Journal of Pharmaceutical and Biomedical Analysis 71 (2012) 45–53

Contents lists available at SciVerse ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Analysis of synthetic cannabinoids in herbal blends by means of nano-liquid chromatography

Gustavo Merola[a,b,c], Zeineb Aturki[b], Giovanni D'Orazio[b], Rossella Gottardo[c], Teodora Macchia[a], Franco Tagliaro[c], Salvatore Fanali[b,*]

[a] Department of Therapeutic Research and Medicines Evaluation, Istituto Superiore di Sanità, Viale Regina Elena 299, Rome 00161, Italy
[b] Institute of Chemical Methodologies, Consiglio Nazionale delle Ricerche, Area della Ricerca di Roma I, Via Salaria Km 29,300, 00015 Monterotondo, Rome, Italy
[c] Department of Public Health and Community Medicine, Unit of Forensic Medicine, University of Verona, P.le L.A. Scuro 10, 37134 Verona, Italy

ARTICLE INFO

Article history:
Received 2 July 2012
Received in revised form 4 August 2012
Accepted 7 August 2012
Available online 16 August 2012

Keywords:
Nano-liquid chromatography
Mass spectrometry
Synthetic cannabinoids
Herbal blends

ABSTRACT

In this study, a rapid and simultaneous separation of 12 synthetic cannabinoids and Δ^9-tetrahydrocannabinol (Δ^9-THC) in herbal blends was obtained by means of nano-liquid chromatography (nano-LC). The nano-LC experiments were performed in a 100 μm i.d. capillary column packed with Cogent® bidentate C_{18} silica particles for 25.0 cm. All compounds were resolved using an isocratic elution mode in less than 30 min. A mobile phase containing ACN/MeOH/H_2O/formic acid 69/5/25/1 (v/v/v/v) was employed for the chromatographic separation. The developed analytical method was validated in terms of precision, linearity, sensitivity and accuracy. Under optimal nano-LC–UV conditions, the resulting RSD percentages for intra-day and inter-day repeatability, related to retention time and peak area, were below 2.98 and 6.40%, respectively. Limits of detection and quantification were 0.2 and 0.5 μg/mL, respectively, for all the studied compounds. Linearity was assessed in the concentration range of interest for all analytes with determination coefficients $r^2 \geq 0.9975$. The method was then applied to the determination of synthetic cannabinoids in herbal blends. Quantitative analyses of the cannabimimetic compounds in six products showed that there was a wide difference in the concentration of the studied compounds among different products. Further, the nano-LC system was coupled with a mass spectrometer measuring the MS and MS–MS spectra to unequivocally identify the cannabinoids present in smoking mixtures.

1. Introduction

In the last few years, different types of herbal products have been sold in the international market via Internet and in some specialized shops. Although these products were declared as incense and not for human consumption, "spice products" are smoked by some users with the purpose to have psychotropic effects similar to those of cannabis [1].

At the end of 2008, the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) (EMCDDA-Europol, 2008. Annual Report on the implementation of Council Decision 2005/387/JHA) reported that analyses of the herbal products found the presence of JWH-018 (naphthalen-1-yl-(1-pentylindol-3-yl)methanone), a synthetic psychoactive compound. Afterwards the homologue JWH-073 (naphthalen-1-yl-(1-butylindol-3-yl)methanone) and a great variety of other aminoalkylindole molecules have been identified in herbal blends. The cannabinoid-like molecules of aminoalkylindole family (e.g. WIN 55-212, JWH-018, JWH-019, JWH-073) were synthesized in 1995 by Huffman [2]. These compounds have been found to act as the agonists of cannabinoid receptors CB1 and CB2 [3,4]. Due to their high receptor affinity, the synthetic cannabinoids have been added to herbal mixtures to achieve psychoactive effects similar to those obtained by smoking cannabis.

Although these compounds were developed as pharmaceutical candidates for the treatment of different diseases, they exhibit the unwanted cannabis-like psychoactive effect in addition to the therapeutical activity.

The administration of the smoking mixtures caused several adverse effects on human health with consequent serious risks including tachycardia, psychosis, intoxications, etc. [5–11].

Because of the lack of specific regulations and widespread use of products with unknown composition in terms of components and dosages, the growing consumption of synthetic cannabinoids as designer drugs of abuse has become a significant trouble for public health institutions.

To counteract the distribution of these products, many of these psychoactive molecules have been included under control by law in

* Corresponding author. Tel.: +39 0690672256; fax: +39 0690672269.
E-mail address: salvatore.fanali@imc.cnr.it (S. Fanali).

0731-7085/$ – see front matter © 2012 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.jpba.2012.08.008

many European countries as well as in Italy. To overcome this ban, new analogues of aminoalkylindoles and other cannabimimetics have been continuously introduced in the market.

For this reason, forensic laboratories are increasingly involved in the analysis of a great number of samples containing both scheduled and not yet identified synthetic cannabinoids.

The development of rapid and efficient analytical tools for the identification of these compounds is of utmost importance both in clinical and forensic toxicology.

Separation techniques so far used for the analysis of synthetic cannabinoids are mainly based on chromatographic ones such as liquid and gas chromatography both hyphenated with mass spectrometry (LC–MS and GC–MS). As reported in literature, they were mostly applied to the determination of these compounds and their metabolites in serum and urine [12–20] as well as for the identification and quantification of synthetic cannabinoids in herbal blends [21–29]. Most of these studies were often focused on the identification of one or few cannabinoids in one single run.

Owing to the great demand for faster, efficient and less expensive analytical methods, miniaturization has emerged as an increasing popular tool in several field also including forensic sciences. Among them, capillary zone electrophoresis (CZE), micellar electrokinetic chromatography and capillary electrochromatography have been successfully applied to the analysis of illegal drugs in seized materials and in biological fluids [30–34]. Recently miniaturization has also been introduced in HPLC with the development of nano-liquid chromatography (nano-LC). This technique, firstly proposed by Karlsson and Novotny [35], allows the separation of a large number and type of compounds in capillary columns of internal diameter (i.d.) $\leq$100 μm employing flow rates in the range of 50–800 nL/min. Compared to conventional LC, nano-LC offers some unique advantages such as high efficiency and selectivity, shorter analysis time, lower sample dilution, need of smaller volumes of samples and reagents as well as small amounts of packing materials. In addition the low flow rates used consent to an easy coupling with mass spectrometry (MS) and makes these tool eco-friendly. In recent years, nano-LC has proved to be a powerful analytical technique in various application fields such as proteomics, pharmaceutical, agrochemical, environmental and enantiomeric analysis [36–39].

Considering all the above mentioned features and the good performances achieved in other application fields, nano-LC can also be considered for practical applications in forensic sciences. However, until now, this miniaturized technique has been rarely applied to the field of forensic toxicology. Indeed, only few papers concerning the analysis of epoetins by nano-LC, for doping control, have been published [40,41].

To the best of our knowledge nano-LC has not been yet applied to the analysis of synthetic cannabinoids.

