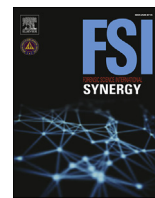




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Instrumental neutron activation analysis (INAA) and liquid chromatography (LC) coupled to high resolution mass spectrometry (HRMS) characterisation of sildenafil based products seized on the Italian illegal market

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ABSTRACT

The commerce of illegal and counterfeit medicinal products on internet is a serious criminal problem. Drugs for erectile dysfunction such as phosphodiesterase type 5 inhibitor are the most commonly counterfeited medicines in Europe. The search of possible toxic chemical substances in seized products is needed. Moreover, the profiling of the material can be the source of relevant forensic information. For the first time a combined approach based on liquid chromatography (LC) coupled to high resolution mass spectrometry (HRMS) and instrumental neutron activation analysis (INAA) is proposed and tested, allowing characterisation of both authentic and illegal pharmaceuticals containing sildenafil seized in Italy. LC-HRMS allowed the detection and identification of unknown impurities not reported on labels in illegal products and the quantitation of the sildenafil. INAA showed to be suitable to provide both qualitative and quantitative information for forensic purposes on 23 elements, allowing discrimination between legal and illegal products.

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1. Introduction

The Internet plays a critical role in illegal trafficking, providing illegal products online from anywhere around the world, including counterfeit goods such as pharmaceuticals [1]. Broséus et al. has recently studied the darknet market and found that most of the sale proposals (63%) concern licit or illicit drugs but in this category there are also prescription drugs and medicines [2]. Illegal and counterfeit medicinal products on internet is a criminal problem that poses a serious threat to public health. This issue pushed the research to develop many analytical tools to allow forensic characterisation of such products [3–5]. Drugs for erectile dysfunction such as phosphodiesterase type 5 (PDE5) inhibitor medications belong to a special class of illegal pharmaceutical products sold on

the Internet [6–8]. They are the most commonly counterfeited medicines in Europe and they were also found in dietary supplements [9,10]. The analysis of these products is important for two reasons: on one hand the search of possible toxic chemical substances is needed to protect public health, on the other hand the profiling of the material can support to infer about the source of illegal materials and other relevant forensic information. PDE5 inhibitor medications were analysed by high-performance liquid chromatography (HPLC) [11], liquid chromatography-mass spectrometry (LC-MS) [12–15], nuclear magnetic resonance (NMR) [16,17], Fourier transformed infrared spectroscopy (FTIR) [18] and Raman micro-spectroscopy [19].

Organic analysis is not enough for forensic characterisation of illegal pharmaceutical products. Heavy metals can be found into pharmaceutical products as impurities, to be monitored because some metals are known to be toxic even in small doses. The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use

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(ICH), the European Pharmacopoeia (EP) and the United States Pharmacopoeia (USP) have made regulations for inorganic impurities. Among the suitable methods are inductively coupled plasma atomic emission spectrometry (ICP-AES) and inductively coupled plasma atomic mass spectrometry (ICP-MS), which have the capability to carry out rapid and accurate multi-element analysis at and below ng/g levels, coupled with excellent performance characteristics such as wide elemental coverage, rapid analysis (all elements at once), wide analytical working range (up to 9 orders), simple spectra and high tolerance to matrix effect [20]. Since 1990s researchers studied heavy metals by atomic absorption spectrometry (AAS), inductively coupled plasma mass spectrometry (ICP-MS), instrumental neutron activation analysis (INAA), in illicit drugs such as heroin and MDMA to establish the profiling and infer about their origins. The classification of seized drugs by impurity profiling is able to provide useful forensic information to identify drug traffic routes, clandestine laboratories and methods of drug preparation. This study has been becoming a great tool for police activities [21]. AAS is still popular because allows reliable determination of metallic impurities but is not multi-element and flame AAS suffers of poor sensitivity. ICP-MS has multi-element capability and shows higher sensitivity, accuracy and precision compared with AAS.

Before the advent of ICP-MS, INAA was used for long time to analyse trace metal elements, not only into illicit drugs [22,23] but also to obtain important information on the concentration levels of several toxic elements (Hg, Cd, As, Se, Sb, U and Th) into radiopharmaceuticals (DTPA, HMPAO, DMSA) [24] and trace elements into drugs samples [25].

Wollein et al. [26] showed the development and validation of the methods used for the determination of 21 selected metals in 113 samples from drug products and their active pharmaceutical ingredients. To analyse metal residues, ICP-MS was used for the determination of Mn, Co, Ni, Mo, Ru, Rh, Pd, Cd, Sn, Sb, Ir, Pt, and Pb, V and Os; Cu, Fe, and Zn were analysed using an ICP optical emission spectroscopy (OES); Hg was analysed by cold vapour atomic absorbance spectroscopy (CV-AAS). Determination of As was carried out using a hydride generation (HG) AAS and Cr by graphite F-AAS. The advantage of ICP-MS was shown, as the majority of the selected elements could be quantified by using this technique with RSD lower than 4.5%. Afterwards, the ICP-MS was applied to quantitative analysis of heavy metal of 190 samples from 31 different excipients and 15 samples from eight drug substances provided through the International Pharmaceutical Excipient Council of the Americas [27]. In each run, 24 elements including Cd, Pb, As, Hg, Co, V, Ni, Tl, Au, Pd, Ir, Os, Rh, Ru, Se, Ag, Pt, Li, Sb, Ba, Mo, Cu, Sn, and Cr were measured, some at multiple isotopic masses to identify and correct for interferences. In the 2000s it has been proposed the ICP-MS as alternative method to the heavy metal test for pharmaceutical material [28,29], which was later tested on selected drugs such as dicydomine-HCl, ethambutol, pyrazinamide and furazolidone [30], and antihypertensive drugs [31]. In a review published in 2007 the ICP-MS resulted as the most used method to find the metal elements into drugs and pharmaceutical material [32] and more applications were published later about ICP-MS [33,34] and laser ablation (LA) ICP-MS [35] in applications meeting Pharmacopoeia requirements for analysis of elemental impurities in pharmaceutical goods. If ICP-MS showed to be perfectly suitable for highly standardised products such as medicines but, when considering the forensic issue of illegal and counterfeit pharmaceutical products, INAA is expected to have some advantages. First of all it allows to avoid representative subsampling and sample preparation difficulties. Then as it is based on completely different physical principles, it can be considered as

validation technique. Moreover, the degree of accuracy and metrological traceability of the values of the measurement can meet the highest international metrological requirements [21].

