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Identification and characterization of the new designer drug 4′-methylethcathinone (4-MEC) and elaboration of a novel liquid chromatography–tandem mass spectrometry (LC–MS/MS) screening method for seven different methcathinone analogs

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ABSTRACT

A fast and simple LC–MS/MS method was developed for screening mephedrone, butylone, methylenedioxypyrovalerone (MDPV), flephedrone, methylone and methedrone in bulk powder samples. Samples were separated on a reverse phase column using gradient elution with mixtures of water, acetonitrile and formic acid. After optimization a limit of detection of about 2 ng mL^{-1} was achieved using multiple reaction monitoring (MRM) mode. Total run time was less than 8 min. Typical fragmentation characteristics of the studied compounds are discussed. The method was successfully applied to several unknown bulk powder samples seized by the Hungarian Customs and Finance Guard. One of the samples contained the new designer drug 4′-methylethcathinone (4-MEC), which was identified and characterized by LC–MS/MS, NMR, FT-IR and LC–TOF-MS techniques. The method is also deemed to be applicable for the screening of simple dosage forms such as tablets and capsules.

1. Introduction

The National Institute of Pharmacy (NIP) functions as the drug control agency of human medicinal products in Hungary and in terms of the national legislation it is responsible for providing expert reports on seized samples presumed to contain active pharmaceutical ingredients. In practice, such suspect "drug-like" samples often turn out to contain illegal compounds such as psychoactive substances or doping agents.

In 2010 the laboratory of the NIP was requested to give expert reports on several unknown bulk powder samples seized by the Hungarian Customs and Finance Guard. Package information and invoices claimed that the samples were pharmaceutical excipients or similar harmless substances. However, analysis of the samples demonstrated that each powder contained the hydrochloride salt of one of the following compounds: mephedrone (4′-methylmethcathinone) (**1**), butylone (beta-keto-MBDB) (**2**), 3,4-methylenedioxypyrovalerone (MDPV) (**3**), flephedrone (4′-fluoromethcathinone) (**4**) and a new potential designer drug, 4′-methylethcathinone (4-MEC) (**7**) (Fig. 1). All substances are chemical derivatives of the psychoactive stimulant methcathinone.

Among others, some of these compounds were mentioned in a 2010 joint report of Europol and the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) on mephedrone [1]. The report concluded that in many countries mephedrone was marketed as a legal alternative to ecstasy or cocaine. Participants reported seizures of mephedrone in the form of bulk powder or tablets and provided evidence of toxicity associated with mephedrone use. The conclusion of the study was confirmed by other works reporting undesired side-effects, symptoms of intoxication as well as fatalities associated with mephedrone abuse [2–7].

As a result, mephedrone has been illegalized by several European countries (including Hungary) recently. Furthermore, the United Kingdom decided to put a generic ban on the entire mephedrone family based on the similarity of their chemical structures [2].

It was assumed that a method of appropriate sensitivity and selectivity for simultaneous detection of methcathinone derivatives

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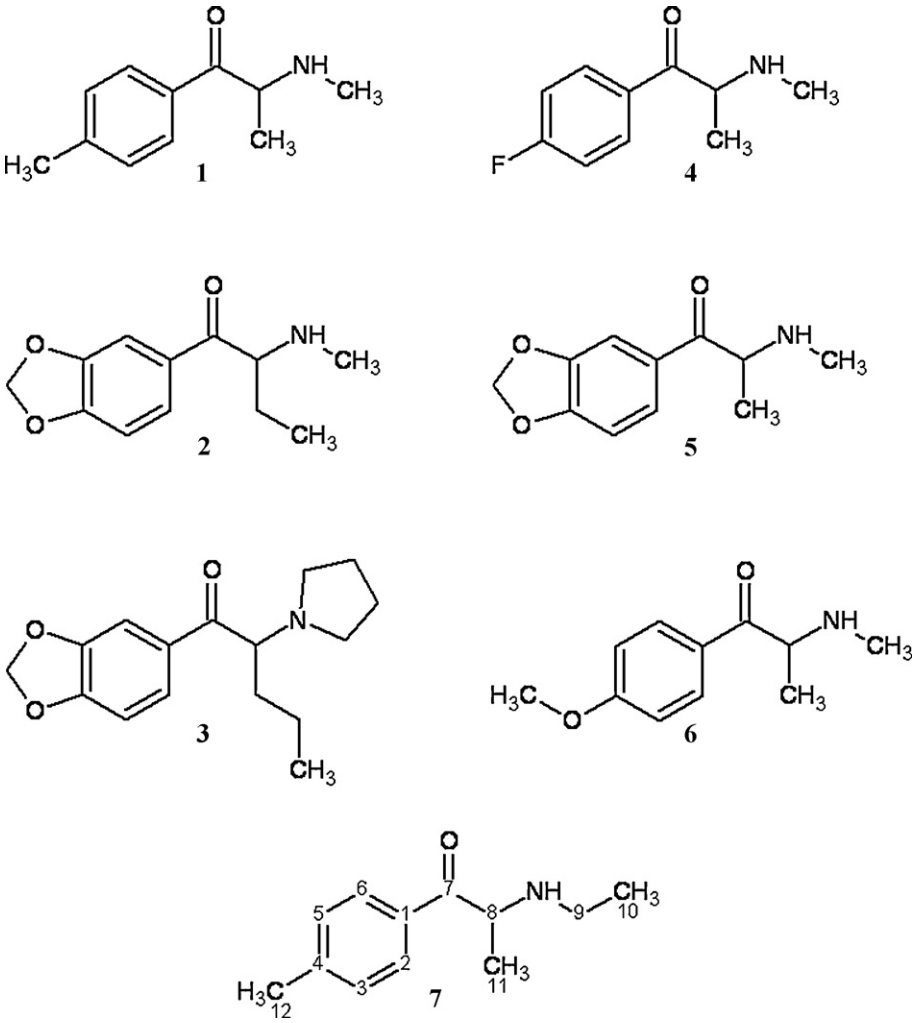