Therefore the aim of this study was to develop and validate a rapid nano-LC method for the analysis of synthetic cannabinoids in herbal mixtures. For this purpose, optimised separation conditions were evaluated to resolve simultaneously 12 aminoalkylindoles and Δ^9-THC, generally found in herbal products. The method was optimized employing UV–vis detection. In addition, mass spectrometry (MS) with electrospray (ESI) interface was used for the identification and characterisation of the studied compounds after measuring the MS and MS/MS spectra.

2. Materials and methods

2.1. Chemicals

All solvents used were of analytical reagent grade. Acetonitrile (ACN), methanol (MeOH) and formic acid were from Carlo Erba (Milan, Italy). Distilled water was deionized with a Milli-Q water purification system (Millipore, Waters, Milford, MA, USA).

The selected synthetic cannabinoids: naphthalen-1-yl-(1-pentylindol-3-yl)methanone (JWH-018), naphthalen-1-yl-(1-hexylindol-3-yl)methanone (JWH-019), naphthalen-1-yl-(1-butylindol-3-yl)methanone (JWH-073), 4-methoxynaphthalen-1-yl-(1-pentylindol-3-yl)methanone (JWH-081), (4-methyl-1-naphthyl)-(1-pentylindol-3-yl)methanone (JWH-122), (1-(2-morpholin-4-ylethyl)indol-3-yl) naphthalen-1-ylmethanone (JWH-200), 2-(2-chlorophenyl)-1-(1-pentylindol-3-yl)ethanone (JWH-203), 2-(2-methoxyphenyl)-1-(1-pentylindol-3-yl)ethanone (JWH-250), 1-[(5-fluoropentyl)-1H-indol-3-yl]-(2-iodophenyl) methanone (AM-694), and (R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-napthalenylmethanone (WIN-55,212-2) were purchased from LGC Standards (Sesto San Giovanni, Milan, Italy). Δ^9-Tetrahydrocannabinol (Δ^9-THC) was acquired from Sigma–Aldrich (Milan, Italy). Owing to standards unavailability, a solution containing both 4-ethylnaphthalen-1-yl-(1-pentylindol-3-yl)methanone (JWH-210) and 1-[(5-fluoropentyl)-1H-indol-3-yl]-(naphthalen-1-yl)methanone (AM-2201), was obtained by extraction of an herbal mixture at unknown concentration. The chemical structures of cannabimimetic molecules are reported in Fig. 1. The analysed commercial herbal blends samples were obtained as part of the project “Smart Search” funded by the Department for Anti-Drug Policies, Presidency of the Council of Ministers, Italy.

Stock solutions of each cannabinoid (1 mg/mL) were prepared in MeOH and stored at +4 °C. Appropriate volumes of stock solutions were mixed and diluted with water to obtain a standard mixture with a final concentration of 25 μg/mL of each compound except for Δ^9-THC (50 μg/mL). Owing to the unknown concentration of JWH-210 and AM-2201, these two compounds were used only for qualitative analysis.

Mobile phases used for nano-LC experiments were daily prepared by mixing proper volumes of the organic solvents and water.

2.2. Instrumentation

Experiments were carried out using a nano-LC system assembled in our laboratory. It included a Rheos 2000 micro-LC pump (Flux Instrumentations, Basel, Switzerland), controlled by a software system (Janeiro II, Version 2.0, Flux Instrumentations, Basel, Switzerland), and a modified injector valve (Enantiosep GmbH, Münster, Germany) with a 50 μL loop, used as reservoir for the mobile phase. Samples were introduced into the column by means of a nano-injector (100 nL) (VICI Valco, Houston, TX, USA). Mobile phase reservoir and nano-injector were connected each other with a polyetheretherketone (PEEK) tube (12 cm × 20 μm i.d.). The capillary column was directly connected to the nano-injector.

To reduce the flow rate from μL/min to nL/min, a split flow system, including a stainless steel T piece (VICI Valco Instruments, Huston, TX, USA), was employed. The pump was connected to one entrance of the T piece through a 50 cm × 130 μm i.d. PEEK capillary. The second entry of the T was coupled to the injection valve via a stainless steel tube of 7 cm × 500 μm i.d. and joined to the waste through a 50 cm × 50 μm i.d. fused silica capillary.

The flow rate of 500 nL/min was estimated connecting a microsyringe to the end of the capillary column through a Teflon tube and measuring the mobile phase volume after 5 min.

Analytes were on-column detected at 214 nm using a Spectra Focus PC 1000 UV–vis detector, Thermo Separation Products (San José, CA, USA). Data were collected by Clarity^{TM} Advanced Chromatography Software (DataApex Ltd., Prague, Czech Republic).

Analytes identification and characterization were performed using an LCQ^{TM} ion-trap electrospray mass spectrometer (Thermo-Finnigan, San José, CA, USA) equipped with an ESI source and

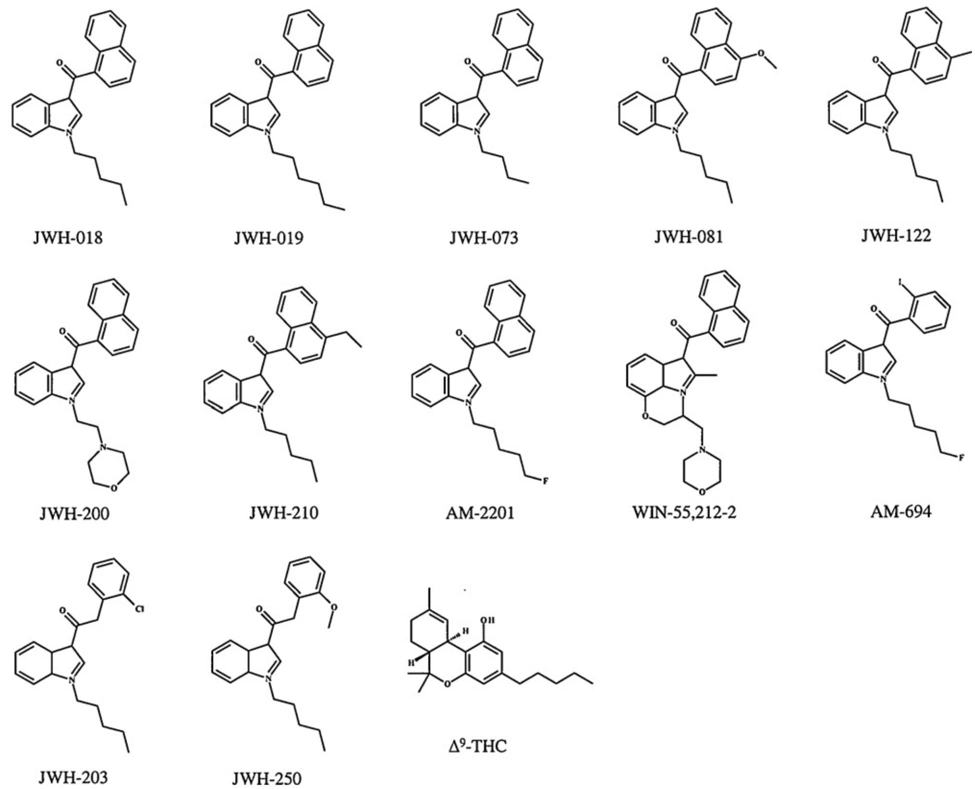

Fig. 1. Chemical structures of the analysed synthetic cannabinoids.

operating in positive ion mode. System control and data collection were performed using Xcalibur^{TM} 1.3 software (Thermo-Finnigan). The chromatographic capillary column was connected to the MS through a commercial nano-spray interface (Thermo-Finnigan).