On the other hand, mass spectrometric techniques, mainly coupled to gas or liquid chromatography, are the golden standard for forensic characterisation of drugs and impurities. About sildenafil characterisation, a comparison among different analytical techniques demonstrated that UPLC-MS ingredients profile was the most reliable technique for distinguish authentic and unauthentic drugs [7].

The aim of the present research was to develop a novel approach based on LC coupled to high resolution mass spectrometry (HRMS) and INAA to characterise both the authentic and illegal pharmaceuticals containing sildenafil seized in Italy and to provide quantitative elemental data for forensic purposes. The specific role of INAA in the research was to obtain information on the presence of both toxic elements and trace elements allowing inferring about the possible common origin of confiscated material.

2. Materials and methods

2.1. Chemicals and reagents

Ultrapure water, acetonitrile, ammonium formate, formic acid, sildenafil were purchased from Sigma-Aldrich (Milan, Italy); methanol was obtained from Merck (Merck KGaA, Darmstadt, Germany).

The standards reference materials used for INAA were NIST-SRM 1633a and NIST-SRM 1547 from the National Institute of Standards and Technology (NIST).

2.2. LC/HRMS equipment

The LC/HRMS system was composed of a Thermo ULTIMATE 3000 equipped with a Thermo Acclaim RSLC 120 C18 analytical column (2.1 × 100 mm, 2.2 μm particle size) coupled to a Thermo single-stage Orbitrap (Exactive) MS system, interfaced with a HESI Ion Max source.

2.3. LC/HRMS conditions

Mobile phase A was ultrapure water with 0.1% formic acid/ammonium formate 5 mM, mobile phase B was methanol/acetonitrile 1:1 with 0.1% formic acid. The analytical column was maintained at 40 °C, and sample injection volume was 10 μL. The flow rate was set at 400 μL/min. The mobile phase gradient was as follows: 100% A for 1 min, gradient to 15% B in 4.1 min, to 50% B in 1.8 min and then to 100% B in 2.1 min and maintained for 3.5 min. A column re-equilibration was performed with a linear gradient to 100% A in 3.0 min and then maintained for 3.0 min. The HESI source was heated at 340 °C. The other following parameters were used: source current, 6 μA; sheath and auxiliary gas (both nitrogen) flow rates, 35 and 18 arbitrary units, respectively; capillary temperature, 275 °C, and capillary voltage 45 V. The data were acquired in full scan mode over a mass range of 110–650 m/z. The instrument was operating in positive ion mode with a resolving power of 100000 FWHM. Mass calibration was performed according to the guidelines provided by the instrument's supplier. The recommended calibration solution was made of MRFA (L-methionyl-arginyl-phenylalanyl-alanine acetate), caffeine and Ultramark® 1621 dissolved in methanol/water (1:1). The automatic calibration feature of the Exactive tune software was used for calibration. The mass scale was calibrated every 2 days over a mass range *m/z* 50–2000. Lock mass

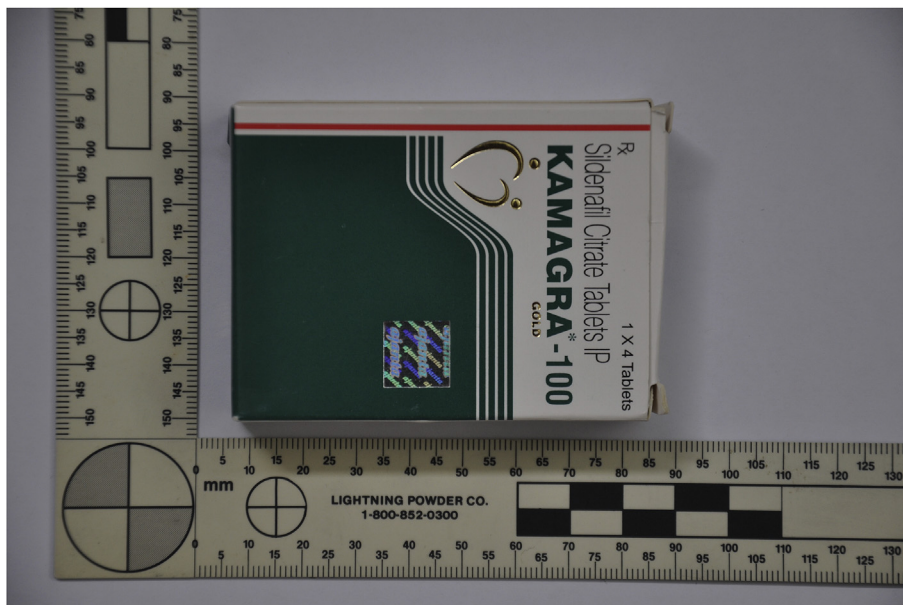


Fig. 1. Package of Kamagra Gold.

was employed during sample analyses to compensate any possible mass axis drifts. The diisodecyl phthalate ion 391.2843, generally present in a laboratory environment, was used as lock mass.

