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Systematic analytical characterization of new psychoactive substances: A case study

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ABSTRACT

New psychoactive substances (NPS) are synthesized compounds that are not usually covered by European and/or international laws. With a slight alteration in the chemical structure of existing illegal substances registered in the European Union (EU), these NPS circumvent existing controls and are thus referred to as "legal highs". They are becoming increasingly available and can easily be purchased through both the internet and other means (smart shops). Thus, it is essential that the identification of NPS keeps up with this rapidly evolving market.

In this case study, the Belgian Customs authorities apprehended a parcel, originating from China, containing two samples, declared as being "white pigments". For routine identification, the Belgian Customs Laboratory first analysed both samples by gas-chromatography mass-spectrometry and Fourier-Transform Infrared spectroscopy. The information obtained by these techniques is essential and can give an indication of the chemical structure of an unknown substance but not the complete identification of its structure. To bridge this gap, scientific and technical support is ensured by the Joint Research Centre (JRC) to the European Commission Directorate General for Taxation and Customs Unions (DG TAXUD) and the Customs Laboratory European Network (CLEN) through an Administrative Arrangement for fast recognition of NPS and identification of unknown chemicals. The samples were sent to the JRC for a complete characterization using advanced techniques and chemoinformatic tools.

The aim of this study was also to encourage the development of a science-based policy driven approach on NPS.

These samples were fully characterized and identified as 5F-AMB and PX-3 using ^{1}H and ^{13}C nuclear magnetic resonance (NMR), high-resolution tandem mass-spectrometry (HR-MS/MS) and Raman spectroscopy. A chemoinformatic platform was used to manage, unify analytical data from multiple techniques and instruments, and combine it with chemical and structural information.

1. Introduction

Designer drugs are "analogues of compounds with proven pharmacological activity manufactured by underground chemists for sale on the street" [1]. New psychoactive substances are designer drugs that intend to mimic controlled substances and are not yet covered by international laws. They pose a worrying threat since they are emerging on the market in increasing numbers every year. About 100 NPS were reported to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) in 2015 alone, a 25% increase from 2014 [2]. By slightly altering their chemical structure these substances circumvent existing legal controls. Often they are also referred to as "legal highs" since they are not yet covered by the country's drug laws and are thus sold as replacements for the illegal classic drugs. NPS may also be commercialized as "natural herbal products" or "research chemicals" and therefore be legally sold on the market [2] distributed by

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"wellness" retailers, smart shops or the internet. The attractiveness of NPS is mainly due to their "legal" aspect, which misleads the buyer into thinking these drugs are not harmful. Their availability, attractive packaging and appealing names also play a role [3].

The EMCDDA and the United Nations Office on Drugs and Crime (UNODC) monitor and categorize these new drugs. To this end, the EMCDDA together with Europol, have started the European Union Early Warning System (EWS) that currently monitors over 450 NPS [2]. They are also responsible for preparing risk assessments on these NPS and informing the member states of their existence so they can take the appropriate legal measures. The European Commission has prepared a proposal for a new Regulation on NPS and a Directive amending Council Framework Decision 2004/757/JHA on illicit drug trafficking as part of the EU response to drugs [4,5]. These proposals aim at strengthening the EU legal framework regarding NPS in line with the EU Drugs Strategy 2013–2020 (2012/C401/01) from the EU Drugs Action Plan for the period of 2013–2016.

The number of NPS seizures was approximately 35,000 in 2013 [2]. Synthetic cannabinoids are amongst the most commonly seized NPS in Europe with 8 out 10 seizures per year, followed by cathinones [2]. They are mainly manufactured in China and India, and purchased online via "darknets" (anonymous networks) [6] without any age restriction. Recent publications have analyzed cathinones and synthetic cannabinoids by NMR, gas-chromatography–mass-spectrometry (GC–MS) and/or high-resolution liquid-chromatography tandem mass-spectrometry (HR-LC–MS/MS) [7–12]. A major challenge is the lack of analytical research information available on these substances and the lack of reference standards since they are new to the market and consequently not yet been characterized [13]. Thus, there is a critical need for the analytical characterization of these NPS to facilitate their detection in biological matrices [14].

Drugs reach Europe through maritime, air and terrestrial borders. Customs authorities are responsible for collecting and safeguarding customs duties[1], and controlling the flow of goods into the EU acting as the first contact point of these NPS. The majority of the customs laboratories are not equipped with advanced analytical tools such as nuclear magnetic resonance (NMR), high-resolution liquid-chromatography tandem mass-spectrometry (HR-LC–MS/MS) and chemoinformatics tools, to enable the identification and characterization of the new and/or relatively unknown NPS. The JRC, together with the CLEN has the role of performing the analytical identification of unknown substances seized by Customs. Since public health laws are country-dependent, the purpose of this research project is to facilitate the law enforcement turn-over procedure through the rapid identification and analytical characterization of NPS.

The Belgian Customs authorities seized a parcel containing two plastic bags, declared as "white pigments", coming from China. One was labelled as 5F-AMB, a synthetic cannabinoid; and the other was not labelled at all. The buyer had intended to purchase the synthetic cannabinoid 5F-AMB and Clonazolam, a new designer benzodiazepine. These are often used together with other drugs to either increase the effects of the drug's high, or to counterbalance the adverse effects of other drugs [15]. Not surprisingly, the number of analysed benzodizepines and synthetic cannabinoids in the Belgian Customs Laboratory increased in 2015 when compared to 2014: in 2014 only 1 whereas in 2015 there were 5. The number of synthetic cannabinoids analysed in 2015 was 68, up from 48 in 2014.