Fig. 1. Chemical structures of mephedrone (**1**), butylone (**2**), MDPV (**3**), flephedrone (**4**), methylone (**5**), methedrone (**6**), and 4′-methylethcathinone (4-MEC) (**7**).

in pharmaceutical formulations like mixed or triturated powders and tablets could considerably help control laboratories to provide expert reports within the usually very strict timelines. After a search in the literature it was found that despite the numerous scientific publications on the analysis of this group of substances none of the studies addressed this particular issue.

Detailed structure elucidation of mephedrone by NMR, MS, IR and molecular modeling was carried out by several research groups [8,9]. Flephedrone [10], methylone and butylone [9], MDPV [11,12] as well as other structurally related 4′-methoxymethcathinone and pyrrolidine derivatives [13–15] were also characterized. Quantification and metabolization studies in biological matrices were published for mephedrone [4,6,16], methedrone [17], methylone [16,18,19], MDPV [20,21] and butylone [16,22].

The present paper describes a rapid and simple target screening method by LC–MS/MS for the detection and identification of seven methcathinone derivatives in bulk powders. The target group includes five compounds identified by the laboratory of the NIP in seized samples (compounds **1**–**4** and **7**) as well as two other frequently encountered structures [17,23,24]: methylone and methedrone (compounds **5** and **6** in Fig. 1). Possible applicability of the method for more complex matrices is discussed. Since no reports were found concerning characterization of 4-MEC, structure elucidation of this compound by LC–MS/MS, NMR, FT-IR and high resolution MS (HRMS) was also carried out and the results are presented.

2. Experimental

2.1. Materials

Certified reference standards of compounds **5** and **6** were purchased from LGC Standards (Teddington, United Kingdom) in the form of hydrochloride salts. Because compounds **1**–**4** derived from seized samples their identity and purity were confirmed by LC–MS/MS, NMR, FT-IR and HRMS techniques.

2.2. Solvents and reagents

Water was produced by a Millipore Elix3 (Billerica, MA, USA) water purifying system. Acetonitrile (ACN), methanol (MeOH) (both from Merck KGaA, Darmstadt, Germany) and formic acid (Sigma–Aldrich GmbH, Seelze, Germany) were of LC–MS grade.

For NMR studies D_2O of 99.9% isotopic purity containing 1% DSS-d_6 (Sigma–Aldrich GmbH, Seelze, Germany) was used.

2.3. Apparatus

LC–MS/MS: Agilent Technologies 1200 Series HPLC equipped with an UV-VIS diode array detector (DAD) and coupled with an Agilent Technologies 6410A Triple Quad mass spectrometer equipped with a multimode ion source (MMI).

FT-IR: Perkin Elmer Spectrum 400 FT-IR/FT-NIR spectrophotometer.

NMR: ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) spectra: 600 MHz Varian VNMRS spectrometer in D_2O solutions; δ in ppm rel. to DSS as internal standard.

HRMS: Agilent Technologies 1260 Infinity HPLC coupled with Agilent Technologies 6230 Time of Flight MS equipped with a Jet Stream ion source operated in positive ion mode. Two reference masses (m/z 121.050873 and 922.009798) were used. Agilent MassHunter Workstation Software B.01.03 was used for data analysis.

Table 1
MRM parameters used in the screening method.