Emitter tips were prepared in our laboratory shaping a fused silica capillary (length, 10.7 cm, 25 μm i.d., 375 μm o.d.) supplied by Composite Metal Services (Hallow, UK) with sand paper on a rotating disk. The tip was positioned at 1–2 mm from the MS orifice using a *x–y–z* translation stage.

The analytes were detected setting the spray voltage, capillary voltage and capillary temperature at 1.8 kV, 34 V, 200 °C, respectively. The acquisition of ion chromatograms was realized in the range of 150–450 *m*/*z* and 90–500 *m*/*z* in full and MS/MS scan mode, respectively. To obtain the MS^2 spectra, the protonated molecular ion of each selected compound was fragmented varying the normalized collision energy (%) in the range of 26–40%.

2.3. Capillary column preparation

Nano-LC experiments were performed in fused silica capillaries (100 μm i.d., 375 μm o.d. purchased from Composite Metal Services (Hallow, UK), packed in our laboratory with Cogent® bidentate C_{18} 4.2 μm stationary phase (Microsolv Technology Corporation, Eatontown, USA). The packing procedure was the same to that reported in a previous work [42].

Briefly, one end of the capillary was connected to a mechanical temporary frit (VICI Valco, Houston, TX, USA) to retain the packing material and the other end to a stainless steel HPLC pre-column (10 cm × 4.1 mm i.d.) which was used as reservoir for the slurry. Particles suspended in acetone were packed into the capillary using a LC pump (Perkin-Elmer series 10 LC, Palo Alto, CA, USA), for a length of about 35.0 cm. Afterwards, the column was flushed for 30 min with water at 20 MPa to remove the packing solvent from the capillary. The inlet and outlet frits were prepared sintering the particles for 6 s at 700 °C using a laboratory made heating wire and flushing the capillary continuously with water. The temporary frit was removed and the excess of packing material was eliminated by flushing with water. The detection window was prepared by removing 0.5 cm of the polyimide layer with a razor.

The capillary columns were packed for 25.0 cm and the effective and total lengths were 25.5 cm and 34.0 cm, respectively.

2.4. Sample preparation

Six herbal mixtures sold for their cannabis-like effects were purchased in Italian “smart shops” and via Internet. All the products were presented in the form of dried leaves.

For quantitative analysis, the whole content of the package was crushed into powder in a mortar. For the extraction of the synthetic cannabinoids, methanol (1 mL) was added to 10 mg of the herbal mixture in glass tubes. The mixture was stirred for 3 min, ultrasonicated (10 min) and centrifuged at 3600 rpm for 3 min. An aliquot (100 μL) of supernatant was withdrawn and diluted 10 times with MeOH. Before injection, the solution was further diluted to the desired concentration with water.

3. Results and discussion

3.1. Optimization of nano-LC cannabimimetics separation

The studied cannabimimetics (JWH-018, JWH-019, JWH-073, JWH-081, JWH-122, JWH-200, JWH-203, JWH-210, JWH-250, AM-694, AM-2201 and WIN-55,212-2) belonging to the class of *N*-alkylindoles, possess similar chemical structures differing from each other for the presence of several substituents such as benzoyl, phenyl–acetyl and naphthoyl groups. Hence, a highly efficient and selective method for the discrimination of such structurally related compounds is needed. From the data reported in literature,

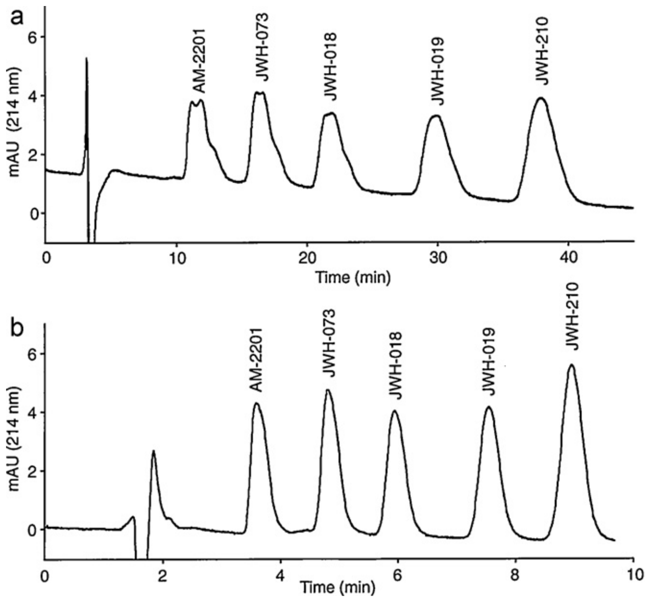

Fig. 2. Comparison of the chromatographic separation of five synthetic cannabinoids achieved with different organic modifiers in the mobile phase. Experimental conditions: capillary columns packed with Cogent® bidentate C_{18} stationary phase, 100 μm i.d. × 25 cm packed length, effective and total lengths 25.5 and 34 cm, respectively; mobile phases: (a) MeOH/H_2O (80/20, v/v) and (b) ACN/H_2O (80/20, v/v) containing both 0.1% formic acid; flow rate 667 nL/min; UV detection at 214 nm and injected volume 100 nL.

reversed-phase chromatographic methods have been developed using a gradient elution mode. However, only few papers deal with the simultaneous separation of a limited number of synthetic cannabinois [12,22].

In order to obtain the greatest column selectivity, an RP packing material called Cogent® bidentate C_{18} was employed for the nano-LC experiments. This stationary phase was previously used, with good results, for the separation of basic drugs by means of nano-LC [43].

In this packing material, silicon hydride groups (Si—H) replace 95% of the silanols on the surface. Good separation efficiency and resolution of complex mixtures were obtained also using this sorbent in the capillary column format with the miniaturized techniques [44].

In order to properly separate all studied compounds, experimental parameters such as mobile phase composition influencing retention and selectivity were evaluated.

For this purpose, several mixtures containing the solvents commonly used in reversed-phase chromatography e.g. ACN, MeOH and H_2O, were investigated. Preliminary experiments were carried out using mobile phases composed by ACN/H_2O and MeOH/H_2O (80/20, v/v) both containing 0.1% (v/v) formic acid.

