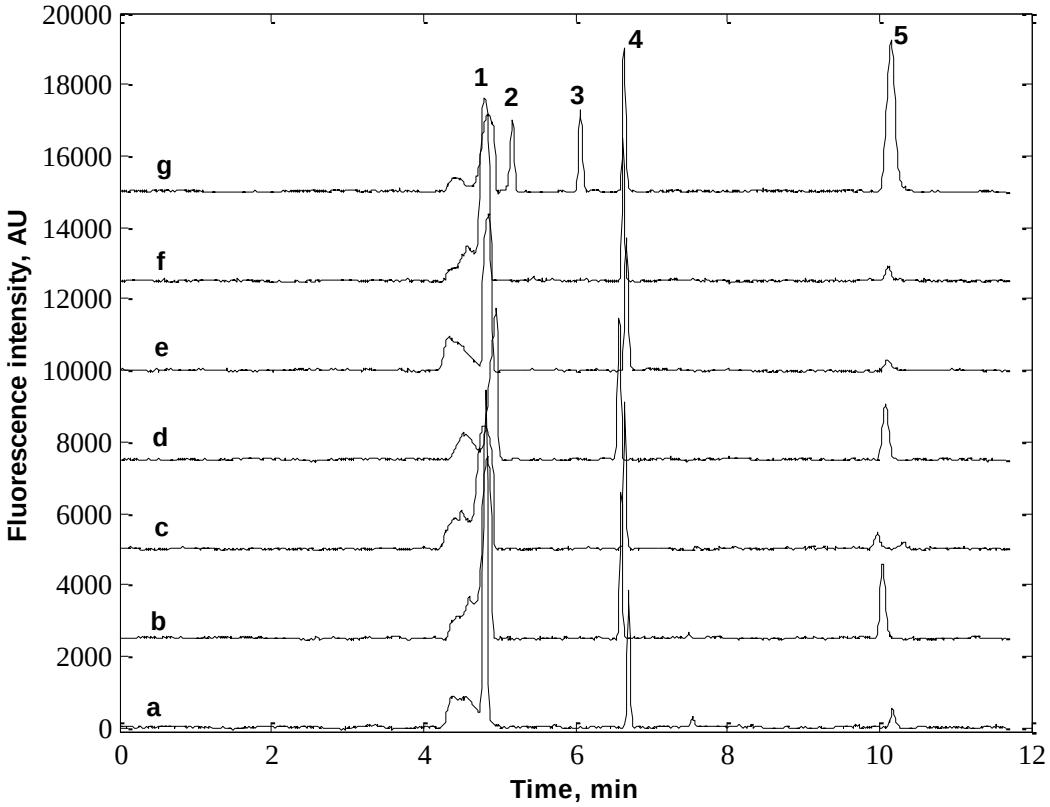


Figure 3. Assessment of the endogenous matrix effect. Experimental conditions as in Figure 2. Electropherograms (a-f) represent the CE analysis of OF samples from six volunteers, (g) – OF sample spiked with standards of CBD, THC and IS. Peaks: 1- neutral compound from Salivette® swab, 2 – CBD, 3 – THC, 4 – IS, 5 – saliva endogenous compound.