Compound	Precursor ion $[M+H]^+$	Product ion[a]	V_{fr} (V)	CE (V)
1	178	Transition 1: 160	90	10
		Transition 2: 144		36
2	222	Transition 1: 174	100	16
		Transition 2: 204		9
3	276	Transition 1: 126	125	27
		Transition 2: 135		27
4	182	Transition 1: 164	100	11
		Transition 2: 149		22
5	208	Transition 1: 160	90	16
		Transition 2: 132		30
6	194	Transition 1: 176	90	8
		Transition 2: 161		18
7	192	Transition 1: 174	100	11
		Transition 2: 144		32

[a] Proposed transitions for quantitative analysis where "Transition 1" and "Transition 2" correspond to the first and second most intensive transitions, respectively.

2.4. Identification and purity assessment of compounds 1–4

For the purpose of MS/MS fragmentation studies each bulk powder sample was dissolved in methanol and their mass spectra were acquired by flow injection analysis (FIA) using electrospray ionization (ESI) with positive polarity in full scan and product ion mode.

Universal attenuated total reflection (UATR) FT-IR spectra were identical with those available in the literature for mephedrone hydrochloride, butylone hydrochloride [25], MDPV hydrochloride [11,25] and flephedrone hydrochloride [10].

HRMS spectra were obtained with the direct injection of aqueous solutions of each sample. The relative differences between measured and calculated exact masses were within ±5.0 ppm for all compounds with isotopic abundance scores greater than 90%.

Chemical structures were confirmed by ^{1}H NMR and ^{13}C NMR. None of the spectra showed signs of any contaminating compound or impurity.

Samples were subjected to further purity assessment by HPLC–MS/MS by applying a gradient elution program where ACN content of the mobile phase was linearly increased from 5% to 95%. Neither the UV chromatograms recorded at 210 nm nor the total ion chromatograms obtained using a mass range of m/z 102–500 showed any significant peaks apart from the main peaks.

2.5. LC–MS/MS parameters for the screening method

Separation was carried out at room temperature on an Agilent Zorbax Eclipse XDB C-18 75 mm × 4.6 mm column (particle size: 3.5 μm). A gradient elution program was applied at a flow rate of 0.6 mL min^{-1} with eluent A consisting of 95% water, 5% ACN and 0.1% formic acid and eluent B consisting of 95% ACN, 5% water and 0.1% formic acid. Gradient elution was used as follows: 0–2 min 10% B; 2–7 min 10 → 40% B; 7–9 min 40% B.

UV chromatogram at 210 nm and UV spectra (210–400 nm) were recorded by the diode array detector (DAD).

MS was operated in multiple reaction monitoring (MRM) mode using positive ESI ionization. Drying gas (nitrogen) temperature was set to 325 °C, gas flow rate to 5 L min^{-1}, nebulizer pressure to 60 psi, capillary voltage to 2500 V. The compound-dependent operating parameters of the MS detector were optimized by evaluating the effectiveness of ionization and fragmentation. FIA was employed to optimize the fragmentor voltage (V_{fr}) and the collision energy (CE) for each MRM transition. The parameters of the optimized MRM transitions used in the screening method are presented in Table 1. Dwell time was 200 ms for each transition.