As a model, a mixture of 5 synthetic cannabinoids (JWH-018, JWH-019, JWH-073, JWH-210, AM-2201) was analysed to evaluate the effect of the organic modifier nature on the chromatographic separation. As shown in Fig. 2, ACN/H_2O mixture allowed a good and rapid separation of all compounds. On the other hand, with MeOH as organic modifier, the compounds were strongly retained with consequent long analysis time and broaden peaks. For this reason, ACN was selected as the organic solvent of choice to optimise the separation of a mixture of 10 selected compounds.

Using 100% ACN as mobile phase, a partial separation of the compounds was achieved in few minutes. Decreasing ACN concentration, stronger interactions between the analytes and the stationary phase with consequent increment of the resolution were achieved.

With 93% (v/v) of ACN almost all the compounds were separated from each other, with the exception of JWH-019 and JWH-122 that co-eluted. To obtain the separation of these two compounds, the content of ACN in the mobile phase was further decreased up to 70% without obtaining any resolution improvement.

Also a step gradient mode using several ACN/H_2O mixtures was applied without good results.

In order to improve the standard mixture separation, the addition of MeOH to the mobile phase was also taken into account. As reported in the preliminary experiments, the analytes eluted slowly when MeOH was used as organic modifier in the mobile phase. A mixture of ACN/H_2O (70/30, v/v) was selected as starting point, varying the concentration of MeOH in the range 4–25% in spite of water. All the mobile phases tested were prepared by adding 0.1% formic acid.

Among all the examined conditions, a mixture of ACN/MeOH/H_2O (70/5/25, v/v/v) allowed the partial resolution of JWH-019 and JWH-122 (R = 0.89). An improved resolution of the two mentioned analytes was obtained increasing the concentration of formic acid from 0.1 to 1.0%.

Other three aminoalkylindoles (JWH-200, JWH-203, WIN-55,212-2) were subsequently added to the standard mixture and analysed in the above chromatographic conditions. The separation of all synthetic cannabinoids considered was achieved, with the exception of JWH-200 that co-eluted with the dead time.

To overcome this problem, a mobile phase containing ACN/MeOH/H_2O/formic acid 69/5/25/1 (v/v/v/v) was employed allowing the resolution of all thirteen compounds within 30 min. In Fig. 3 the nano-LC chromatogram of the separated compounds is depicted.

Owing to the high selectivity and efficiency of nano-LC technique, for the first time the simultaneous separation of a high number of synthetic cannabinoids is presented.

3.2. Method validation

Under the optimal conditions, in order to perform the quantitative analysis of the studied cannabimimetics, the nano-LC method was validated in terms of precision, sensitivity, linearity range and recovery. In the validation study, a standard mixture containing 11 cannabimimetics with the exception of JWH-210 and AM-2201 (unknown concentration) was employed.

3.2.1. Precision and reproducibility column to column

The analytical precision was performed by assessing a mixture at a concentration of 25 μg/mL for each analyte and 50 μg/mL for Δ^9-THC. Intra-day and inter-day precision were calculated for six consecutive runs in the same day and over three different days (n = 9). The relative standard deviation (RSD) was taken as a measure of precision.

In Table 1, inter-day, and intra-day precision data concerning retention time (t_r) and peak areas, expressed as the relative standard deviation percentage (RSD), are reported. The RSD values for retention time were lower than 1.68% and 2.98% for intra-day and inter-day precision, respectively. Good results were also achieved for peak areas with RSD ≤ 4.33% and 6.40% for intra- and inter-day experiments.

Column-to-column reproducibility was also evaluated performing the analysis of the cannabimimetics in three different capillary columns packed with the same procedure.

For column-to-column reproducibility, satisfactory results were obtained with RSD values of retention time and peak area in the range 3.14–5.12% and 3.77–7.93%, respectively (results shown in Table 1).

Table 1
Analytical nano-LC–UV precision data expressed as intra-day, inter-day and column reproducibility RSD% of retention time (t_r) and peak areas.

Analytes	Precision				Reproducibility	
	Intra-day ($n = 6$), RSD%		Inter-day (3 days, $n = 9$), RSD%		Column-to-column (3 columns, $n = 9$), RSD%	
	t_r	Peak area	t_r	Peak area	t_r	Peak area
JWH-200	1.41	2.96	2.98	3.65	3.73	4.54
WIN-55,212-2	1.16	4.33	1.96	5.81	3.14	6.21
AM-694	1.31	4.01	1.78	4.41	3.64	4.69
AM-2201	1.44	4.14	1.89	4.46	3.77	4.50
JWH-250	1.47	2.48	1.96	3.54	3.99	3.77
JWH-073	1.68	3.17	2.03	3.86	3.93	3.99
JWH-203	1.39	3.33	2.02	6.40	4.35	7.93
JWH-018	1.50	2.78	2.23	5.86	4.34	6.97
JWH-081	1.59	4.11	2.20	6.00	4.55	7.69
JWH-122	1.44	1.48	2.17	2.49	4.73	4.59
JWH-019	1.35	3.45	2.17	4.12	4.81	6.52
Δ^9-THC	1.55	2.48	2.18	3.85	4.88	4.55
JWH-210	1.57	2.88	2.21	3.75	5.12	6.75

Table 2
Linearity and sensitivity parameters.

Analytes	Regression line				Sensitivity	
	Linearity range (μg/mL)	Slope	Intercept	Determination coefficient r^2	LOD (μg/mL)	LOQ (μg/mL)
JWH-200	0.5–50.0	10.101 ± 0.045	3.598 ± 0.983	0.9998	0.2	0.5
WIN-55,212-2	0.5–50.0	7.181 ± 0.092	1.507 ± 2.274	0.9988	0.2	0.5
AM-694	0.5–50.0	6.085 ± 0.059	1.053 ± 1.393	0.9987	0.2	0.5
JWH-250	0.5–50.0	6.363 ± 0.031	2.057 ± 0.705	0.9990	0.2	0.5
JWH-073	0.5–50.0	11.015 ± 0.064	3.404 ± 1.503	0.9992	0.2	0.5
JWH-203	0.5–50.0	4.068 ± 0.042	4.459 ± 0.995	0.9975	0.2	0.5
JWH-018	0.5–50.0	11.162 ± 0.071	2.870 ± 1.673	0.9990	0.2	0.5
JWH-081	0.5–50.0	6.007 ± 0.037	2.171 ± 0.879	0.9984	0.2	0.5
JWH-122	0.5–50.0	10.378 ± 0.059	4.526 ± 1.404	0.9989	0.2	0.5
JWH-019	0.5–50.0	8.494 ± 0.045	3.684 ± 1.056	0.9991	0.2	0.5
Δ^9-THC	1.0–100.0	6.345 ± 0.089	2.369 ± 1.543	0.9982	0.4	1.0

3.2.2. Sensitivity and linearity

To investigate the nano-LC method sensitivity under the proposed conditions, limits of detection (LOD) and quantification (LOQ) were determined at a signal to-noise ratio of 3 and 10 times, respectively. LODs and LOQs values of 0.2 μg/mL and 0.5 μg/mL, for all selected cannabimimetics and 0.4 μg/mL and 1.0 μg/mL for Δ^9-THC, respectively, were found (see Table 2). The limits of detection obtained were in accord with the sensitivity requirements for forensic analyses of synthetic cannabinoids in herbal mixtures.

