Analytical Methods

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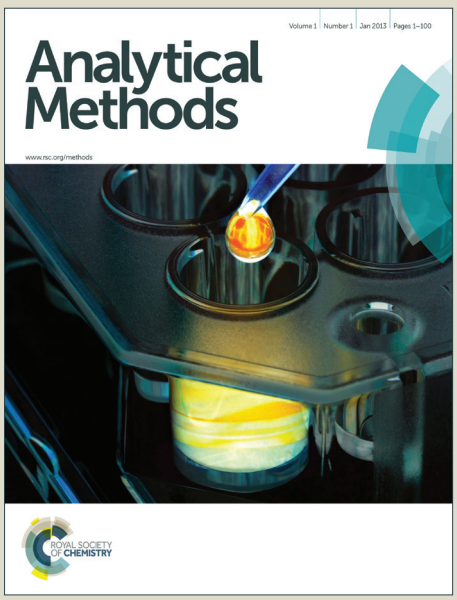

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Supercritical fluid chromatography method for the systematic toxicology analysis of cannabinoids and their metabolites†

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A novel, simple and rapid supercritical fluid chromatography method was developed as a screening tool for natural and synthetic cannabinoids and their metabolites in biological samples.

Numerous narcotic analogs have been widely distributed as psychotropic substances in recent years.[1] Synthetic cannabinoids (SCs) are a diverse group of compounds that are derived from indole, indene and pyrrole structures and bind to one or both cannabinoid receptors with different affinities. They were chemically designed for enhancing the pharmacological potency of Δ^9-tetrahydrocannabinol (THC, the active component of cannabis). Many of them are not structurally related to the naturally occurring cannabinoids based on dibenzopyran. The SCs have code names (*e.g.* JWH-, AM-) which are mostly derived from the initials of the name of the scientists who first synthesized them or the given names can be also derived from their long chemical names. European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) registered 134 SCs till December 2014 and the number of detected SCs on illegal drug scene grows year on year.[2] SCs are available at the market as smoking blends or collectibles and gained popularity due to their easy accessibility and psychoactive effects.[3,4] Cases of abuse of SCs with threat to life or even fatal were reported in the last decade.[5] Some methods were reported for determination of SCs in herbal products and in different biological matrices including whole blood, serum, plasma, hair, oral fluids or urine.[6-8] The analysis of urine samples is further complicated by the fact that SCs are rapidly biotransformed into a large number of metabolites.[9] Moreover, the conventional cannabinoid immunochemical screening tests are ineffective in detecting this class of compounds with sufficient specificity. For systematic toxicology analysis of SCs fast and efficient analytical methods are required. Determination of original, unchanged drugs as well as their metabolites in biological fluids is requisite. At present, the reported methods are primarily based on GC or LC coupled with MS or high resolution accurate MS.[10-15] Few works are focused on analyzing of SCs using MEKC or LC connected with UV detection, respectively.[16,17] However, by now no SFC method for determination of SCs has been published. In different laboratory applications, SFC could be used because of its high separation efficiency, short analysis time and last but not least lower contribution to environmental pollution.[18,19] The aim of this work was to develop and validate simple and fast SFC method for the simultaneous analysis of natural cannabinoids and a wide group of the SCs and their metabolites in human urine. The influence of mobile phase (MP) composition and the effect of temperature, back pressure (BP) and flow rate were evaluated for optimizing the separation process. In addition, the performance of various extraction techniques for extraction from urine samples was examined and compared with respect to recovery and simplicity of extraction. The potential of the developed method was demonstrated by analysis of real urine sample obtained from patient after herbal blends abusing contain JWH-073 (naphthalen-1-yl-(1-butylindol-3-yl)methanone). The structures of the studied analytes are depicted in Fig. S1, †. The chosen analytes contained the most abused SCs in the European Union and their main metabolites and also two natural cannabinoids (THC and CBD) for comparison. Detailed information about instrumentation, experimental conditions, standards, calibration and sample preparation are stated in the Experimental part in the ESI S1,†.