The aim of this study was to (a) fully characterize both samples in order to confirm their true identity using a consolidated analytical strategy and chemoinformatics tools and (b) to disseminate the results and encourage the development of a science-based policy driven approach on NPS.

An earlier publication this year [10] has made available the analytical profile of 5F-AMB which we are complementing with the FTIR and the Raman spectra. The full analytical profile for the PX-3 sample is published here.

This paper presents an analytical strategy which allows the characterization of unknown compounds, based on the scientific experience of the JRC, in the use of advanced analytical techniques (high-resolution and chemoinformatics tools). These approaches have been successfully tested on samples provided by the CLEN, through the successful identification of approximately 100 samples between 2013 and 2015. This is further demonstrated in this case study which was accomplished in collaboration with the Belgian Customs Laboratory.

2. Materials and methods

2.1. Chemicals and reagents

For the NMR, HR-MS/MS and Raman analysis, the reagents were all obtained from Sigma-Aldrich® (Milan, Italy). The solvents were all LC–MS Chromasolv® grade from Fluka Analytical, purchased via Sigma-Aldrich®. The deionised water used was taken from a Milli-Q Purifier with a Millipak®40, 0.22 μm filter (18.2 mΩ cm, 25 °C). These analytical techniques were performed at the JRC.

For the GC–MS analysis, reagent grade solvents (methanol and dichloromethane) were obtained from Sigma-Aldrich®. For the FTIR analysis, no solvents or reagents were used. These analyses have been performed by the Belgian Customs Laboratory.

2.2. Sample preparation and instrumentation

2.2.1. GC–MS

For the GC–MS measurement, a small aliquot of each sample (ca. 10–20 mg) was dissolved in a 50:50 mixture of methanol and dichloromethane.

An Agilent® G1530N Gas Chromatograph equipped with a 5973N Mass Detector (EI mode) was used for the determination of the mass spectrum of the substances. The GC-column was a DB5-MS (Phenyl Arylene polymer stationary phase) from Agilent J&W (30 m, 0.250 mm, 0.25 μm) with helium as the carrier gas. An initial oven temperature of 100 °C was set (no isothermal period), ramping at 15 °C/min until it reached 320 °C and held for 15.33 min. The total run time was 30 min.

The injection volume was 1 μL in split mode (20:1) and the injector temperature set at 280 °C.

2.2.2. FTIR

A Spectrum One FTIR from PerkinElmer®, equipped with an ATR-accessory was used and no sample preparation was needed as the powdered samples were measured as such on the ATR-unit. The FTIR spectrum of the sample was recorded from 4000 down to 650 cm^{-1}.

2.2.3. NMR

For the acquisition of a ^{1}H NMR spectrum a 10 mg aliquot of the samples was mixed with 600 μL DMSO-d_6, which was used as an internal lock and chemical shift reference at 2.50 ppm.

The ^{1}H NMR experiments were performed at 300 K on a Bruker® (Rheinstetten, Germany) spectrometer Avance III HD 600 (nominal proton frequency 600.13 MHz) equipped with a 5 mm QCI cryo-probe (^{1}H, ^{13}C, ^{15}N and ^{19}F). The ^{1}H and ^{13}C chemical shifts are expressed in d scale (ppm), referenced to the proton signal of the DMSO-d_6 (2.50 ppm for proton and 39.52 for ^{13}C).

[1] A custom duty is a payment due to the revenue of a state, levied by force of law. It is used to describe a tax on certain items purchased abroad.

Compounds were characterized by one-dimensional 1H, 13 C and APT as well as two-dimensional $^{1}H/^{1}H$ COSY, $^{1}H/^{1}H$ TOCSY, $^{1}H/^{13}C$ HSQC, $^{1}H/^{13}C$ HMBC and $^{1}H/^{15}N$ HMBC experiments.

2.2.4. HR-MS/MS

For the HR-MS/MS analysis, 1 mg of each sample was dissolved in 5 mL of MeOH to obtain a 200 μg/mL solution. This was then diluted with mobile phase (50:50 MeOH:H_2O + 0.1% formic acid) to obtain a sample concentration of 2 μg/mL for infusion.

The HR-MS/MS analyses were performed on a hybrid quadrupole time-of-flight mass spectrometer (Q-TOF Premier, Waters®) equipped with an electrospray ionization source (ESI). The operating conditions of the Q-TOF mass spectrometer were as follows: capillary voltage, 3.5 kV (+); sample cone voltage, 38 V; extraction cone, 3.0 kV; source temperature, 80 °C; desolvation temperature, 150 °C; cone flow, 25 L/h; desolvation flow, 450 L/h. For the quadrupole and the collision cell, the parameters were as follows: LM resolution, 5.0; HM resolution, 15.0; ion energy, 1.5 V; cell entrance, 2.0 V; gas flow 0.10 mL/min. Time-of-flight (TOF) was performed in the continuous extraction mode. In the non-linear mode (W mode), an accelerating voltage of 9.0 kV and a reflectron voltage of 2.0 kV were used for the TOF. The micro-channel plate (MCP) value of the detector was set to 650 V. The instrument was operated in the positive ESI mode. The TOF analyzer was calibrated using a solution of sodium formate in the *m/z* range of 50–1000. Mass spectra (MS mode) were collected over the full *m/z* range with a scan time of 0.5 s and an interscan time of 0.05 s. The resolution used was 17,000 FWHM (*i.e.*, an *m/z* 500 ion peak has a width at half height of *m/z* 0.03) in the W mode. The HR-MS/MS system was operated using MassLynx 4.1 software (Waters®). Raw data files (*.RAW) were generated and converted into *.CDF files using the Data Bridge tool (Waters®).