Method linearity was evaluated in the concentration range of 0.5–50.0 μg/mL for all the analytes and 1.0–100.0 μg/mL for Δ^9-THC, assessing six concentration levels in triplicate runs for each analyte. The calibration curves were obtained plotting the peak areas as a function of analytes concentration. The regression analysis was performed by calculating the coefficient of determination r^2 which ranges between 0.9975 and 0.9992 confirming a good linearity (Table 2).

3.3. Analysis of commercial herbal blends

The developed nano-LC method was applied to the analysis of cannabimimetics in herbal blends purchased in several "smart shops" and via Internet.

The herbal products were subjected to a liquid extraction procedure testing two different organic solvents (ACN and MeOH). Employing ACN, the analytes were extracted with a maximum yield of 76% with respect to the extraction performed with MeOH. The extraction procedure performed with the latter solvent was described in Section 2.4. The extraction procedure recovery was estimated fortifying an herbal blend sample with a standard

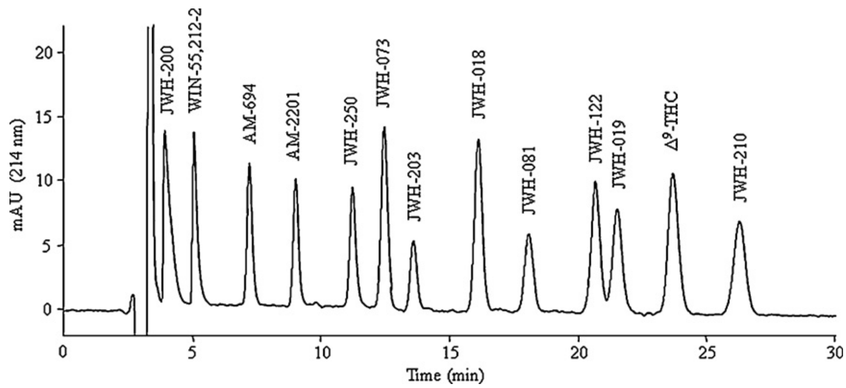

Fig. 3. Nano-LC separation of synthetic cannabinoids and Δ^9-THC in the optimum conditions: mobile phase ACN/MeOH/H_2O/formic acid 69/5/25/1 (v/v/v/v); flow rate 500 nL/min; concentration of all analytes 25 μg/mL and Δ^9-THC 50 μg/mL. Other conditions as given in Fig. 2.

Table 3
Mean recovery data.

Compounds	Spiked concentration (μg/mL)	Recovery (%)	RSD %
JWH-200	5	85.5	4.75
	25	93.2	3.18
WIN-55,212-2	5	83.9	3.52
	25	98.1	2.86
AM-694	5	88.3	5.26
	25	102.5	3.82
JWH-250	5	86.3	5.86
	25	98.4	2.85
JWH-073	5	93.4	4.87
	25	101.9	4.12
JWH-203	5	92.1	3.94
	25	105.5	2.45
JWH-018	5	88.7	5.17
	25	100.4	2.95
JWH-081	5	90.1	3.51
	25	102.3	2.17
JWH-122	5	86.6	4.87
	25	98.5	2.24
JWH-019	5	91.1	3.87
	25	99.8	1.41
Δ^9-THC	10	89.7	5.27
	50	101.9	3.82

mixture of the 11 selected analytes at two different concentration levels (5.0 and 25.0 μg/mL, final concentration added) and calculating the average values of three repeated runs. Recovery data with the RSD values are reported in Table 3 showing the good efficiency of the extraction method.

The estimated amounts of synthetic cannabinoids found in six different herbal blends analysed are shown in Table 4. As shown, each herbal product contained at least two of the cannabinoids tested. The psychoactive ingredients in the analysed samples varied in a wide range of concentrations between 3.3 and 81.4 mg/g. Furthermore, psychoactive substances amounts can vary from batch to batch within the same brand name. For example, the two samples of "Orange Alesya New", contained different concentrations of the same cannabinoids (JWH-073 and JWH-081).

In Fig. 4, the chromatograms of two herbal products labelled "Jamaican Spirit" and "n'joy" containing JWH-250, JWH-081 and JWH-073, JWH-018, respectively, are shown. The peak identification was done by comparing retention times and spiking the herbal products with standard solutions of cannabimimetics to confirm the identity of the peaks.

The synthetic cannabinoids found in the herbal products analysed in the present work were in accordance with the results published by Gottardo et al. [26] concerning the screening of synthetic cannabinoids in herbal blends by MALDI–TOF MS.

3.4. Synthetic cannabinoids characterization by nano-LC–MS

With the aim to certainly identify the analysed compounds, the nano-LC system was coupled with the ion-trap mass spectrometer detector through a nano-LC interface. Nano-LC–MS experiments were performed using the optimised conditions with UV detector, as described above. The cannabimimetics were analysed in positive ion mode.

In Fig. 5, the total ion and base peak chromatograms of a standard mixture containing the 12 studied synthetic cannabinoids and Δ^9-THC are reported. The method developed allowed the chromatographic separation of all compounds including JWH-019 and JWH-122 which possess the same molecular masses, resulting in identical MS spectra. To unambiguously characterize the two isomers, tandem MS (MS^2) experiments were carried out by applying a normalised collision energy in the range 26–40%.

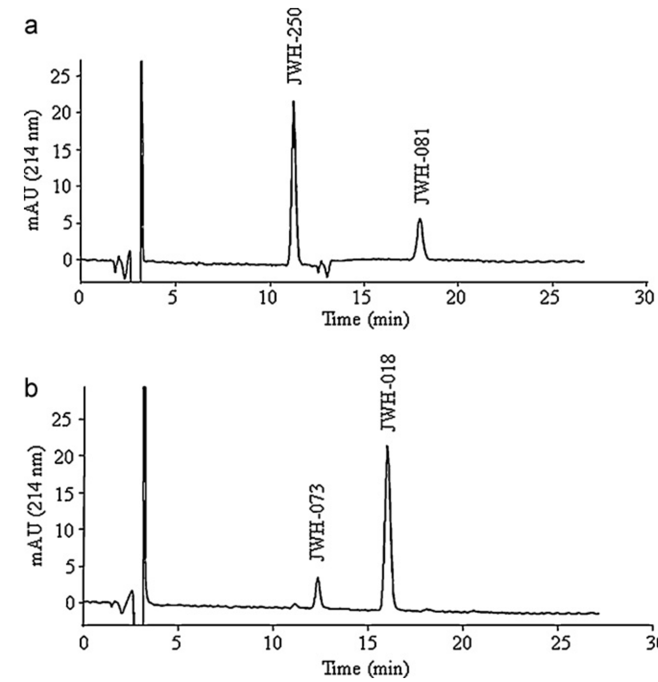

Fig. 4. Nano-LC profiles of two different herbal blends: chromatograms obtained after the extraction procedure: (a) Jamaican Spirit and (b) n'joy. Experimental conditions as in Fig. 3.