At first, various MPs differing in the type and amount of organic modifier (OM), acetonitrile (ACN), methanol (MeOH), propane-2-ol (IPA), added to supercritical CO_2 were evaluated. No significant differences among the values of retention factors and resolutions of the studied analytes in the MPs with the same OM contents were found. Nevertheless, MPs composed of CO_2 and ACN showed better peak shapes and higher response. As expected, the retention factors and resolution values decreased with increasing ACN concentration in the MPs (data not shown), as the MP polarity increased. The most promising MP contained 7% (*v/v*) of ACN in CO_2, therefore, this MP composition was studied in detail. The flow rate of the optimized MP was examined first. As a compromise between resolution values and analysis time a flow rate of 2.5 mL/min was used. The effect of column temperature was studied under the optimized MP conditions in the range of 25-40 °C.

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Concerning retention and resolution values, the optimum temperature for the analysis was 40 °C (data not shown). The influence of the BP was tested in the range of 80-150 bars. Higher BP resulted in lower retention that was accompanied by a certain decrease of resolution values whereas lower BP provided conditions for better separation. Therefore, BP of 95 bars was chosen.

Three extraction techniques including solid-phase extraction (SPE), liquid-liquid extraction (LLE)[11] and salting out assisted liquid-liquid extraction (SALLE)[20] were employed for determination of natural cannabinoids, SCs and their metabolites in urine samples. Sample preparations by SPE with Supel™-Select SCX SPE Tube (1 mL x 30 mg) and LLE were associated with lower recovery values (data not shown), compared to SALLE. Therefore, SALLE procedure was applied in further study. Detailed description of this procedure is given in ESI S1,†.

Validation of the method was carried out under the optimized separation conditions. The newly developed method was validated in terms of precision, selectivity, sensitivity, linearity, extraction recovery and robustness according to forensic analysis standards published by Peters et al. [21] Intra-day precision was evaluated by extracting and analyzing eight urine samples spiked at three concentration levels by target analytes (3.0 µg/mL, 5.0 µg/mL, 10.0 µg/mL). Inter-day precision was evaluated by preparing and analyzing eight urine samples spiked with the analytes at 5.0 µg/mL final concentration within three consecutive days. The values, expressed as RSD of retention times and peak areas are summarized in Table S1,†. The RSD values for retention time were ≤0.16% and 0.19% for intra-day and inter-day precision, respectively. Satisfactory results were also achieved for peak areas with RSD ≤ 5.12% and 6.58% for intra- and inter-day measurements. The selectivity was assessed by comparing the chromatographic data of eight different blank human urines with the corresponding spiked urine. Fig. 1 shows the typical chromatograms of blank urine and blank urine spiked with 5 µg/mL of the mixture of the tested analytes. Chromatographic data are shown in Table S2,†. Blank samples showed no significant interference from the matrix at the retention times (peak positions) of the analytes. Calibration solutions were prepared by spiking of studied analytes standards into negative urine (100 µL) and then extracted by described salting out protocol (see ESI S1,†). Linearity was verified over the concentration range of 0.5 – 20.0 µg/mL, 1.0 – 20.0 µg/mL, 1.5 – 20.0 µg/mL and 2.0 – 20.0 µg/mL respectively, by injection of calibration solutions containing known concentrations of a mixture of THC, CBD, SCs and their metabolites (see Table S3,†). Six point calibration method was used, each point was measured three times. The calibration curves were obtained by plotting the peak areas as a function of analytes concentrations. The regression analysis was performed by calculating the coefficients of determination R^2, which ranged between 0.9980 and 0.9998. These values clearly confirm good linearity in the given calibration range (Table S3,†). LOD, expressed as a concentration at a signal-to-noise ratio 3:1, was calculated based on the baseline noise, which was evaluated by recording the detector response over a period approximately ten times the widths of the peaks. LOQ was taken as a concentration of analyte where signal-to-noise ratio was 10:1. The obtained LOD and LOQ values ranged between 0.15-0.52 µg/mL and 0.50-1.73 µg/mL, respectively.