2.2.5. Raman

Pellets were prepared directly using the sample powder using the KBr pellet technique.

Samples were analysed using a DXR Smart Raman instrument from Thermo Scientific (Madison, WI, US), with a single exposure of the charged coupled device (CCD), and controlled by the Thermo Scientific OMNIC 9.1 Raman Software. The excitation laser, at 780 nm, operated between 150 and 3400 cm^{-1} for all measurements using a laser power of 50 mW on each sample and a full range grating. Spectra were collected with a focused laser beam of 50 μm 'pinhole' diameter, spectral resolution of 5 cm^{-1} and a scanning time of 60 s. Calibration of the instrument was carried out automatically using both neon and white light.

2.2.6. Chemoinformatic tools

The ACD/Spectrus Platform database was used, together with Mass Frontier (Thermo Fisher Scientific®) for structural elucidation.

With ACD/Spectrus, it is possible to confirm the consistency of proposed chemical structures with NMR experimental data (1D and 2D) and assign experimental spectra with an identified structure and easily build a central fully searchable repository of the assigned NMR spectra.

Mass Frontier software predicts and returns MS fragmentation pathways based on a set of general ionization (protonation in ESI+ mode), fragmentation, rearrangement (maximum number reaction steps: 5) and resonance (electron transfer and charge stabilization) rules. The software was used to predict fragmentation, confront experimental with theoretical data and annotate MS and MS/MS spectral ion trees.

ACD Labs Spectrus Platform combined with the database module allows the creation of a library of spectra from different analytical sources, and the search and match of spectra available.

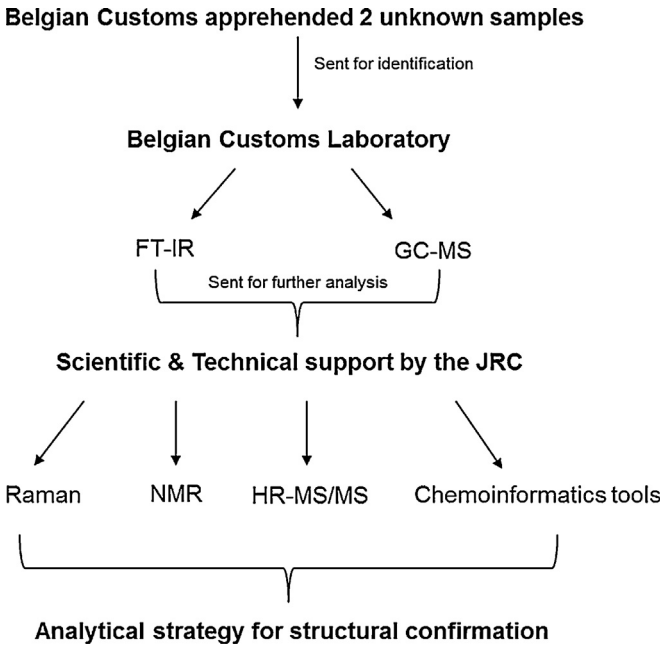

Fig. 1. Experimental design. A combination of advanced analytical techniques and chemoinformatics tools allowed sample confirmation.

3. Results and discussion

A schematic outline of the experimental design may be found in Fig. 1.

3.1. GC–MS and FTIR

The GC–MS spectra of both samples are shown in Figs. 2 and 3, respectively, and together with the HR-MS/MS spectra, and the corresponding FTIR spectra can be found in Fig. 4.

For the first substance, the GC–MS spectrum as well as the FTIR spectrum corresponded well with the analytical data for 5F-AMB available on the Scientific Working Group for the Analysis of Seized Drugs (SWGDRUG) Website [16].

For the second sample however, no match with existing spectra could be found. The GC–MS and the FTIR spectra showed similarities with the ones of AB-CHMINACA, an indazole-based synthetic cannabinoid, but there were also significant differences. By looking at the differences between the GC–MS spectrum in Fig. 3 (with *m/z* 360 and 404) and that of AB-CHMINACA (with *m/z* 312 and 356) it could be deduced that the methylpropyl-group (2-butanyl-group) that is present in AB-CHMINACA was replaced in the substance under investigation by a group with an *m/z* that is 48 units lager than for methylpropyl.

These two techniques may be used for confirmatory purposes if the initial structure is previously known, or by spectral library search and match.

3.2. NMR

The NMR analysis allows structural elucidation as a stand-alone technique.

The observed NMR, ^{1}H and ^{13}C NMR, spectroscopic data is presented below and the results of the correlation presented in Fig. 5 for both samples.