2.4. INAA equipment and conditions

INAA was performed using the standard techniques adopted in the Radiochemistry Laboratory of the Department of General Chemistry of the University of Pavia. Irradiation has been carried out in the Triga Mark II 250 kW research reactor of Pavia University at a thermal neutron flux of about $1 \cdot 10^{12} \text{ n} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$ for 12 h. Induced radioactivity was measured by γ -ray spectrometry using a

HPGe detector coupled to a computer assisted Ortec spectral analysis system. Counting started 3 days after the end of irradiation and was repeated after 6, 12 and 24 days.

2.5. Samples

15 samples of illegal products containing sildenafil sold through the net and confiscated by Carabinieri (see three of the illegal products analysed in Figs. 1–5) were analysed for characterisation, identification and quantification of the active compound [36]. The samples were then analysed by INAA, in order to obtain information on the presence of both toxic elements and trace elements and

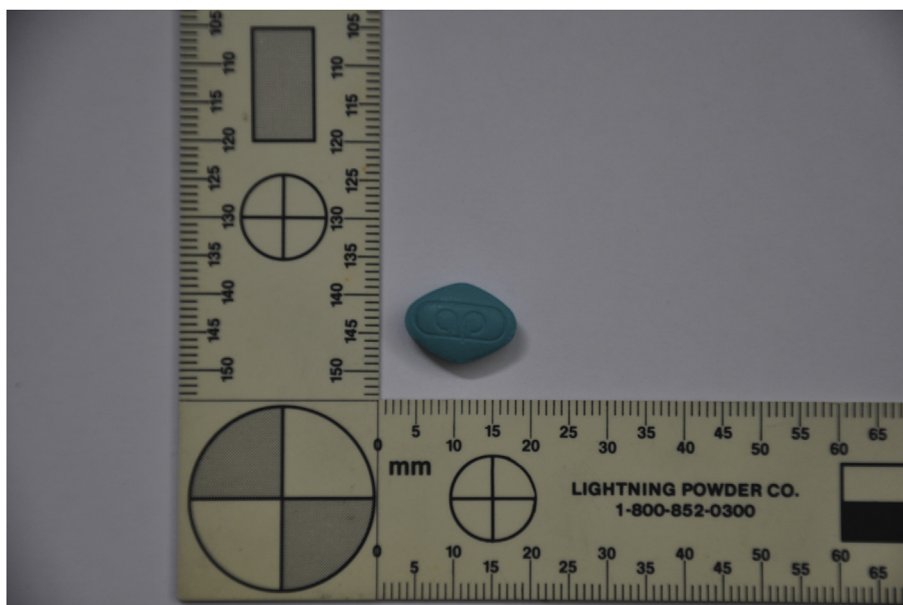


Fig. 2. Kamagra Gold tablet.

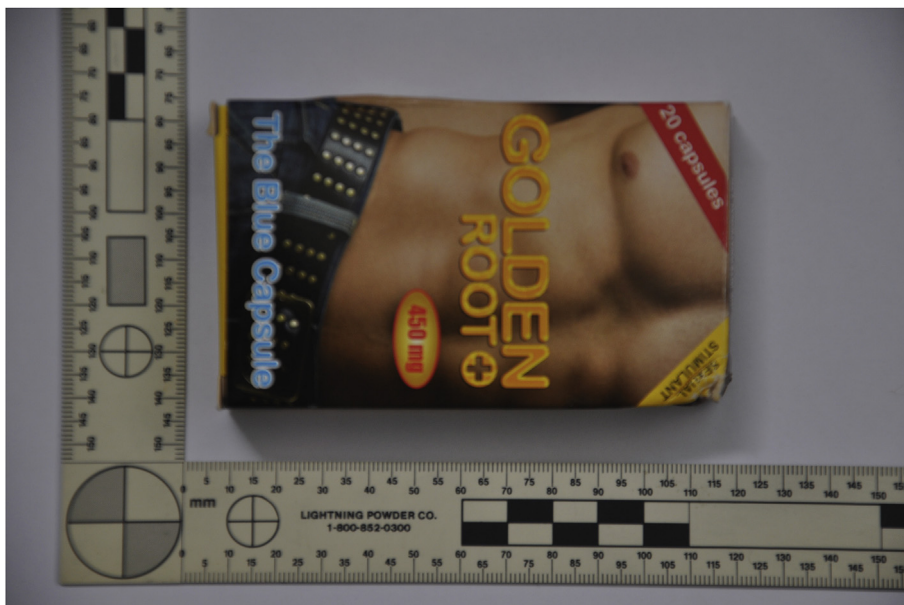


Fig. 3. Package of Golden Root.

to allow inferring about the possible common origin of confiscated material.

Many of the products were confiscated as blisters containing the tablets without boxes or package insert and no indication on the composition of the product, except for the sildenafil amount (generally claimed as 100 mg).

As reference, 10 Viagra[®] 25 mg, 1 sildenafil DOC 25 mg, 1 sildenafil TEVA 100 mg, and powder of pure sildenafil were analysed.

2.6. Sample preparation

For LC-HRMS characterisation, both pharmaceutical products and confiscated samples were grinded and homogenized in a mortar; 10 mg of the obtained powder were dissolved in 10 mL of

methanol under sonication for 10 minutes and centrifuged. 10 μ L of the supernatant were subsequently diluted in 1 mL of methanol and 10 μ L directly injected in the LC-HRMS instrument. For LC-HRMS quantitation of active compound, samples were prepared and analysed as reported elsewhere [36]. For INAA all samples were grinded and homogenized in a mortar. Preliminarily aliquots of 200 mg were submitted to homogeneity tests. Variance analysis, taken as an evaluation of the sample variability due to sampling, provided evidence aliquots of 70 mg or greater are homogeneous and representative of the investigated materials.