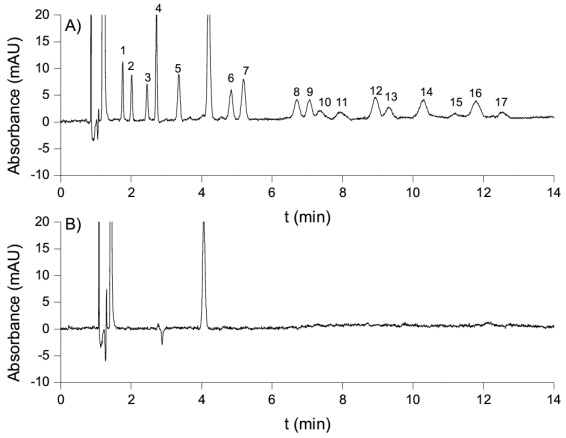

Fig. 1 Typical chromatograms of blank urine sample spiked with 5.0 µg/mL working solution of analytes (A) and blank urine (B) under optimized experimental conditions. MP composition: CO_2/ACN 93/7 (*v*/*v*); flow rate 2.5 mL/min; column temperature 40 °C; 95 bars as BP; UV detection at 210 nm, injection volume 5 µL. Elution order: 1. THC, 2. CBD, 3. JWH-250, 4. JWH-073, 5. AM-2201, 6. JWH-019 5-hydroxyindole, 7. JWH-073 5-hydroxyindole, 8. JWH-018 6-hydroxyindole, 9. JWH-073 6-hydroxyindole, 10. JWH-210 *N*-(5-carboxypentyl), 11. JWH-018 *N*-pentanoic acid, 12. AM-2201 M1, 13. RCS-4 *N*-(4-hydroxypentyl), 14. JWH-018 *N*-(4-hydroxypentyl), 15. JWH-019 *N*-(6-hydroxyhexyl), 16. JWH-200, 17. JWH-200 4-hydroxyindole.

The extraction recoveries were calculated by comparing the experimental results of two sets of solutions at two concentration levels. In the first set, eight blank urine samples were spiked with all analytes at 5.0 and 10.0 µg/mL final concentrations before the extraction step, while in the second set the spiked standard solutions (at the same concentrations) were made on the blank urine samples. Recovery data with the RSD values are reported in Table S4,†. The percentage recovery for analytes at 5.0 µg/mL final concentrations was in the range of 74.2-124.3% and at 10.0 µg/mL final concentrations was in the range of 71.2-96.9%.

One-way ANOVA statistical method was used for robustness testing. Selected variable method parameters were: ACN content in the MP (7.0 ± 0.2 vol%), column temperature (39 °C, 40 °C and 41 °C) and BP (90 ± 5 bars). The robustness was determined for triplicate injections of 5.0 µg/mL of the mixture of tested analytes. The effects of method parameters on peak areas and retention times were calculated. The hypothesis that errors resulted from a normal distribution was tested first. This hypothesis was accepted in all cases at significance level (α = 0.05). Consequently, the robustness of the method was examined using the one-way ANOVA. The null hypothesis (all medians are equal) was accepted in all cases (obtained *p*-values were higher than 0.05, data not shown), so the robustness of the selected parameters was verified.

The developed method for separation and detection of natural cannabinoids, SCs and their metabolites was used for screening of these compounds in urine sample suspicious of JWH-073 abuse to demonstrate the potential of the method. The sample

preparation and extraction was performed according to the procedure described in ESI S1, †. Representative chromatograms of the urine sample analysis obtained from patient after JWH-073 administration and blank urine obtained from healthy volunteer are shown in Fig. 2. The identification of JWH-073 5-hydroxyindol and JWH-073 6-hydroxyindol metabolites in urine was carried out by standard addition method, the comparison of retention times and DAD spectra with standards. As it can be seen from Fig. 2, two hydroxy-metabolites were identified in urine. However, the absence of detailed information such as dosage, duration of administration and time delay between intoxication and sample collection makes difficult to interpret the concentration ratio between identified metabolites. The concentrations of JWH-073 5-hydroxyindol and JWH-073 6-hydroxyindol were determined as 2.5 ± 0.2 μg/mL and 1.0 ± 0.2 μg/mL, respectively.

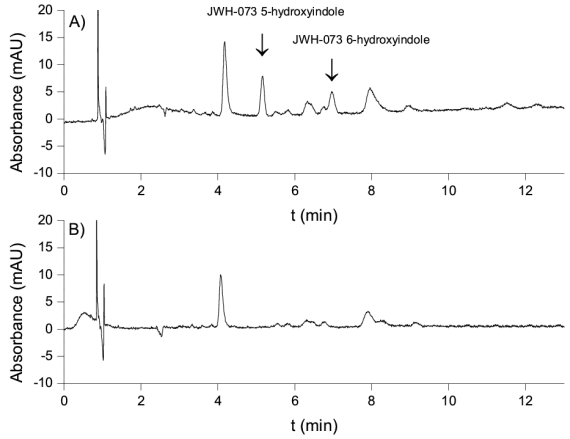

Fig. 2. Chromatograms of analysis of urine obtained from patient after JWH-073 abusing (A) and blank urine (B). See caption to Fig. 1 for details.