The types of multiplicity and the coupling constants of the peaks were determined automatically in ACD/Spectrus 2014 software (Advanced Chemistry Development, Inc., Toronto, Canada), in which

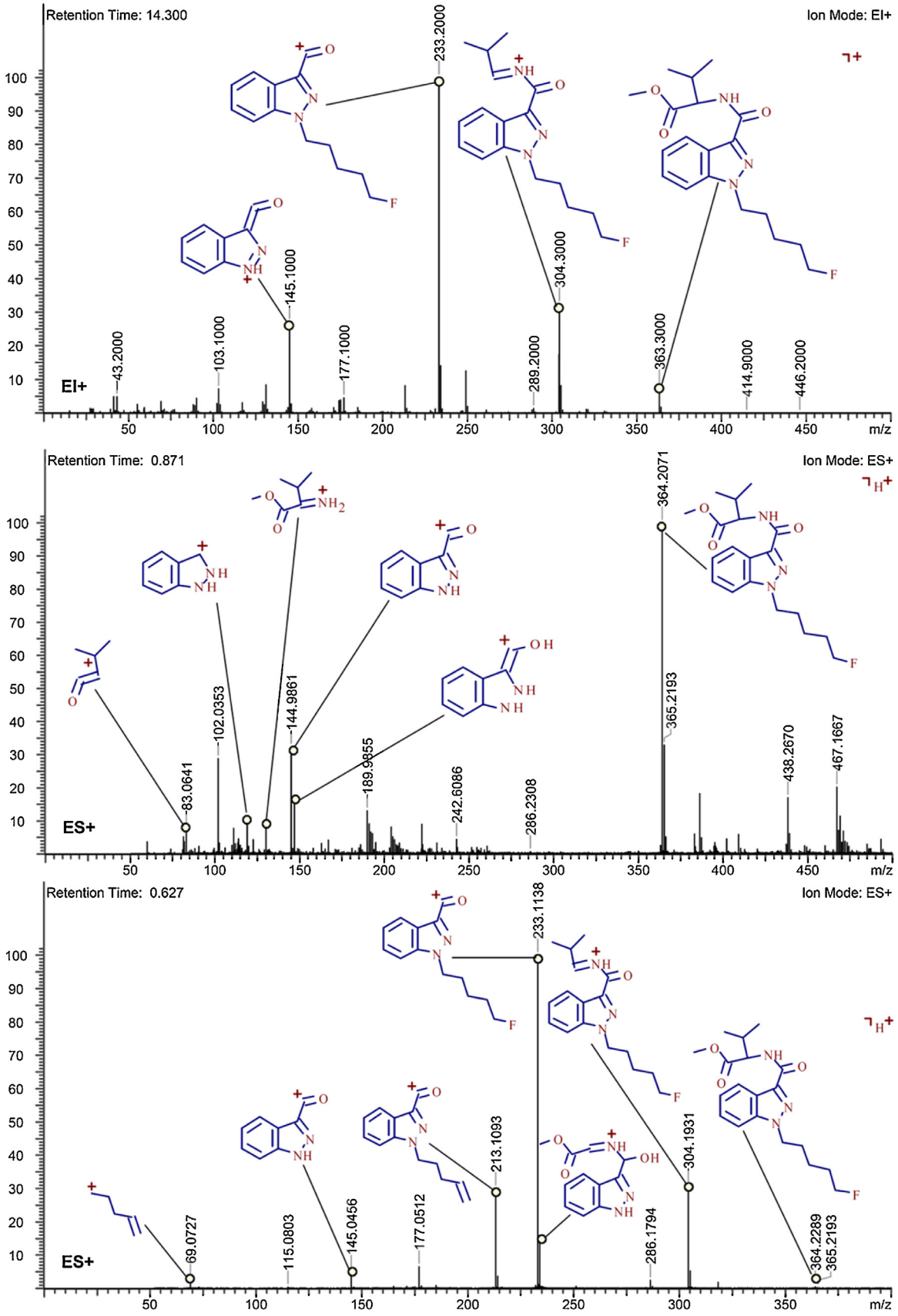

Fig. 2. GC–MS and HR-MS/MS spectra for sample 5F-AMB (A).