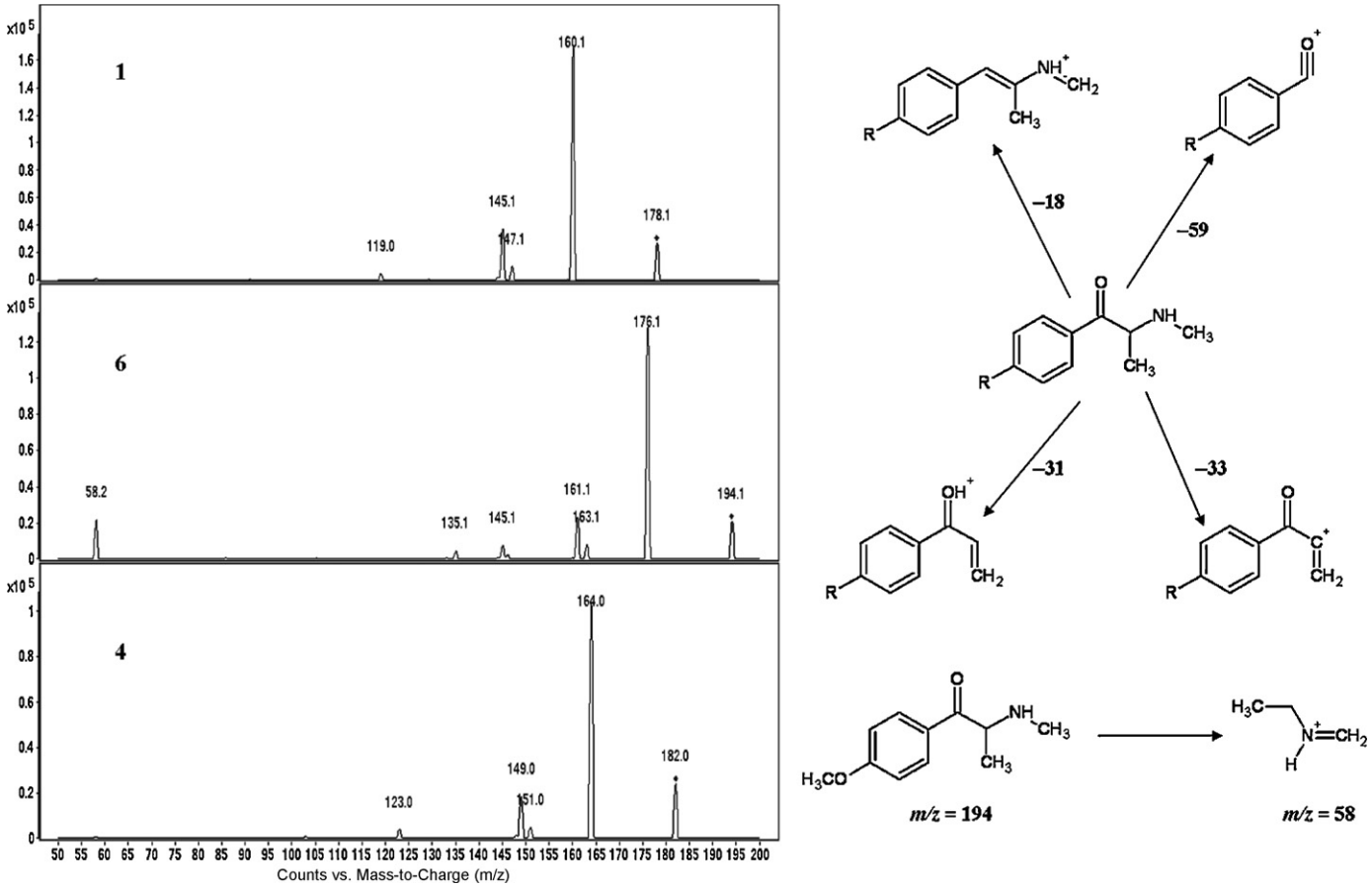

Fig. 2. Product ion spectra and proposed fragmentation mechanisms of **1**, **4** and **6** (direct injection; flow: 0.3 mL min^{-1}; eluent: ACN + water + formic acid 50:50:0.1 (v/v); V_{fr} = 90 V; CE = 10 V).