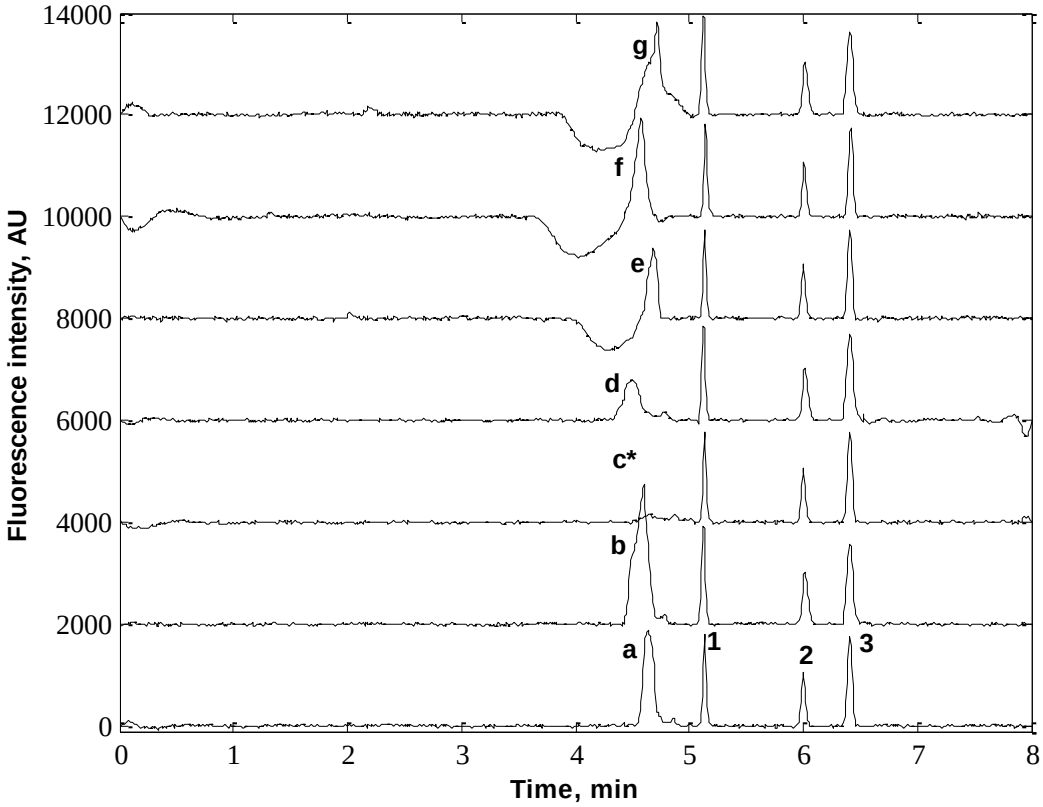


Figure 4. Assessment of exogenous matrix effect. Experimental conditions as in Figure 2.

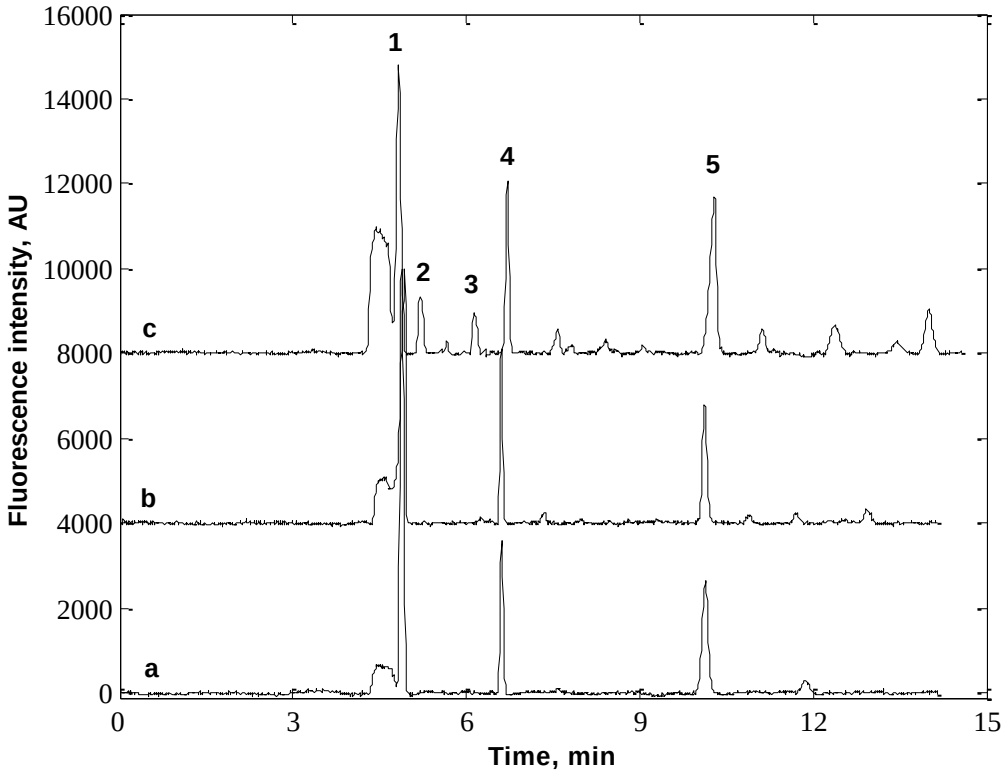
Peaks: a - tramadol, b - metoprolol, c* – omeprasol peak at 14 min, d - venlavaxin, e - estsitalopram, f - tsitalopram and g - codeine (1 µg/mL of each drug); 1- CBD (1 µg/mL), 2 - THC (1 µg/mlL) and 3 – IS (0.5 µg/mL).