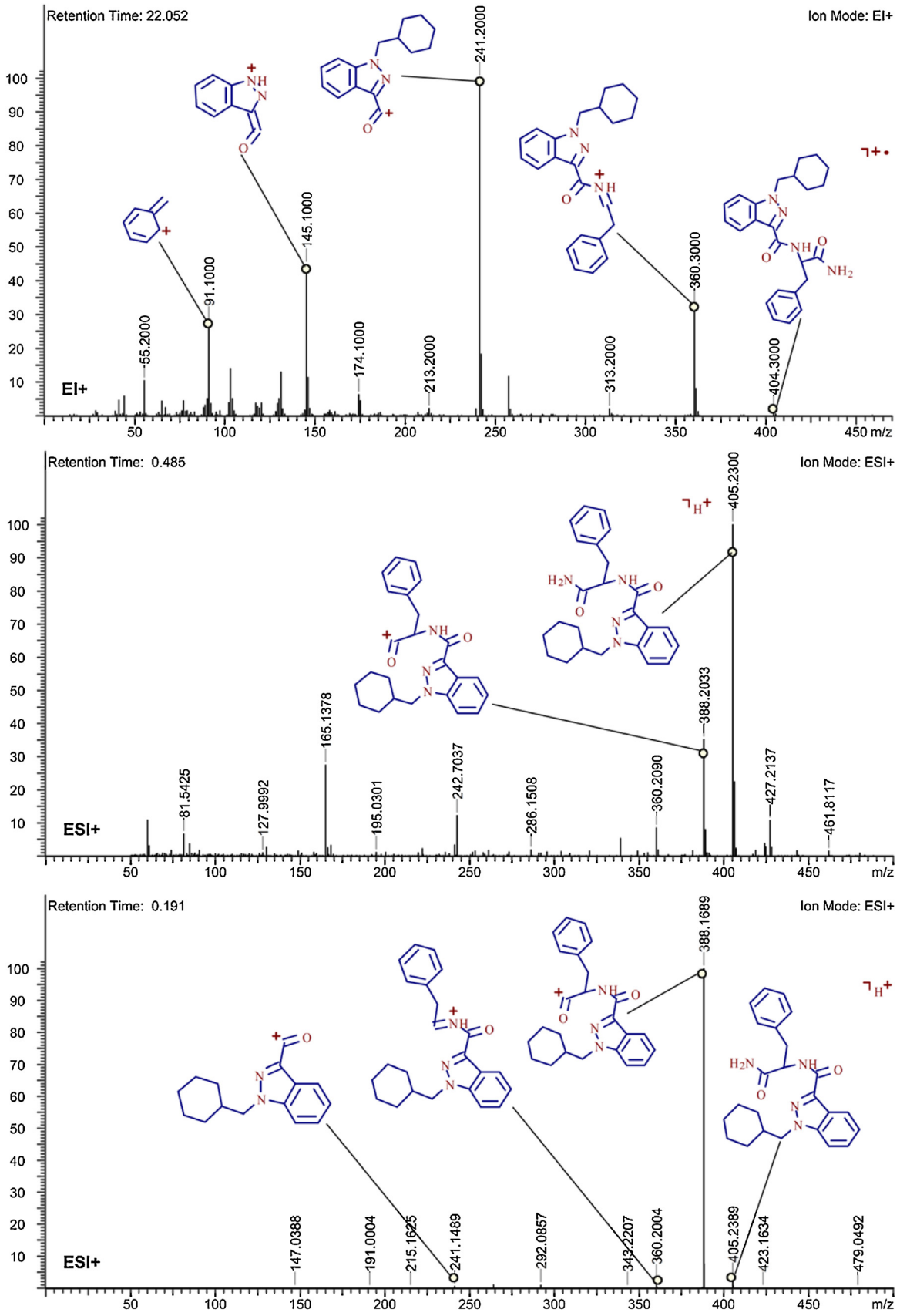

Fig. 3. GC–MS and HR-MS/MS spectra for sample PX3 (B).