3. Results

Sildenafil was identified and quantified in all the illegal products

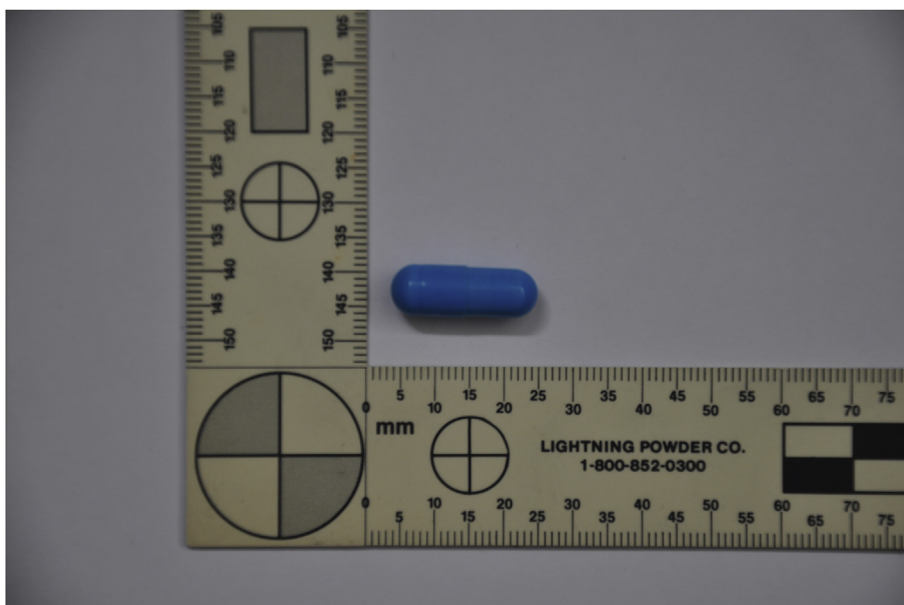


Fig. 4. Golden root capsule.

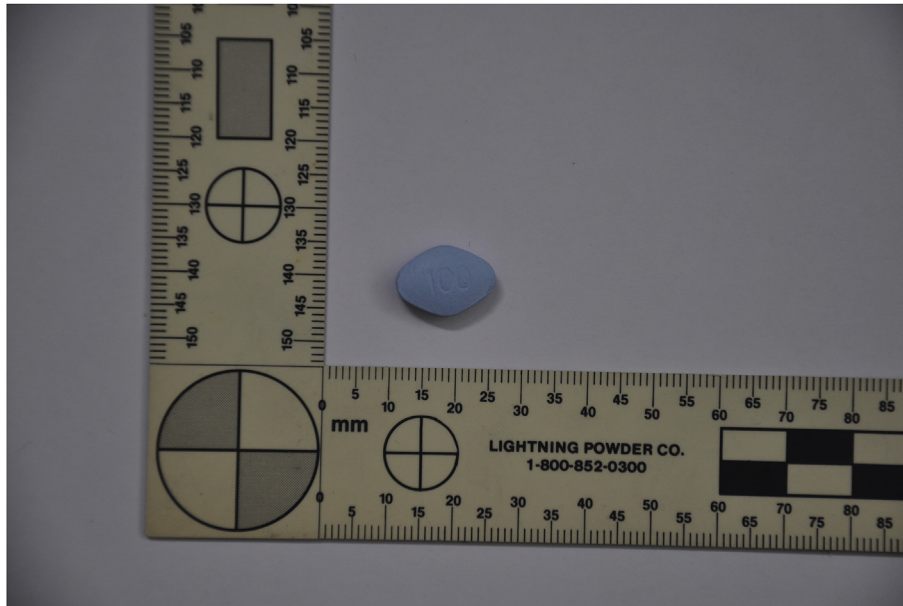


Fig. 5. Cenforce tablet.