Exogenous interferences caused by the smoking of tobacco cigarettes were also evaluated. Figure 5 (a) and (b) demonstrates a volunteer's blank OF sample and saliva sample analyses after the smoking of one "LM blue" cigarette. No quantifiable peaks were detected at the specific retention time for both analytes.

Inter-day reproducibility (precision) and accuracy were assessed using a fortified drug-free matrix at three concentration levels (Table 1). Inter-day reproducibility results expressed as residual standard deviation (RSD%) were constantly < 6%. Accuracy calculated as the percent difference between mean and target concentrations of each analyte ranged from 98 to 108% for all concentration levels.

Extraction recovery was calculated by comparing mean corrected peak areas (n=5) of analytes in drug-free OF fortified prior to and after the extraction procedure with the Salivette® device. Mean extraction efficiencies ranged between 80 and 85%, which are very good compared to LLE (70%)[4] or MEPS (50%)[35] saliva pre-treatment methodologies.

With regard to the matrix effect in neat OF, both CBD and THC showed fluorescence signal enhancement: +26% and +40%, respectively (Table 1). Therefore, the quantitative determination of these cannabinoids in saliva must be conducted by a standard addition method into a sample matrix.



Figue 5. CE analysis of real OF specimens. Experimental conditions as in Figure 2. Electropherograms: a – blank OF, b - saliva sample after the smoking of one "LM blue" cigarette, c - saliva sample after the smoking of one cannabis cigarette. Peaks: 1- neutral compound from a Salivette® swab, 2 – CBD, 3 – THC, 4 – IS, 5 – saliva endogenous compound.

Table 1. Performance characteristics of the CE-LED method for the quantification of THC and CBD in saliva

Analytes	Linear range, µg/mL	Equation ($Area_{analyte}/Area_{IS}$)	R^2	LOD, µg/mL	LOQ, µg/mL	Inter-day precision (n=30), RSD%	Accuracy ±SD, % (n=30)	Extraction recovery ±SD, % (n=30)	Matrix effect (ME)
CBD	0.5-10	y=(0.127±0.002)x +(0.006±0.009)	0.9955	0.17	0.36	0.5: 5.6 2.5: 5.2 10: 4.0	108±3 98±2 100±3	83±4 81±3	1.26
THC	0.5-10	y=(0.158±0.003)x +(0.013±0.013)	0.9943	0.19	0.41	0.5: 2.8 2.5: 5.6 10: 3.7	107±5 100±3 100±6	81±3 80±3	1.40

Negative samples injected immediately after samples containing 100 µg/ml of THC and CBD showed no evidence of carry-over (the signal was below the LOD for both analytes).

1.8 Application to real OF specimens

The developed CE-LED-IF method was employed to quantify THC and CBD in OF specimens collected with the Salivette® device after the controlled *al libitum* smoking of one cannabis cigarette containing approximately 20 mg THC and 15 mg CBD. The protocols were approved by the National Medical Research Ethics Committee, and the participants provided written informed consent. OF was collected 10 min before (blank) and 20 min after the start of smoking. A representative electropherogram demonstrating a selective separation of analytes from a participant's OF after smoking is presented in Figure 5 (c). The measured concentrations of THC and CBD were 3.04 µg/mL and 4.05 µg/mL, respectively.

Conclusions

A rapid and simple method of CE with ultraviolet light-emitting diode-induced native fluorescence (UV-LEDIF) detection was presented to determine THC and CBD in OF. The proposed separation protocol in combination with highly selective native fluorescence detection made it possible to quantify these cannabinoids in a biological matrix with a minimum sample pretreatment. No separate precipitation of proteins or derivatisation was needed. The procedure of saliva sample collection and clean-up was accomplished with the Salivette® sampling device and the whole analysis, including CE separation of analytes, took 10 minutes.

The validation results show that the detection limits of the CE-LEDIF method are less reliable than GC-MS LODs, but can be considered acceptable for determination of cannabinoids in OF during a short time after smoking (a couple of hours). Moreover, the lack of detection

sensitivity can be overcome by using an excitation source at the first $\lambda_{ex}/\lambda_{em}$ maximum of cannabinoids (230/307 nm). The implementation, for example, of a miniature flash Xe-lamp instead of an LED for the excitation of analytes provides an approximate 50-fold increase in sensitivity. In respect to the simplified operating conditions, excellent selectivity and miniaturization benefits of the proposed CE technology, it seems to be a very promising and reliable alternative to conventional laboratory GC/LC-MS for on-site analysis of drug abuse. The construction of a novel portable CE instrument with a 230 nm excitation source would be an interesting direction for further development.

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The authors have declared no conflict of interest.

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