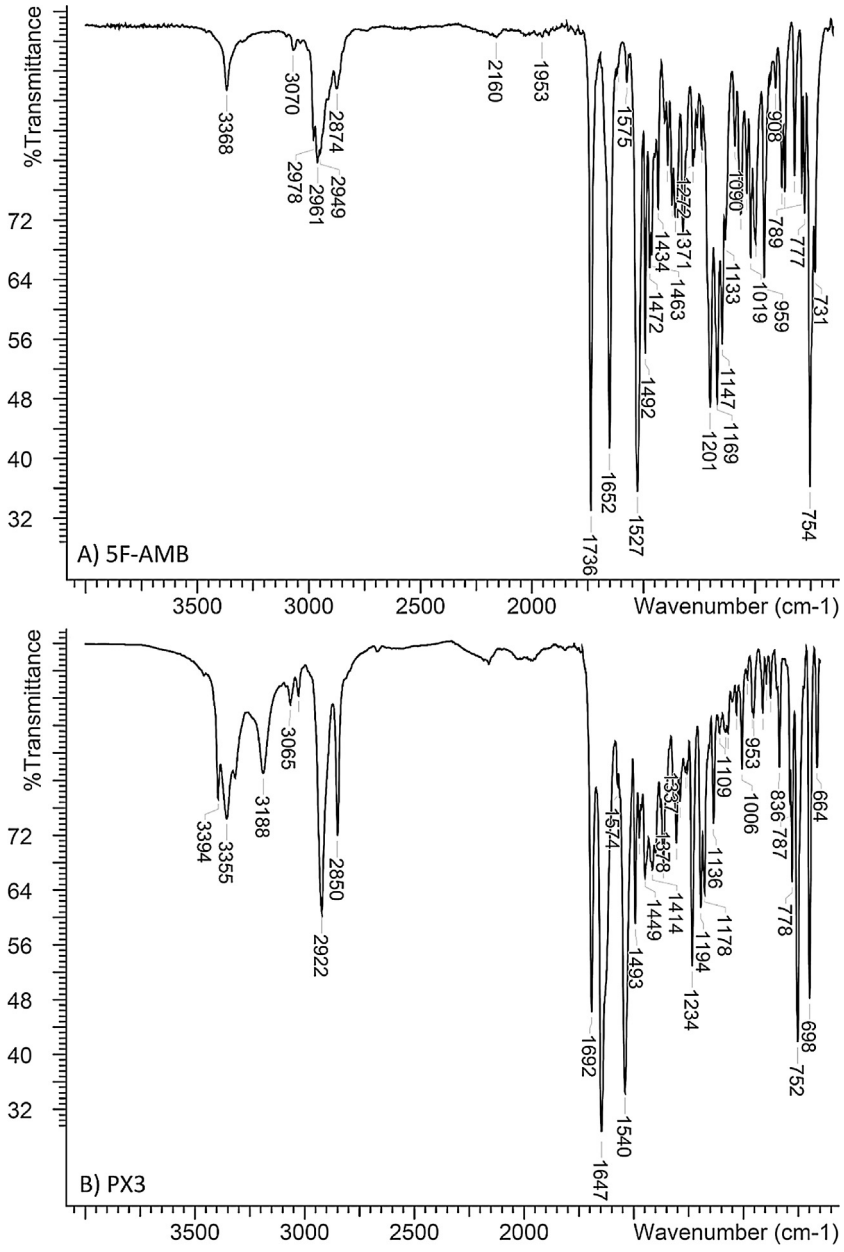

Fig. 4. FTIR spectrum for samples 5F-AMB (A) and PX3 (B).

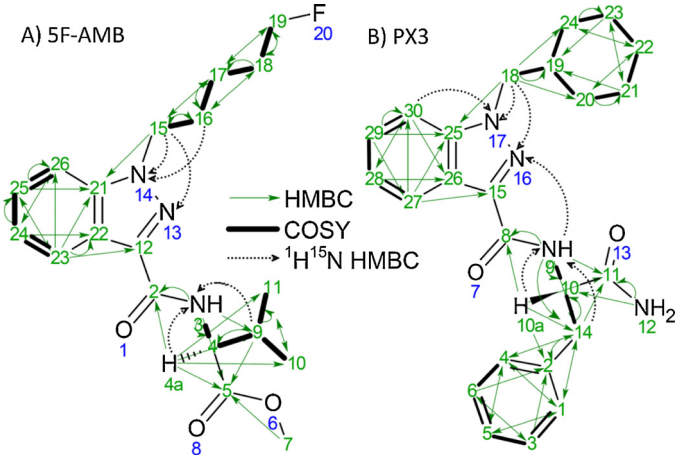

Fig. 5. NMR correlation for samples 5F-AMB (A) and PX3 (B).

the assignment of the peaks with corresponding atoms of the structures was also performed.

3.2.1. 5F-AMB (A)

δ_H (DMSO-d_6): 8.14 (1H, d, J = 8 Hz, atom 23), 8.04 (1H, d, J = 8 Hz, atom 3), 7.80 (1H, d, J = 9 Hz, atom 26), 7.47 (1H, ddd, J = 8, 7, 1 Hz, atom 25), 7.29 (1H, t, J = 8 Hz, atom 24), 4.53 (2H, t, J = 7 Hz, atom 15), 4.43–4.47 (2H, m, atom 4a, 19), 4.37 (1H, t, J = 6 Hz, atom 19), 3.66–3.74 (3H, m, atom 7), 2.22–2.31 (1H, m, atom 9), 1.93 (2H, quin, J = 7 Hz, atom 16), 1.61–1.74 (2H, m, atom 18), 1.37 (2H, quin, J = 8 Hz, atom 17) and 0.94–1.00 (6H, m, atom 10, 11).

δ_C (DMSO-d_6): 172.5 (C-5), 162.4 (C-2), 141.1 (C-21), 136.7 (C-12), 127.1 (C-25), 123.0 (C-24), 122.5 (C-22), 122.1 (C-23), 110.9 (C-26), 84.1 (C-19), 57.7 (C-4), 52.3 (C-7), 49.1 (C-15), 40.0 (C-), 30.4 (C-9), 29.8 (C-18), 29.4 (C-16), 22.5 (C-17), 19.5 (C-11) and 19.1 (C-10)

3.2.2. PX3 (B)

δ_H (DMSO-d_6): 8.11 (1H, d, J = 8 Hz, atom 27), 7.89 (1H, d, J = 8 Hz, atom 9), 7.77 (1H, d, J = 9 Hz, atom 30), 7.65 (1H, s, atom

Table 1
Raman spectroscopic data for sample 5F-AMB (A).

Position	Intensity	Possible functional group
763.44	37.148	–C–halogens
779.81	63.751	
882.74	22.351	
908.95	32.149	–C=C–
1005.61	59.671	C–O–C esters
1136.23	26.042	C–N in aromatic compound
1178.95	23.548	
1214.77	25.593	Branched chain in hydrocarbons
1240.12	36.770	
1273.01	26.264	C–N in aromatic amines
1324.16	33.366	–C–F in aliphatic compounds
1361.24	51.548	Isopropyl group
1376.13	38.125	$-CH_3$
1405.57	110.571	C–N in primary amides
1441.51	35.976	$-CH_2CH_3$
1472.66	60.965	N aromatic
1575.51	64.968	Possible N=N aliphatic
1653.89	50.390	Possible C=N
1738.41	17.001	Carbonyl group
2915.07	78.751	Aliphatic chain
2959.30	71.425	Aliphatic chain
2981.67	60.548	Aliphatic chain
3070.33	72.038	Aromatic ring–possibly substituted

Table 2
Raman spectroscopic data for sample PX3 (B).