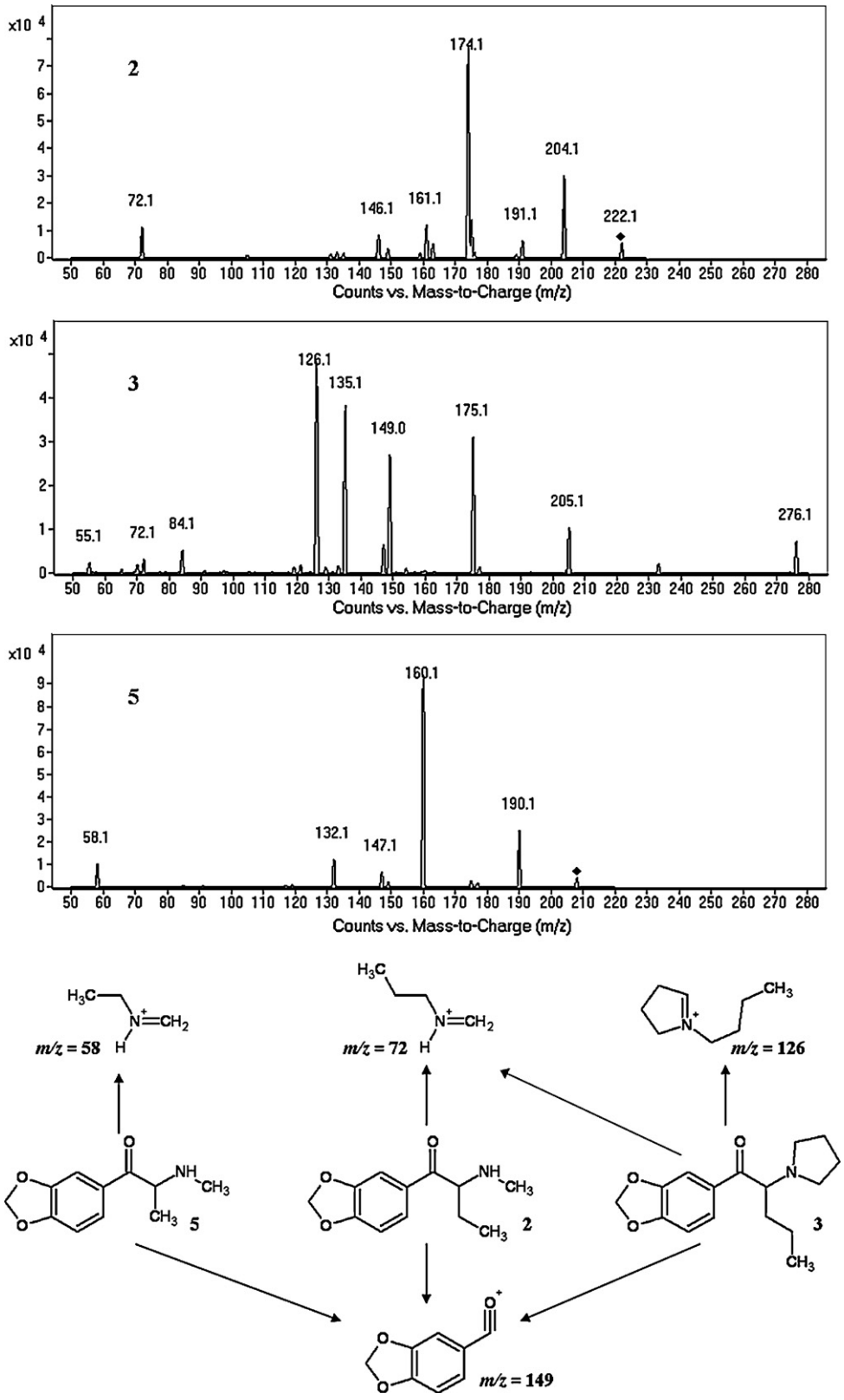

Fig. 3. Product ion spectra and some proposed fragmentation mechanisms of compounds **2**, **3** and **5** (direct injection; flow: 0.3 mL min^{-1}; eluent: ACN + water + formic acid 50:50:0.1 (*v*/*v*); V_{fr} = 90 V; CE = 10 V for compounds **2** and **5**; CE = 25 V for compound **3**).

3. Results and discussion

3.1. Fragmentation studies and LC–MS/MS screening method for compounds 1–6

Although fragmentation studies of the analytes have already been published it is believed that a comparison of their fragmentation characteristics may help to identify other methcathinone derivatives which are unknown for the time being but may be reported as designer drugs in the future. Full scan mass spectra were obtained using ESI positive mode. Electrospray ionization gave better responses for the compounds under investigation compared to atmospheric pressure chemical ionization (APCI). Under the employed conditions besides the protonated molecular ions no other adducts such as sodium, potassium or ammonium were visible in the spectra. By employing the default fragmentation voltage of 135 V, compounds **1**, **2**, **4**, **5** and **6** showed signs of in-source fragmentation resulting in loss of H_2O (18 Da) and a less clearly visible loss of CH_5N (31 Da).

For compounds **1**, **4** and **6** loss of C_3H_9N (59 Da) was also observed. On the other hand, for compounds **2** and **5** loss of CH_4O_2 (48 Da) was typical, which can be attributed to the methylenedioxy group. Compound **3** appeared to be more stable and showed no in-source fragmentation under the employed conditions.

The parameters for recording the product ion spectra were chosen so as to obtain fragmentation patterns which are found to be useful for the interpretation of the chemical structures. In the product ion spectra of compounds **1**, **4** and **6** analogous fragmentation mechanisms were observed resulting in similar, though more abundant fragments, compared to those produced by in-source fragmentation (Fig. 2).

Mass differences between each corresponding fragment were the same as those between the respective precursor ions. The benzoylcations were present at *m*/*z* 119, 123 and 135 for **1**, **4** and **6**, respectively. However, in the case of compound **6** an additional peak at *m*/*z* 58 was also observed and a cluster of fragments at *m*/*z* 144–146.

For compounds **2** and **5** fragments of neutral losses of 18 Da (H_2O), 31 Da (CH_5N) and 48 Da (CH_4O_2) appeared to be typical in the product ion spectra. A fragment peak at *m*/*z* 58 was observed for compound **5** (the same as for **6**) and a mass peak at *m*/*z* 72 for compound **2**. The mass difference of 14 Da indicated that these fragments were the corresponding immonium ions. This assumption was confirmed by the product ion spectrum of compound **3** where a fragment at *m*/*z* 126 appeared, corresponding to the