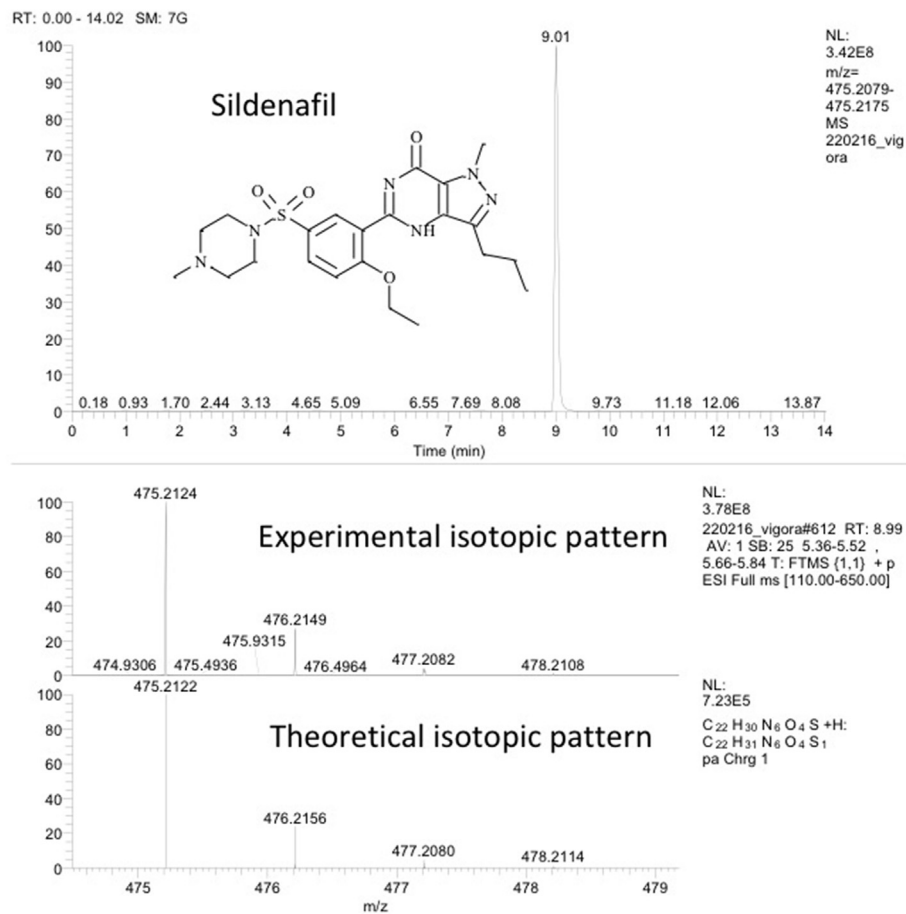


Fig. 6. Extracted ion chromatogram of sildenafil (exact mass $[M+H]^+ = 475.2122$) in an authentic sample (above plot), the related experimental isotopic pattern of sildenafil $[M+H]^+$ ionic species (middle plot) and theoretical isotopic pattern of ionic species with the elemental composition $C_{22}H_{31}N_6O_4S$ (bottom plot).

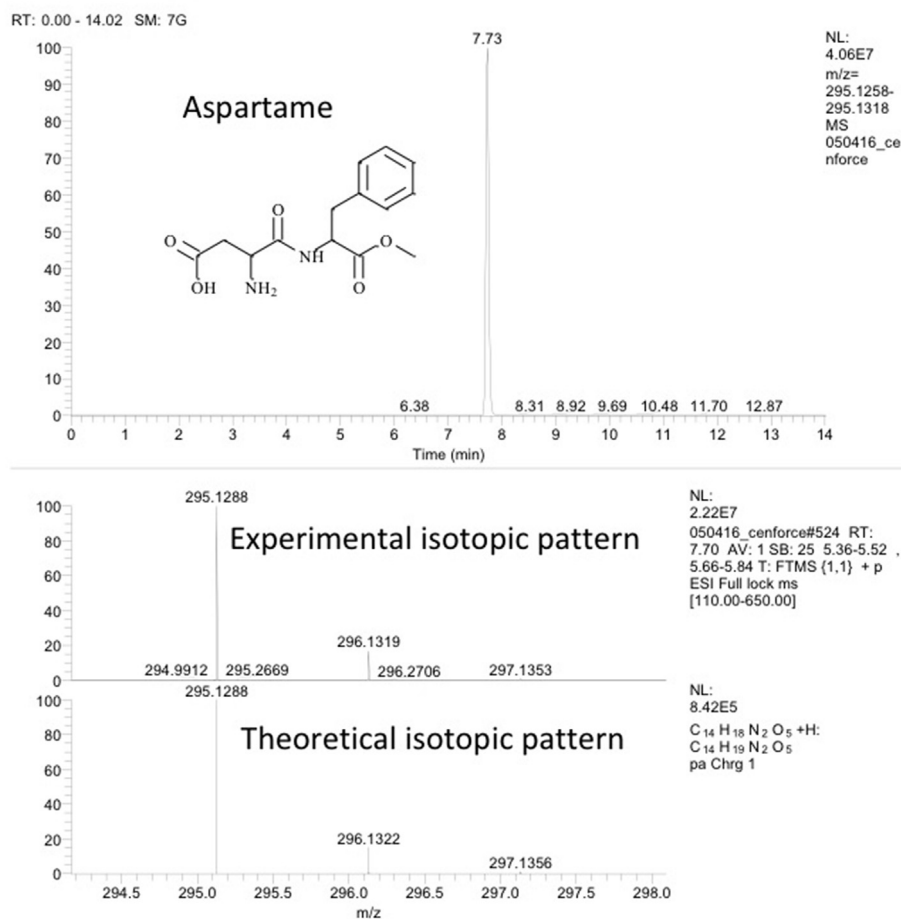


Fig. 7. Extracted ion chromatogram of aspartame (exact mass $[M+H]^+ = 295.1288$) in an authentic sample (above plot), the related experimental isotopic pattern of aspartame $[M+H]^+$ ionic species (middle plot) and theoretical isotopic pattern of ionic species with the elemental composition $C_{14}H_{19}N_2O_5$ (bottom plot).

by LC/HRMS. Fig. 6 shows the HRMS spectrum with the accurate mass of an illegal sildenafil and the theoretical spectrum.

The amount of sildenafil determined in the tablets/capsules was always different from that declared, spanning from 36 to 221 mg per unit. In the product “golden root”, claimed to contain only natural extracts, both sildenafil and thiosildenafil were present. LC/HRMS analyses identified also the chemical substance aspartame (L-aspartil-L-phenylalanine methylester) in 5 samples. Other main impurities found were imidazosagatriazinone (5-(2-Ethoxyphenyl)-1-methyl-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-7(6H)-one) in 6 of the 15 illegal products analysed and 5-chloroimidazosagatriazinone in 11 of the 15 illegal products analysed, as shown in Figs. 7–9. The analytical findings obtained by LC/HRMS are depicted in Table 1.

The minimum detectable amounts (MDA) by INAA for the 23 elements monitored are reported in Table 2. Analytical results from authentic samples from the legal market are reported in Table 3. In the original Viagra® samples INAA allowed to measure concentrations above the MDAs only for 5 elements: Na, Ca, Br, La and Cl. Table 4 shows analytical results from the 15 illegal products seized in Italy by Carabinieri and analysed by INAA determining a set of the eleven elements (Na, Cl, K, Ca, Cr, V, Fe, Co, Zn, Br, La) whose signal was above MDAs values. Cenforce a and b refer to illegal products seized in different times, as Kamagra a, b and c. 15a, 15b, 15c and 15d Sildigra refer to four different pills belonging to the same seizure. In all the illegal samples analysed more than 5

elements were identified and quantified except for Cockfoster, Cenforce b, Kamagra b, Kamagra c, Kamagra oral jelly and Blue. A set of eleven elements (Na, Cl, K, Ca, Cr, V, Fe, Co, Zn, Br, La) was able to show significant differences by comparing authentic Viagra® and illegal products.