Position	Intensity	Possible functional group
778.40	429.929	Subst. benzenes
1003.80	422.594	Carbon ring in cyclic compound
1032.55	169.303	$C–NH_2$
1181.14	122.289	C–C–N in amines
1235.86	195.035	C–O–C in ethers
1267.64	128.401	Alicyclic chain
1363.77	306.634	–C–N in amines
1412.96	278.267	–C–N in amides
1443.05	124.081	N=N aromatic
1476.47	218.405	$-CH_2$ in aliphatic chain
1541.50	160.304	–C=C–
1576.02	231.569	NH_2 in alkyl amide
1645.45	366.643	C=O in amides
2851.11	266.381	–CH attached to N
2938.70	316.625	$-CH_2$ and $-CH_3$ in aliphatic chains
2988.05	151.952	$-CH_2$ and $-CH_3$ in aliphatic chains
3067.17	315.546	C–H aromatic

12), 7.44 (1H, ddd, J = 8, 7, 1 Hz, atom 29), 7.19–7.29 (6H, m, atom 1, 3, 4, 6, 12, 28), 7.15–7.18 (1H, m, atom 5), 4.76 (1H, td, J = 8 and 5 Hz, atom 10a), 4.28–4.38 (2H, m, atom 18), 3.17 (1H, dd, J = 14 and 5 Hz, atom 14), 3.05–3.13 (1H, m, atom 14), 1.93 (1H, dtt, J = 15, 7, 4 Hz, atom 19), 1.63–1.72 (2H, m, atom 21, 23), 1.61 (1H, br d, J = 8 Hz, atom 22), 1.43–1.54 (2H, m, atom 20, 24), 1.11–1.21 (3H, m, atom 21, 22, 23) and 0.98–1.09 (2H, m, atom 20, 24)

δ_C (DMSO-d_6): 173.1 (C-11), 161.7 (C-8), 141.6 (C-25), 138.1 (C-2), 136.8 (C-15), 129.8 (C-4, 1), 128.5 (C-6, 3), 127.0 (C-29), 126.8 (C-5), 122.8 (C-28), 122.3 (C-26), 122.1 (C-27), 111.1 (C-30), 54.9 (C-18), 53.6 (C-10), 38.8 (C-19), 38.2 (C-14), 30.5 (C-24, 20), 26.3 (C-22) and 25.7 (C-21, 23)

3.3. HR-MS/MS

The MS and MS/MS data were imported to Mass Frontier (Thermo Fisher Scientific®) for structural elucidation and fragmentation prediction confirmation. This software allows to predict fragmentation, confront experimental with theoretical data and annotate MS and MS/MS spectral ion trees (Figs. 2 and 3).

The accurate mass spectrum obtained by the Q-TOF Premium for samples A and B gave a parent ion peak at m/z 364.2071 and 405.2301, respectively, in agreement with the NMR spectroscopic data. The monoisotopic mass of the compounds are (A) 363.1958 and (B) 404.2212. The fragmentation of the parent ions on both compounds yields a match of 9 fragments for A, and 5 for B with a 5% peak height minimum requirement (threshold of 5% of the highest peak). The use of HR-MS/MS allows the precise determination of the monoisotopic mass of a compound up to 4 decimal places. Previously knowing the theoretical structure by the techniques employed beforehand, allows confirmation of the molecule by fragmentation matching.

3.4. Raman

The combination of Raman spectroscopy with other analytical tools is promising. It is a fast, non-destructive tool to differentiate among very similar unknown chemicals. The use of peak table results (Tables 1 and 2), in addition to the NMR assignments is confirmatory of the proposed structure and facilitates the characterisation and identification of NPS. The spectra (Fig. 6) for both samples were acquired in the region of 3400.00–150.00 cm^{-1}. For sample A, the absolute threshold was 14 and the sensitivity of 35, whereas for sample B the absolute threshold was of 81 of Raman intensity and the sensitivity of 48. The data is presented in Table 1 (sample A) and Table 2 (sample B).

Analysis with GC–MS and FTIR is suitable for routine control as it can be performed within a relatively short time (few minutes to one hour) and with reasonable running costs. For relatively pure samples, a GC–MS analysis together with a FTIR-spectrum can lead to full identification of the substance if both spectra give a match with existing data. However, the MS-instruments that are used for routine control are generally low-resolution single-MS instruments and the analytical data obtained does not allow a full confirmation of the chemical structure of a new unknown substance on its own rendering it insufficient to be used as a stand-alone technique.

The present work introduces a complete integrated analytical strategy, together with chemoinformatics tools allowing the complete characterization of unknown substances.

After analysis by GC–MS, FTIR, NMR, HR-MS/MS and Raman, the samples were correctly identified as 5F-AMB, also known as 5F-AMB-PINACA (systematic name: *Methyl 2-(amino)-3-methylbutanoate)* [17,18] and PX-3, or APP-CHMINACA (systematic name: *N-(2-amino-1-benzyl-2-oxo-ethyl)-1-(cyclohexylmethyl)indazole-3-carboxamide)*, and not clonazolam.

The compound PX-3 is a synthetic cannabinoid developed and patented by Pfizer® with claimed binding affinity for CB_1.