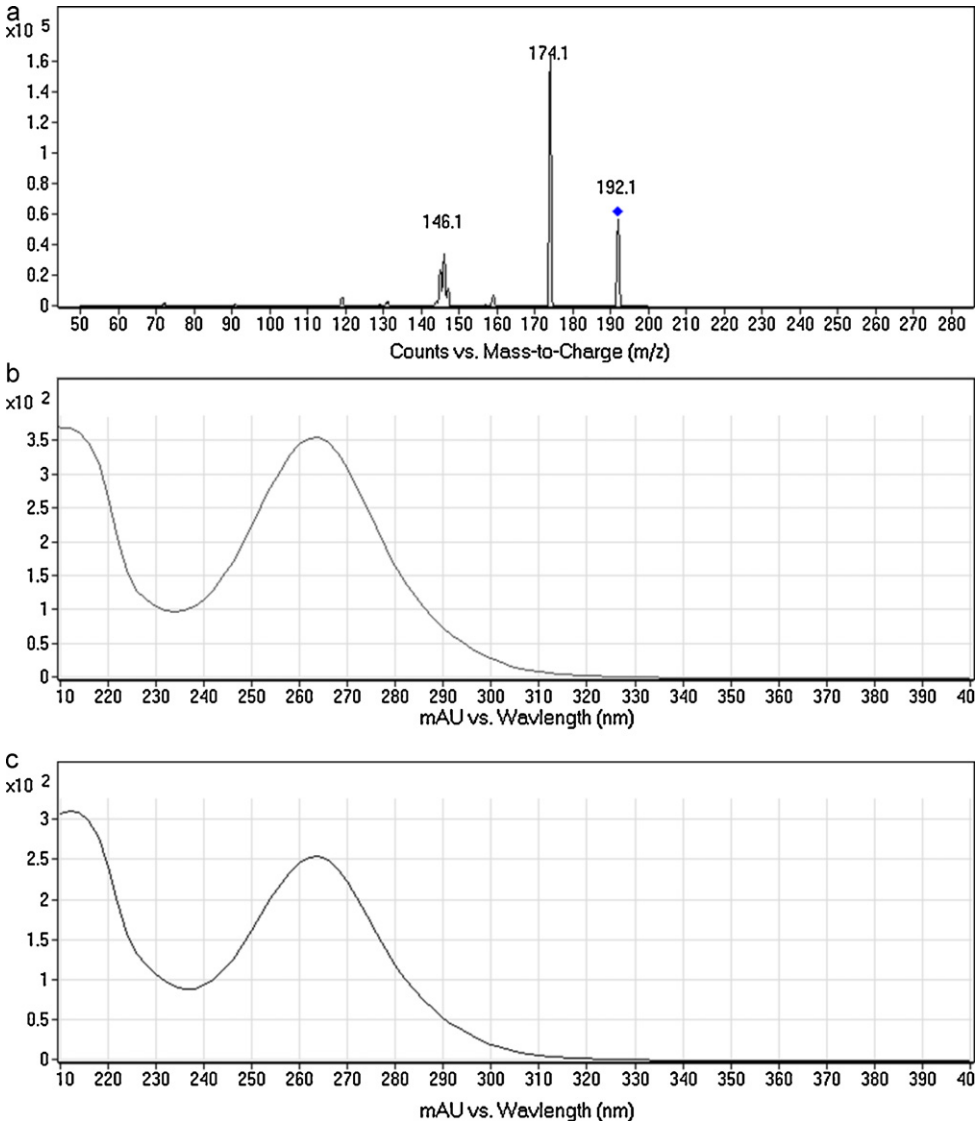

Fig. 4. (a) Product ion spectrum of compound **7** (testing parameters same as for compounds **1**, **4** and **6**) and UV-DAD spectra of (b) compound **1** and (c) compound **7**.

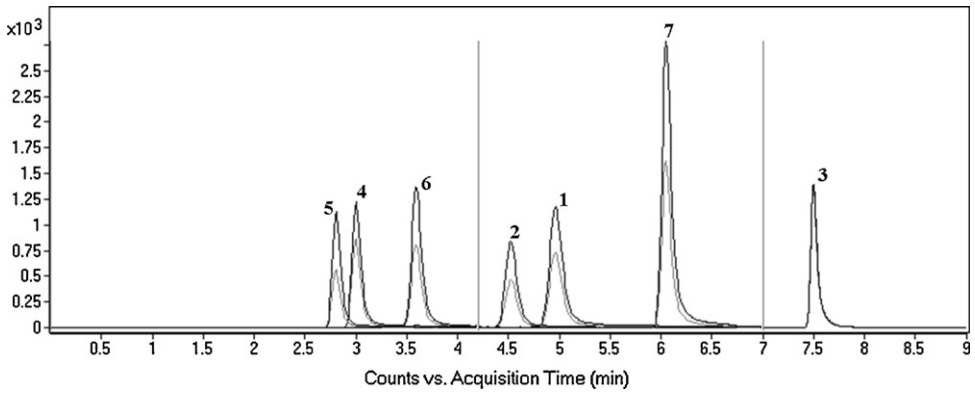

Fig. 5. Overlaid MRM chromatograms of the 2 transitions of each of the 7 compounds obtained under optimized conditions (concentration: about 100 ng mL^{-1} of each HCl salt).

respective immonium ion (Fig. 3). Furthermore, the fragment at *m*/*z* 72 in case of compound **3** can be explained as another immonium ion identical with that observed for compound **2** as a result of the opening of the pyrrolidine ring. Since the intensity of these fragments was considerably lower in the case of compounds **1** and **4**, it was assumed that the presence of an *O*-alkyl group located at the C-4 of the aromatic ring may enhance the production of immonium fragments under the used conditions.