4. Discussion

The combination of the two specific analytical techniques allowed the characterisation of illegal medicaments not only by identification and quantitation of the active compound sildenafil but by identification of other chemical substances and elements of interest. One interesting compound identified by LC/HRMS in 5 samples was aspartame. In 2013 the European Food Safety Authority (EFSA) conducted a comprehensive review of the evidence about aspartame and concluded that it was safe for human consumption, including pregnant women and children, but EFSA also reported that the acceptable daily intake recommendations did not apply to people with phenylketonuria (PKU), a rare genetic disorder where the body cannot break down phenylalanine [37]. Only the product “Kamagra gold”, containing aspartame, had its packaging reporting the ingredients, where the presence of aspartame was anyway not reported. Other products had not packaging or package insert, and the blister didn't report the presence of this substance. Another chemical substance found by LC/HRMS in 6 samples was

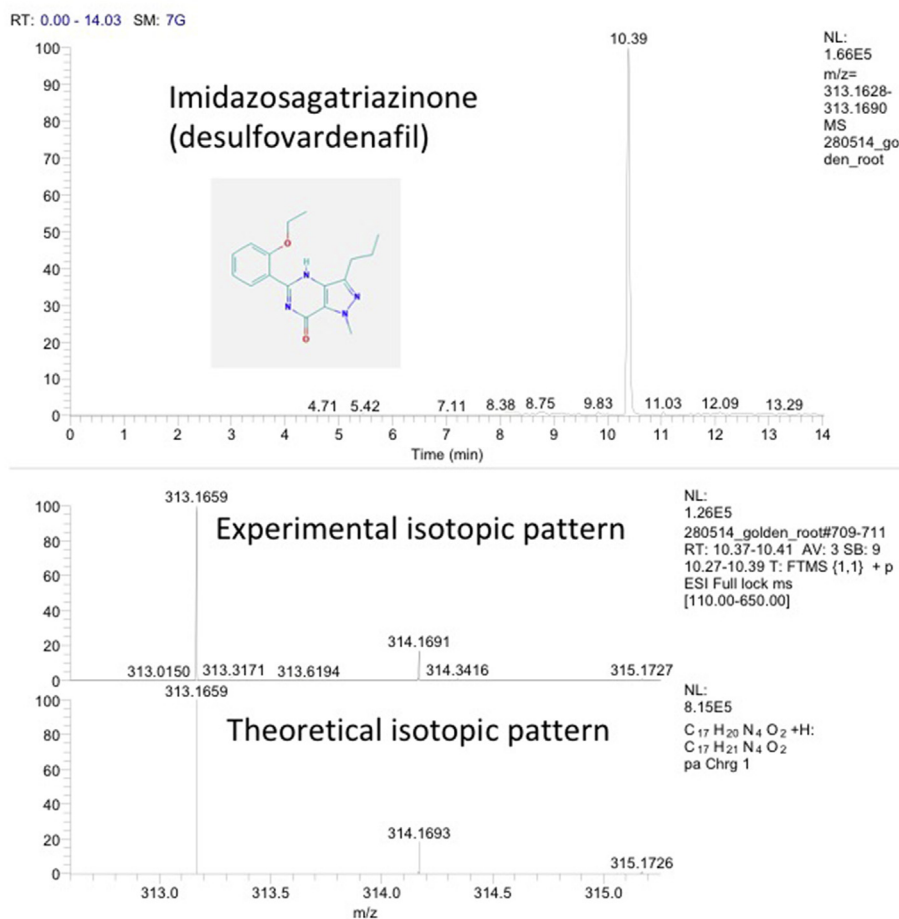


Fig. 8. Extracted ion chromatogram of imidazosagatriazinone (exact mass $[M+H]^+ = 313.1659$) in an authentic sample (above plot), the related experimental isotopic pattern of imidazosagatriazinone $[M+H]^+$ ionic species (middle plot) and theoretical isotopic pattern of ionic species with the elemental composition $C_{17}H_{21}N_4O_2$ (bottom plot).

imidazosagatriazinone, a possible by-product. A Warning Signal for Globally Harmonized System (GHS) Hazard is reported for this substance, as can provoke skin, eye and respiratory tract irritation [38]. A third chemical substance found by LC/HRMS in 11 samples was chloro-imidazosagatriazinone. No toxicity data was found for this substance. It has to be noted that chloro-imidazosagatriazinone was also present in Sildenafil DOC and in sildenafil pure powder, used for galenic preparations. These by-products are probably due to the poor manufacturing processes, in which a poor purification (or no purification at all) is performed after the synthesis of sildenafil. The presence of one or more of these compounds can be hence be presumptive of illegal sildenafil. Another interesting result about the illegal products, resulting in possible danger for health, is the unpredictable amount of active principle present. In fact, it varies from 36 mg, to 220 mg, over twice the declared dose, which was always 100 mg, with inherent health risks for the users. It is interesting to consider that some of the authors already reported sildenafil in illegal products associated with new psychoactive substances (NPS) in Italy [39].