The analytical results are the collaborative effort of the Belgian Customs Laboratory, who performed a preliminary analysis, and of the JRC which allowed the rapid identification and confirmation of these two NPS. These were then reported by the Customs Laboratory to its corresponding National Focal Point, who in its turn sent an official notification to the EMCDDA who then includes them in the European Drug Network Database (EDND), a European information system and database on new drugs.

For the sample 5F-AMB, the results support previous published findings [10,16], complemented here with FTIR and Raman spectra. For the sample PX-3, the analytical data is presented.

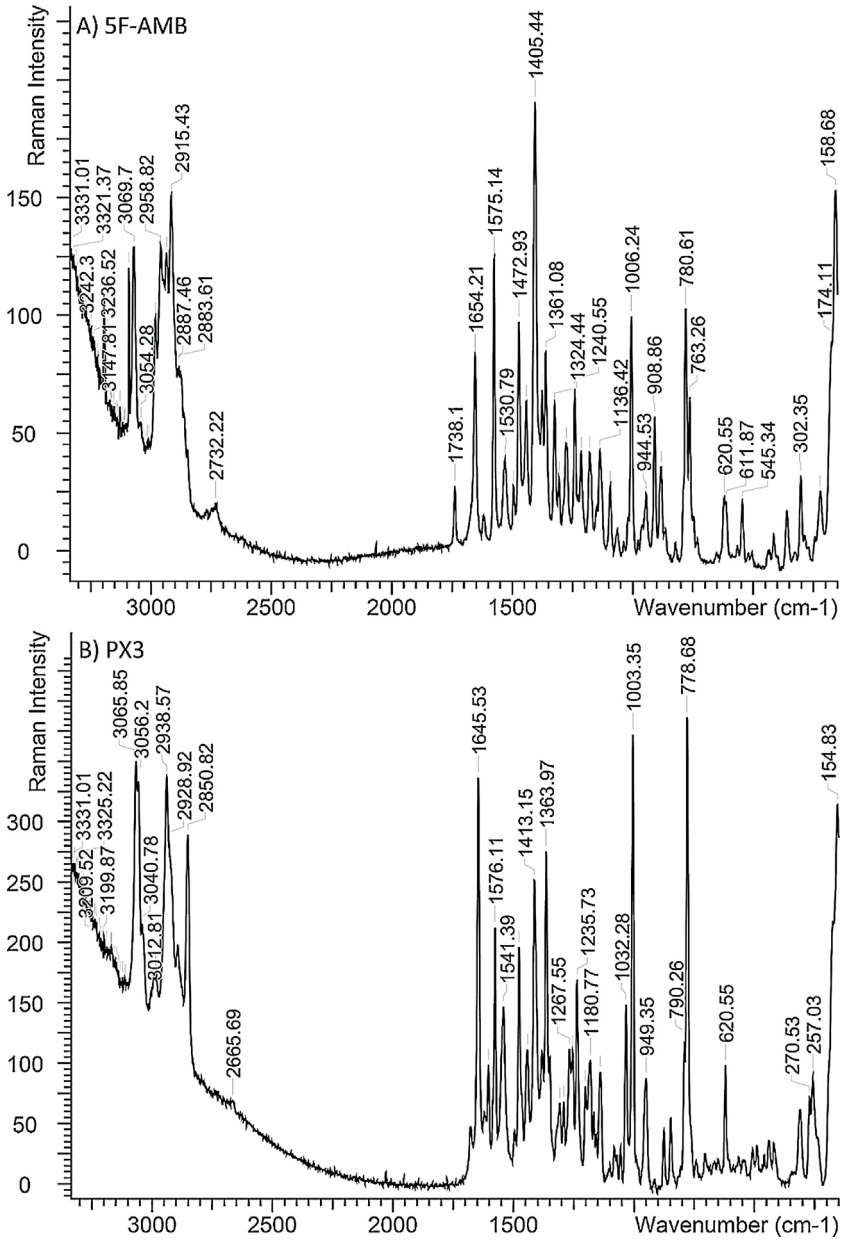

Fig. 6. Raman spectra for samples A and B.

4. Conclusion

The systematic analytical approach used, together with the chemoinformatics tools allowed us to structurally define and successfully confirm the identification of both samples employing different powerful and complementary analytical techniques.

The confirmation of these NPS remains challenging and even though GC-MS and FTIR are elucidative techniques, for the full analytical confirmation of completely new substances additional techniques are required. The collaboration between the network of European Customs laboratories and the JRC is being successfully applied for the rapid identification of these emerging NPS thus and facilitated the identification of these two samples as synthetic cannabinoids, not one synthetic cannabinoid and one benzodiazepine as ordered by the purchaser.

Synthetic cannabinoids mimic the effects of cannabis in the endocannabinoid system but are more toxic than cannabis [19]. Benzodiazepines are anxiolytics with sedative properties that increase the effect of the neurotransmitter gamma-amino butyric acid (GABA) at the $GABA_A$ receptor. They are often used together with other drugs of abuse to either heighten the effects the drug's high, or to counterbalance the adverse effects of other drugs [15].

In this short case study concerning NPS from Belgium, we illustrate the possible vulnerability of consumers who purchase the NPS and who rely on the precarious trustworthiness of web sellers. Instead of a synthetic cannabinoid and a benzodiazepine, the purchaser was sent two synthetic cannabinoids which, if taken together, could have led to severe intoxication.

Conflict of interest

The authors declare that there are no conflicts of interest.

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