The fragmentation pattern of compound **3** seemed to be substantially different compared to the other compounds. Loss of the pyrrolidine group (71 Da) resulting in a fragment at *m*/*z* 205 was unique to this group while the methylenedioxybenzoylcation fragment at *m*/*z* 149 (even though not very intensive for compounds **2** and **5**) was common for the methylenedioxy derivatives (Fig. 3).

Because of the different functional groups present in the molecules it was suggested that LC separation of the six compounds can be achieved in a single run. However, the use of an isocratic separation did not seem to be feasible due to the longer alkyl chains attached to the carbonyl group and thus presumably giving rise to higher log *P* value of compound **3** compared to the other molecules. With the gradient method described under *2.5* it was possible to separate compounds **1**–**6** in less than 8 min. Although baseline separation of compounds **4** and **5** was not achieved, this was not a limitation and could be overcome by evaluating the corresponding MRM chromatograms.

Limit of detection (LOD) of the method was estimated using the 3σ approach [26]. Signal-to-noise ratios for the proposed transitions were calculated using the MRM chromatograms. Peak-to-peak noise was determined in a 30 s range in a region close to each peak. It was found that the sensitivity of the method was similar for all compounds resulting in an average LOD of about 2 ng mL^{-1}. Background interference was checked by injecting a blank solution containing the solvent only. No interfering peaks were observed.

3.2. Identification of compound 7

The full scan mass spectrum of one of the bulk powder samples contained two intensive mass peaks at *m*/*z* 192 and 174. Based on previous observations with methcathinone analogs, it was concluded that the difference of 18 Da between the two peaks could be attributed to the loss of a single water molecule and thus *m*/*z* 192 may correspond to the $[M+H]^+$ molecular ion of a substance with a relative molecular mass of 191 Da. Fragmentation of the compound (Fig. 4a) carried out under similar conditions as for the other analytes (except for compound **3**) resulted in a product ion spectrum similar to that of compound **1** indicating that the substance might be a methcathinone analog. However, it was found that no such molecule with a molecular mass of 191 Da had been described within the mephedrone family in the scientific literature before. Compound **7** was then evaluated by the screening method elaborated for compounds **1**–**6**. Retention time was higher than that of **1** but lower than that of **3** (Fig. 5). From these results it can be concluded that the +14 Da mass difference with respect to mephedrone may be explained by an additional methylene group, which increases retention time with respect to compound **1** in a reverse phase chromatographic system due to its higher log *P* value. According to previous experiences, the lack of a distinct immonium fragment (the peak at *m*/*z* 72 appeared with a very low intensity under the employed conditions) and the presence of the fragment at *m*/*z* 119 indicated that the aromatic ring does not