Conclusions

A new, simple, rapid SFC method for simultaneous separation of the natural cannabinoids, SCs and their metabolites in urine was developed and validated. It offers advantages such as short analysis time, high separation efficiency and low consumption of organic solvents. The method is precise, selective and robust with satisfactory linearity within the calibration range. The developed method of separation of SCs and their metabolites holds the potential for systematic toxicology analysis particularly in case of hyphenation with sensitive detection, namely mass spectrometry.

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Notes and references

1 M. Johansson, D. Fransson, T. Rundlöf, N. H. Huynh and T. Arvidsson, *J. Pharm. Biomed. Anal.* 2014, **100**, 215.
2 http://www.emcdda.europa.eu/topics/pods/synthetic-cannabinoids (downloaded 4th June 2015).
3 J. Lapoint, L. P. James, C. L. Moran, L. S. Nelson, R. S. Hoffman and J. H. Moran, *Clin. Toxicol.* 2011, **49**, 760.
4 G. L. Buser, R. R. Gerona, B. Z. Horowitz, K. P. Vian, M. L. Troxell, R. G. Hendrickson, D. C. Houghton, D. Rozansky, S. W. Su and R. F. Leman, *Clin. Toxicol.* 2014, **52**, 664.
5 A. Pourmand, P. Armstrong, M. Mazer-Amirshahi and H. Shokoohi, *Hum. Exp. Toxicol.* 2014, **33**, 993.
6 J. Znaleziona, P. Ginterová, J. Petr, P. Ondra, I. Válka, J. Ševčík, J. Chastina and V. Maier, *Anal. Chim. Acta* 2015, **874**, 11.
7 H. Koskela, U. Hakala. L. Loiske, P. Vanninen and I. Szilvay, *Anal. Methods* 2011, **3**, 2307.
8 C. E. J. Emanuel, B. Ellion and C. E. Banks, *Anal. Methods* 2010, **6**, 614.
9 M. Mazzarino, X. de la Torre and F. Botrè, *Anal. Chim. Acta* 2014, **837**, 70.
10 D. Favretto, J. P. Pascali and F. Tagliaro, *J. Chromatogr. A* 2013, **1287**, 84.
11 A. Grigoryev, S. Savchuk, A. Melnik, N. Moskaleva, J. Dzhurko, M. Ershov, A. Nosyrev, A. Vedenin, B. Izotov, I. Zabirova and V. Rozhanets, *J. Chromatogr. B* 2011, **879**, 1126.
12 J. Kim, Y. Park, M. Park, E. Kim, W. Yang, S. Baeck, S. Lee and S. Han, *J. Pharm. Biomed. Anal.* 2015, **102**, 162.
13 S. Beuck, I. Möller, A. Thomas, A. Klose, N. Schlörer, W. Schänzer and M. Thevis, *Anal. Bioanal. Chem.* 2011, **401**, 493.
14 K. B. Schweidweiler and M. A. Huestis, J. Chromatogr. A 2014, **1327**, 105.
15 C. Montesano, M. Sergi, S. Odoardi, M. C. Simeoni, D. Compagnone and R. Curini, *J. Pharm. Biomed. Anal.* 2014, **91**, 169.
16 R. Gottardo, A. Bertaso, J. Pascali, D. Sorio, G. Musile, E. Trapani, C. Seri, G. Serpelloni and F. Tagliaro, *J. Chromatogr. A* 2012, **1267**, 198.
17 G. Merola, Z. Aturki, G. D´Orazio, R. Gottardo, T. Machia, F. Tagliaro and S. Fanali, *J. Pharm. Biomed. Anal.* 2012, **71**, 45.
18 C. Radcliffe, K. Maguire and B. Lockwood, *J. Biochem. Biophys. Methods* 200, **43**, 261.
19 K. Kalíková, T. Šlechtová, J. Vozka and E. Tesařová, *Anal. Chim. Acta* 2014, **821**, 1.
20 E. G. Yanes and D. P. Lovett, *J. Chromatogr. B* 2012, **909**, 42.
21 F.T. Peters, O.H. Drummer and F. Musshoff, *Forensic Sci. Int.* 2007, **165**, 216.