INAA analyses allowed to show that in most cases the illegal products contain more than the 5 elements present in authentic Viagra[®]. Cockfoster contained V and Co, while Br and La were absent. Cenforce b contained K and Cr, while Cl and La were absent. Kamagra c contained V, while La was absent. Kamagra oral jelly contained K, while Ca, Br and La were absent. Blue contained K and

Cr, while Cl, Ca and La were absent. Qualitative differences can be examined by considering the samples 15a, 15b, 15c and 15d Sildigra, referring to four different pills belonging to the same seizure. In Kamagra b the only qualitative feature which differentiate the sample from the authentic Viagra[®] was the absence of La and therefore we examined quantitative values. In this case a *t*-test allowed to demonstrate significant differences ($p < 0,05$) for Cl, Ca and Br compared to authentic Viagra[®].

5. Conclusion

The research based on LC/HRMS and INAA to characterise both authentic and illegal pharmaceuticals containing sildenafil seized in Italy showed that the two techniques effectively provided complementary information. LC/HRMS not only allowed identification and quantitation of sildenafil in all the products but was used to search possible toxic chemical substances and other compounds that could be useful to infer about the origin of the product. Aspartame and two impurities of possible forensic interest were identified. Cheaper analytical methods such as LC/DAD can be used to provide the concentration of the active principle in the tablet but LC/HRMS was preferred to other cheaper analytical approaches because it allowed the identification of unknown molecules based on exact mass.

INAA did not identified elements particularly toxic but always

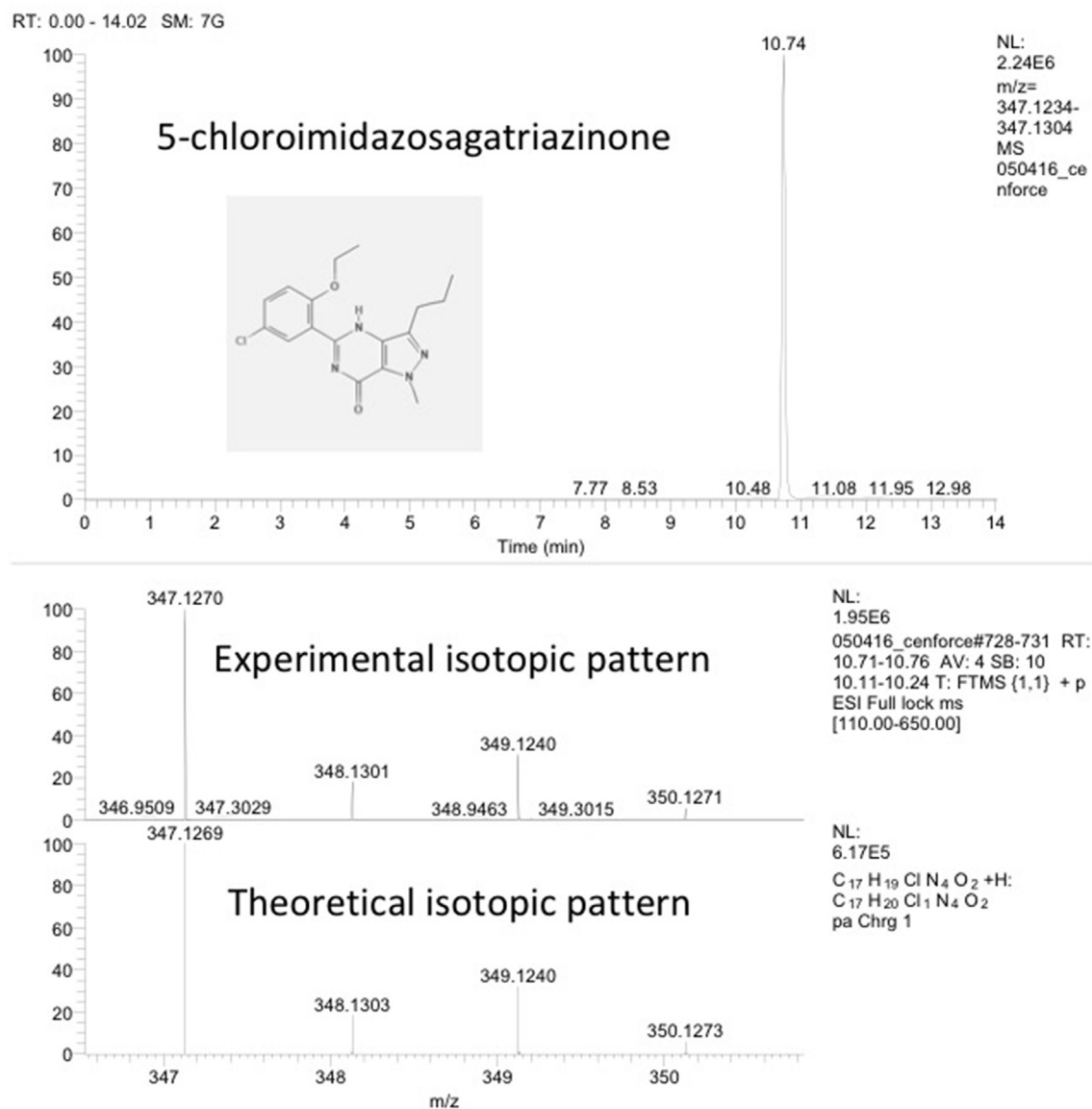


Fig. 9. Extracted ion chromatogram of 5-chloroimidazosagatriazinone (exact mass $[M+H]^+ = 347.1269$) in an authentic sample (above plot), the related experimental isotopic pattern of 5-chloroimidazosagatriazinone $[M+H]^+$ ionic species (middle plot) and theoretical isotopic pattern of ionic species with the elemental composition $C_{17}H_{20}ClN_4O_2$ (bottom plot).

Table 1

Impurities found by LC-HRMS and quantitative results. * Authentic samples.