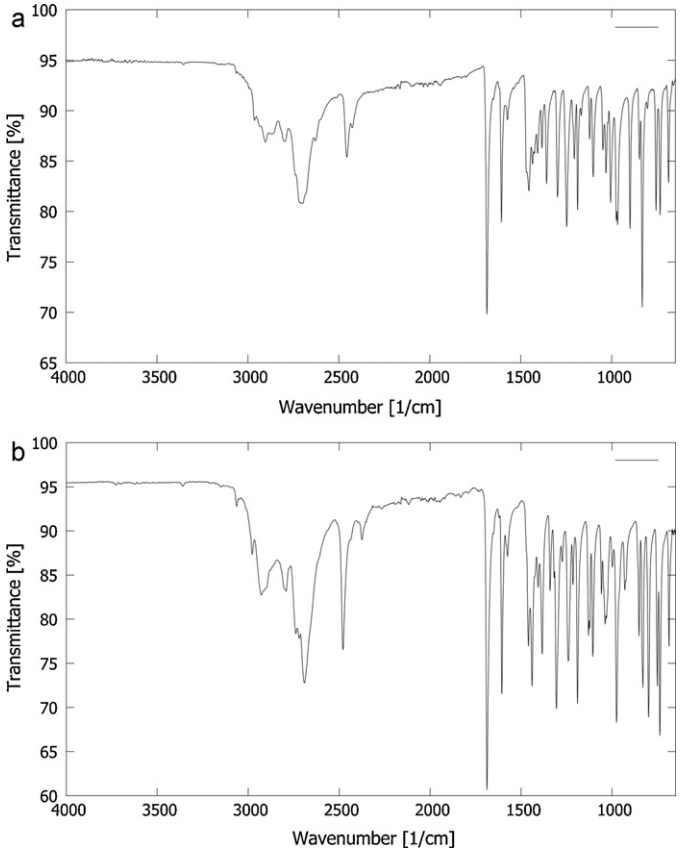

Fig. 6. Infrared UATR spectra of (a) compound **1** and (b) compound **7**. The spectra were obtained from the hydrochloride salts.

Table 2
NMR chemical shifts and NOE cross-peaks for compound **7** (see Fig. 1 for numbering of the carbon atoms).

Number	δ ^{1}H	δ ^{13}C	NOESY
1		150.18	
2	7.96 *d*, 1H	131.86	H-3
3	7.46 *d*, 1H	132.74	H-2
4		132.44	
5	7.46 *d*, 1H	132.74	H-6
6	7.96 *d*, 1H	131.86	H-5
7		199.82	
8	5.19 *q*, 1H	60.71	H-2/H-6, H-9, H-11
9	3.10–3.36 *m*, 2H	44.26	H-8, H-10
10	1.44 *t*, 3H	13.64	H-9
11	1.66 *d*, 3H	18.69	H-8
12	2.47 *s*, 3H	23.85	H-3/H-5

contain an *O*-alkyl group at the 4′ position and possibly remained unchanged with respect to mephedrone. A loss of 45 resulting in a fragment at *m*/*z* 147 (as a part of the cluster at *m*/*z* 145–147) was observed instead of the loss of *m*/*z* 31 (no peak detected at *m*/*z* 161). It was therefore concluded that the methylene substitution might affect either the chiral carbon atom or the nitrogen. This assumption was confirmed by the similarity between the UV spectra of compounds **1** and **7** (Fig. 4b and c) indicating that the chromophore groups (i.e. the aromatic ring and the carbonyl) were not affected by the methylene substituent.

Final structure elucidation was carried out by means of NMR spectroscopy. The signals in the ^{1}H and ^{13}C spectra were assigned on the basis of one- and two-dimensional homo- and heteronuclear experiments (HSQC, HMBC and NOESY). The HMBC and NOESY experiments have unambiguously shown that the additional methylene is part of an ethyl substituent attached to the *N*-atom of the molecule. Chemical shifts and NOE cross-peaks are shown in Table 2.

The accurate mass measurement by HRMS for compound **7** was 191.13162, for which the molecular formula generator calculated one possible composition within ±5 ppm accuracy, when restricting possible elements to C, H, N, and O, resulting in the formula of $C_{12}H_{17}NO$ (accuracy −3.2 ppm). Calculated isotopic abundance score was 93.9%.

The FT-IR spectrum of compound **7** was compared to that of compound **1** (Fig. 6). Although the spectra were similar the differences in the fingerprint regions were self explanatory.

Considering these results the analytical structure presented in Fig. 1 was proposed, which corresponds to 4-MEC, a substance sold as a "research chemical" by several on-line distributors.

4. Conclusions

The method described in this paper was developed using a very pragmatic approach primarily considering the problems usually encountered in the laboratories of forensic or drug control agencies. For the purpose of this paper, the term "screening method" was used for an analytical procedure developed in order to provide a preferably simple and fast method for the detection of certain target molecules or groups of target molecules (in this case methcathinones). The method was successfully applied to illegal powder samples which were found to be of high purity and water-soluble. This latter fact indicates that extraction of the compounds from drug formulations may be easily accomplished with water if they are in the form of water-soluble salts or with dilute acid solutions if they occur as bases, even for the purpose of detection of trace amounts due to the low LOD. After identification and characterization by means of different analytical techniques, the method was successfully applied to the new designer drug 4-MEC.

It is also noted, however, that regioisomers of some methcathinone derivatives like 3′-fluoromethcathinone for compound **4** or *N*,*N*-dimethylmethcathinone for compound **7** are also known. MRM in itself is usually not sufficient for unambiguous identification of these isomers, even though, if a chromatographic separation is carried out, differences in retention times may occur. Should the latter not be the case, further analytical techniques such as IR and NMR spectroscopy need to be involved. Nevertheless, it is noted that from a practical point of view it is not always relevant to distinguish between regioisomers as long as not justified by legislation.

It is assumed that the comprehensive data concerning the fragmentation characteristics of the compounds may be useful for the identification of unknown related compounds of the mephedrone family. Further application of the method to include such new substances seems to be possible.

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