In Fig. 6 the MS and MS^2 spectra of JWH-019 and JWH-122, respectively, are depicted. As shown, the two synthetic cannabinoids have the identical MS spectrum characterised by the same precursor ion m/z 356.13 (Fig. 6a). The selection and subsequent excitation of the precursor ion produced characteristic fragments

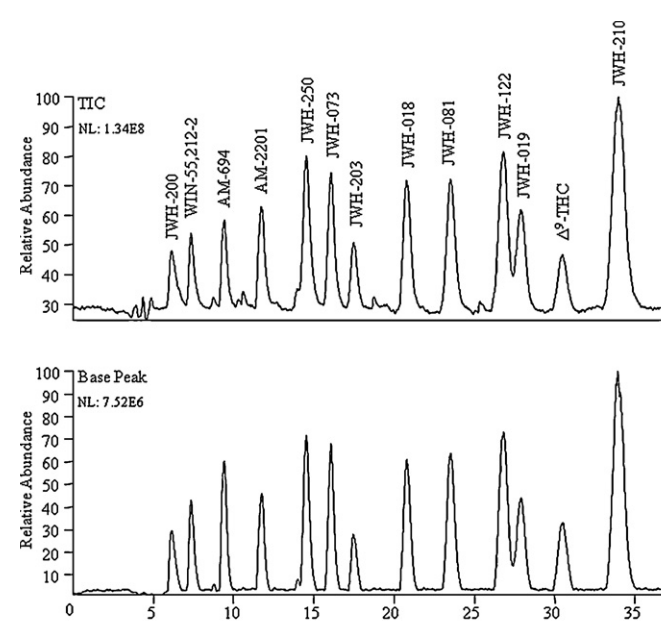

Fig. 5. Total ion and base peak chromatograms of the separation of synthetic cannabinoids and Δ^9-THC: chromatographic conditions as in Fig. 3; nano-ESI-MS conditions: spray voltage 1.8 kV, capillary voltage 34 V and capillary temperature 200 °C.

Table 4
Synthetic cannabinoids content in herbal blends.

Commercial brand	Net weight (g/pack)	Content	
		Synthetic cannabinoids	Amount (mg/g ± SD)
Jamaican Spirit	3	JWH-250	81.4 ± 2.34
		JWH-081	36.3 ± 1.87
n'joy	2	JWH-073	9.0 ± 3.03
		JWH-018	55.4 ± 1.94
Orange Alesya New	4	JWH-073	16.9 ± 2.35
		JWH-081	55.0 ± 1.32
Lucy	3	AM-694	3.3 ± 3.41
		JWH-081	30.6 ± 4.02
Maya	2	AM-2201	n.q.
		JWH-122	23.0 ± 2.57
		JWH-210	n.q.
Orange Alesya New	25	JWH-073	15.8 ± 1.33
		JWH-081	46.6 ± 2.64

n.q.: not quantified.

Table 5
Collision energy values, m/z of molecular ions and their main fragments.

Analytes	Collision energy (%)	M.W. (g/mol)	Precursor ion $[M+H]^+$	MS/MS fragments		
JWH-200	29	384.5	385.0	155.0	298.2	145.2
WIN-55,212-2	29	426.5	427.0	155.1	145.1	127.1
AM-694	32	435.3	435.9	309.0	230.9	234.3
AM-2201	36	359.4	360.1	155.1	232.2	145.1
JWH-250	31	335.4	336.0	121.0	188.1	148.5
JWH-073	35	327.4	328.1	155.1	200.1	145.1
JWH-203	37	339.9	340.1	188.1	125.1	132.1
JWH-018	34	341.5	342.0	155.0	214.1	145.1
JWH-081	34	371.5	372.0	185.1	214.0	156.9
JWH-122	39	355.5	356.1	169.1	214.1	141.2
JWH-019	38	355.5	356.1	155.1	228.1	127.1
Δ^9-THC	32	314.5	315.1	259.2	193.2	233.1
JWH-210	36	369.5	370.1	183.1	214.1	155.0

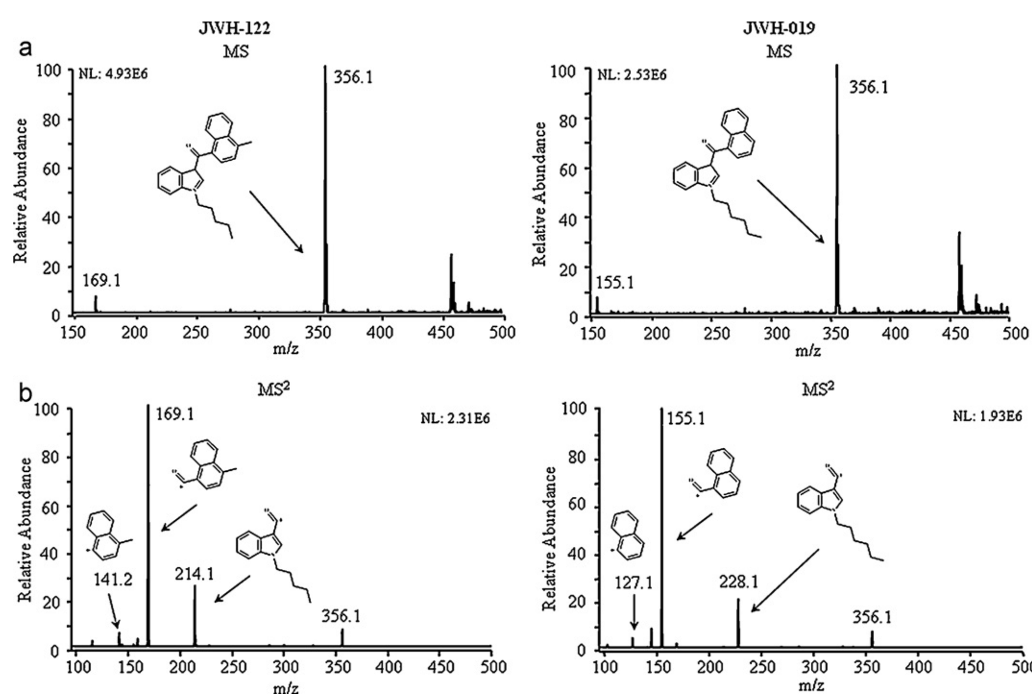

Fig. 6. MS and MS^2 spectra of JWH-122 and JWH-019. For MS conditions see Fig. 5; for MS^2 conditions, range scan (m/z) 90–500, normalised collision energy, 39 and 38%, respectively.

for the two different isomers. JWH-019 and JWH-122 differ in the length of the *N*-alkyl chain and for the methyl substituent on the naphthalene moiety. Those structural differences can be easily detected in the MS^2 spectra of the two cannabinoids. JWH-122 was characterised by three main product ions *m/z* 169.1, 214.1, 141.2 corresponding to 4-methylnaphtalene-1-carbaldehyde, 1-penthylindol-3-carbaldehyde and 1-methylnaphthalene, respectively (Fig. 6b). While, three representative fragments were detected in the MS^2 spectrum of JWH-019 including naphtalene-1-carbaldehyde, *m/z* 155.1, 1-hexylindole-3-carbaldehyde *m/z* 228.1 and naphthalene *m/z* 127.1 (Fig. 6b). Thus the discrimination of the two isomers previously obtained by the nano-LC separation, was confirmed by MS.

Ion trap collision induced fragmentation was carried out for all the studied cannabimimetics providing fragment patterns similar to that reported in literature. The results are shown in Table 5.

4. Conclusion

A rapid nano-LC method for the simultaneous separation of 12 synthetic cannabinoids and Δ^9-THC in a single run was developed. A laboratory assembled nano-LC system was used for chromatographic experiments employing capillary columns packed with Cogent® bidentate C_{18}. The studied cannabimimetics were separated in less than 30 min using an isocratic elution mode.

The nano-LC method developed showed satisfactory results concerning precision and accuracy with good linearity within the calibrated range. The chromatographic performance allowed the determination of synthetic cannabinoids in herbal mixtures. The quantification was carried out with a nano-LC–UV system while an ion-trap mass spectrometer was coupled with the chromatographic apparatus to certainly identify the studied compounds.

The positive results reached have proved that nano-LC can be used as an alternative to well-established LC–MS and GC–MS methods, for the monitoring of “herbal incenses” because of high chromatographic resolution. Besides, other advantages including short analysis time, minimal consumption of reagents and samples, make it a cost effective and eco-friendly analytical system.

Further studies will be carried out to increment method sensitivity for the analysis of synthetic cannabinoids in biological fluids such as plasma and serum.

References

[1] I. Vardakou, C. Pistos, Ch. Spiliopoulou, Spice drugs as a new trend: mode of action, identification and legislation, Toxicol. Lett. 197 (2010) 157–162.

[2] J.W. Huffman, D. Dai, B.R. Martin, D.R. Compton, Design, synthesis and pharmacology of cannabimimetic indoles, Bioorg. Med. Chem. Lett. 4 (1994) 563–566.

[3] D.R. Compton, K.C. Rice, B.R. De Costa, R.K. Razdan, L.S. Melvin, M.R. Johnson, B.R. Martin, Cannabinoid structure–activity relationships: correlation of receptor binding and in vivo activities, J. Pharmacol. Exp. Ther. 265 (1993) 218–226.

[4] J.W. Huffman, Cannabimimetic indoles, pyrroles, and indenes: structure–activity relationships and receptor interactions, in: P.H. Reggio (Ed.), The Cannabinoid Receptors, Humana Press, New York, 2009, pp. 49–94.

[5] U.S. Zimmermann, P.R. Winkelmann, M. Pilhatsch, J.A. Nees, R. Spanagel, K. Schulz, Withdrawal phenomena and dependence syndrome after the consumption of “spice gold”, Dtsch. Arztebl. Int. 106 (2009) 464–467.

[6] S. Every-Palmer, Warning: legal synthetic cannabinoid-receptor agonists such as JWH-018 may precipitate psychosis in vulnerable individuals, Addiction 105 (2010) 1859–1860.

[7] H. Müller, W. Sperling, M. Khrmann, H.B. Huttner, J. Kornhuber, J.M. Maler, The synthetic cannabinoid spice as a trigger for an acute exacerbation of cannabis induced recurrent psychotic episodes, Schizophr. Res. 118 (2010) 309–310.

[8] A.B. Schneir, J. Cullen, B.T. Ly, “Spice girls”: synthetic cannabinoid intoxication, J. Emerg. Med. 40 (2011) 296–299.

[9] J. Simmons, L. Cookman, C. Kang, C. Skinner, Three cases of “spice” exposure, Clin. Toxicol. 49 (2011) 431–433.

[10] S. Every-Palmer, Synthetic cannabinoid JWH-018 and psychosis: an explorative study, Drug Alcohol Depend. 117 (2011) 152–157.

[11] D.M. Benford, J.P. Caplan, Psychiatric sequelae of spice, k2, and synthetic cannabinoid receptor agonists, Psychosomatics 52 (2011) 295.

[12] C.L. Moran, V.H. Le, K.C. Chimalakonda, A.L. Smedley, F.D. Lackey, S.N. Owen, P.D. Kennedy, G.W. Endres, F.L. Ciske, J.B. Kramer, A.M. Kornilov, L.D. Bratton, P.J. Dobrowolski, W.D. Wessinger, W.E. Fantegrossi, P.L. Prather, L.P. James, A. Radominska-Pandya, J.H. Moran, Quantitative measurement of JWH-018 and JWH-073 metabolites excreted in human urine, Anal. Chem. 83 (2011) 4228–4236.

[13] T. Sobolevsky, I. Prasolov, G. Rodchenkov, Detection of JWH-018 metabolites in smoking mixture post-administration urine, Forensic Sci. Int. 200 (2010) 141–147.

[14] M.A. ElSohly, W. Gul, K.M. Elsohly, T.P. Murphy, V.L. Madgula, S.I. Khan, Liquid chromatography-tandem mass spectrometry analysis of urine specimens for K28 (JWH-018), J. Anal. Toxicol. 35 (2011) 487–495.

[15] S. Beuck, I. Möller, A. Thomas, A. Klose, N. Schlörer, W. Schänzer, M. Thevis, Structure characterisation of urinary metabolites of the cannabimimetic JWH-018 using chemically synthesised reference material for the support of LC–MS/MS-based drug testing, Anal. Bioanal. Chem. 401 (2011) 493–505.

[16] J. Teske, J.P. Weller, A. Fieguth, T. Rothämel, Y. Schulz, H.D. Tröger, Sensitive and rapid quantification of the cannabinoid receptor agonist naphthalene-1-yl-(1-pentylindol-3-yl)methanone (JWH-018) in human serum by liquid chromatography–tandem mass spectrometry, J. Chromatogr. B 878 (2010) 2659–2663.

[17] A. Grigoryev, A. Melnik, S. Savchuk, A. Simonov, V. Rozhanets, J. Chromatogr. B 879 (2011) 2519–2526.

[18] M. Hutter, S. Broecker, S. Kneisel, V. Auwärter, Identification of the major urinary metabolites in man of seven synthetic cannabinoids of the aminoalkyindole type present as adulterants in ‘herbal mixtures’ using LC–MS/MS techniques, J. Mass Spectrom. 47 (2012) 54–65.

[19] S. Dresen, S. Kneisel, W. Weinmann, R. Zimmermann, V. Auwärter, Development and validation of a liquid chromatography–tandem mass spectrometry method for the quantitation of synthetic cannabinoids of the aminoalkylindole type and methanandamide in serum and its application to forensic samples, J. Mass Spectrom. 46 (2011) 163–171.

[20] I. Möller, A. Wintermeyer, K. Bender, M. Jübner, A. Thomas, O. Krug, W. Schänzer, M. Thevis, Screening for the synthetic cannabinoid JWH-018 and its major metabolites in human doping controls, Drug Test. Anal. 3 (2011) 609–620.

[21] S. Dresen, N. Ferreirós, M. Pütz, F. Westphal, R. Zimmermann, V. Auwärter, Monitoring of herbal mixtures potentially containing synthetic cannabinoids as psychoactive compounds, J. Mass Spectrom. 45 (2010) 1186–1194.

[22] J. Nakajima, M. Takahashi, T. Seto, C. Kanai, J. Suzuki, M. Yoshida, T. Hamano, Identification and quantitation of two benzoylindoles AM-694 and (4-methoxyphenyl)(1-pentyl-1*H*-indol-3-yl)methanone, and three cannabimimetic naphthoylindoles JWH-210, JWH-122, and JWH-019 as adulterants in illegal products obtained via the Internet, Forensic Toxicol. 29 (2011) 95–110.

[23] S. Hudson, J. Ramsey, L. King, S. Timbers, S. Maynard, P.I. Dargan, D.M. Wood, Use of high-resolution accurate mass spectrometry to detect reported and previously unreported cannabinomimetics in “herbal high” products, J. Anal. Toxicol. 34 (2010) 252–260.

[24] N. Uchiyama, R. Kikura-Hanajiri, J. Ogata, Y. Goda, Chemical analysis of synthetic cannabinoids as designer drugs in herbal products, Forensic Sci. Int. 198 (2010) 31–38.

[25] F. Westphal, F.D. Sönnichsen, S. Thiemt, Identification of 1-butyl-3-(1-(4-methyl)naphthoyl)indole in a herbal mixture, Forensic Sci. Int. 215 (2012) 8–13.

[26] R. Gottardo, A. Chiarini, I. Dal Prà, C. Seri, C. Rimondo, G. Serpelloni, U. Armato, F. Tagliaro, Direct screening of herbal blends for new synthetic cannabinoids by MALDI–TOF MS, J. Mass Spectrom. 47 (2012) 41–46.

[27] L. Ernst, H.M. Schiebel, C. Theuring, R. Lindigkeit, T. Beuerle, Identification and characterization of JWH-122 used as new ingredient in “Spice-like” herbal incenses, Forensic Sci. Int. 208 (2011) e31–e35.

[28] N. Uchiyama, M. Kawamura, R. Kikura-Hanajiri, Y. Goda, Identification and quantitation of two cannabimimetic phenylacetylindoles JWH-251 and JWH-250, and four cannabimimetic naphthoylindoles JWH-081, JWH-015, JWH-200, and JWH-073 as designer drugs in illegal products, Forensic Toxicol. 29 (2011) 25–37.

[29] S. Hudson, J. Ramsey, The emergence and analysis of synthetic cannabinoids, Drug Test. Anal. 3 (2011) 466–478.

[30] J.P. Pascali, F. Bortolotti, F. Tagliaro, Recent advances in the application of CE to forensic sciences, an update over years 2009–2011, Electrophoresis 33 (2012) 117–126.

[31] R. Gottardo, A. Fanigliulo, F. Bortolotti, G. De Paoli, J.P. Pascali, F. Tagliaro, Broad-spectrum toxicological analysis of hair based on capillary zone electrophoresis–time-of-flight mass spectrometry, J. Chromatogr. A 1159 (2007) 190–197.

[32] R. Gottardo, A. Polettini, D. Sorio, J.P. Pascali, F. Bortolotti, E. Liotta, F. Tagliaro, Capillary zone electrophoresis (CZE) coupled to time-of-flight mass spectrometry (TOF–MS) applied to the analysis of illicit and controlled drugs in blood, Electrophoresis 29 (2008) 4078–4087.

[33] Z. Aturki, G. D’Orazio, S. Fanali, A. Rocco, F. Bortolotti, R. Gottardo, F. Tagliaro, Capillary electrochromatographic separation of illicit drugs employing a cyano stationary phase, J. Chromatogr. A 1216 (2009) 3652–3659.

[34] Z. Aturki, G. D’Orazio, A. Rocco, F. Bortolotti, R. Gottardo, F. Tagliaro, S. Fanali, CEC–ESI ion trap MS of multiple drugs of abuse, Electrophoresis 31 (2010) 1256–1263.

[35] K.E. Karlsson, M. Novotny, Separation efficiency of slurry-packed liquid chromatography microcolumns with very small inner diameters, Anal. Chem. 60 (1988) 1662–1665.
[36] J. Hernández-Borges, Z. Aturki, A. Rocco, S. Fanali, Recent applications in nano-liquid chromatography, J. Sep. Sci. 30 (2007) 1589–1610.
[37] T. Dingyin, Z. Lihua, S. Yichu, L. Zhen, Z. Yukui, Recent advances in micro-scale and nano-scale high-performance liquid-phase chromatography for proteome research, Anal. Bioanal. Chem. 399 (2011) 229–241.
[38] Z. Aturki, G. D'Orazio, A. Rocco, K. Si-Ahmed, S. Fanali, Investigation of polar stationary phases for the separation of sympathomimetic drugs with nano-liquid chromatography in hydrophilic interaction liquid chromatography mode, Anal. Chim. Acta 685 (2011) 103–110.
[39] C. Fanali, A. Rocco, Z. Aturki, L. Mondello, S. Fanali, Analysis of polyphenols and methylxanthines in tea samples by means of nano-liquid chromatography utilizing capillary columns packed with core–shell particles, J. Chromatogr. A 1234 (2012) 38–44.
[40] S.M.R. Stanley, A. Poljak, Matrix-assisted laser-desorption time-of flight ionization and high-performance liquid chromatography–electrospray ionization mass spectral analyses of two glycosylated recombinant epoetins, J. Chromatogr. B 785 (2003) 205–218.
[41] H. Yu Nola, N.M. Ho Emmie, S.M. Wan Terence, S.Y. Wong April, Doping control analysis of recombinant human erythropoietin, darbepoetin alfa and methoxy polyethylene glycol-epoetin beta in equine plasma by nano-liquid chromatography–tandem mass spectrometry, Anal. Bioanal. Chem. 396 (2010) 2513–2521.
[42] S. Fanali, E. Camera, B. Chankvetadze, G. D'Orazio, M.G. Quaglia, Separation of tocopherols by nano-liquid chromatography, J. Pharm. Biomed. Anal. 35 (2004) 331–337.
[43] S. Fanali, Z. Aturki, G. D'Orazio, A. Rocco, Separation of basic compounds of pharmaceutical interest by using nano-liquid chromatography coupled with mass spectrometry, J. Chromatogr. A 1150 (2007) 252–258.
[44] J.J. Pesek, M.T. Matyska, D. Sukul, Capillary liquid chromatography and capillary electrochromatography using silica hydride stationary phases, J. Chromatogr. A 1191 (2008) 136–140.