Name of sample	5-chloroimidazosagatriazinone	imidazosagatriazinone	aspartame	mg sildenafil per unit
1 Aurogra	present	present	present	221
2 Cockfoster	present	present	present	136
3 Cenforce a	present		present	167
4 Cenforce b	present		present	170
5 Golden root	present			10 + thiosildenafil
6 Kamagra a				95
7 Kamagra b	present			80
8 Kamagra c				75
9 Kamagra oral jelly				95
10 Silagra	present	present		140
11 Sildenafil citrate	present			95
15 Sildigra	present	present		60
12 Vigora	present	present		36
13 Blue				40
14 Kamagra gold	present		present	71
*Viagra Pfizer 25 mg	absent	absent	absent	24
*Sildenafil DOC 25 mg	present	absent	absent	24
*Sildenafil TEVA 100 mg	absent	absent	absent	101
*Sildenafil powder	present	absent	absent	

Table 2

Minimum detectable amount (MDA) for the 23 elements monitored. Values are expressed in $\mu\text{g/g}$ if not specified.

	Element	MDA
1	Na	0,005 (%)
2	Cl	50
3	K	0,01 (%)
4	Ca	0,01 (%)
5	Sc	0,05
6	V	0,1
7	Cr	1
8	Fe	250
9	Co	0,5
10	Zn	10
11	Br	1
12	Rb	15
13	Cs	1
14	Ba	200
15	La	0,1
16	Ce	1
17	Sm	0,05
18	Eu	0,2
19	Tb	0,5
20	Yb	2
21	Lu	1
22	Hf	0,2
23	Th	0,2

found significant differences between legal and illegal products on the market.

The presence of unexpected inorganic elements in counterfeit products can be assumed to derive from poor management of the production line and a lack of adequate quality controls. In this case the use of the INAA technique allows a qualitative investigation suitable to identify these problems that afflict the entire matrix and certainly not due to the preparation of the sample.

We consider that the use of such powerful analytical techniques is expensive but the identification of toxic threats is the primary aim of the method we propose. A similar approach has been recently proposed to study amoxicillin drugs in Ghana, using both LC and proton induced X-ray emission (PIXE) [40].

Future studies with more samples are needed to allow inferring about the common origin of confiscated material, possibly with samples having different known geographical origin. The results of our approach demonstrated to allow effective discrimination of illegal samples compared to authentic pharmaceutical products. The profiling approach has been successfully studied to infer about the source of several illegal products, including cocaine, ecstasy pills, hashish, heroin, opium [21,41–44]. The results of this pilot study, based on 23 elements monitored by INAA plus LC/HRMS analysis, is expected to support further development to obtain forensic characterisation of other illegal products, including new psychoactive substances, from different countries.

Table 3

INAA elemental analytical results of authentic Viagra®.

	Na (%)	Ca (%)	Br ($\mu\text{g/g}$)	La ($\mu\text{g/g}$)	Cl ($\mu\text{g/g}$)
Viagra Pfizer 25 A	0.26	1.1	0.40	0.17	94
Viagra Pfizer 25 B	0.27	1.2	0.52	0.18	98
Viagra Pfizer 25 C	0.27	1.4	0.48	0.20	102
Viagra Pfizer 25 D	0.26	1.3	0.38	0.14	90
Viagra Pfizer 25 E	0.27	1.4	0.48	0.21	107
Viagra Pfizer 25 F	0.27	1.3	0.37	0.22	94
Viagra Pfizer 25 G	0.28	1.3	0.41	0.19	100
Viagra Pfizer 25 H	0.28	1.4	0.50	0.20	106
Viagra Pfizer 25 I	0.28	1.1	0.50	0.18	105
Arithmetic mean	0.27	1.3	0.45	0.19	99.6
Standard deviation	0.01	0.1	0.06	0.02	6.0
CV%	2.88	9.4	13.0	12.7	6.0

Table 4

INAA elemental analytical results for illegal samples.

Name of sample	Na (%)	Cl ($\mu\text{g/g}$)	K (%)	Ca (%)	Cr ($\mu\text{g/g}$)	V ($\mu\text{g/g}$)	Fe ($\mu\text{g/g}$)	Co ($\mu\text{g/g}$)	Zn ($\mu\text{g/g}$)	Br ($\mu\text{g/g}$)	La ($\mu\text{g/g}$)
1 Aurogra	0.11	193	–	0.40	–	0.17	310	0.64	–	1.9	–
2 Cockfoster	0.12	810	–	0.30	–	0.29	–	0.52	–	–	–
3 Cenforce a	0.71	565	1.1	–	2.3	1.1	304	–	–	4.8	0.71
4 Cenforce b	0.16	–	0.05	0.20	1.8	–	–	–	–	1.9	–
5 Golden root	0.03	664	0.19	–	1.0	0.63	313	–	–	–	0.33
6 Kamagra a	0.21	1553	–	0.30	2.6	0.24	271	2.0	–	6.5	–
7 Kamagra b	0.29	2908	–	0.30	–	–	–	–	–	4.4	–
8 Kamagra c	0.31	2059	–	0.30	–	0.32	–	–	–	4.8	–
9 Kamagra oral jelly	0.24	2610	0.44	–	–	–	–	–	–	–	–
10 Silagra	0.03	248	–	1.7	1.2	0.22	–	2.1	–	–	–
11 Sildenafil citrate	0.05	270	–	0.40	–	0.45	–	–	5633	–	0.12
12 Vigora	0.09	555	–	2.5	2.7	8.6	528	0.5	–	–	0.26
13 Blue	0.04	–	0.04	–	1.5	–	–	–	–	3.7	–
14 Kamagra gold	0.33	–	–	0.30	–	–	500	0.80	17	5.8	–
15a Sildigra	0.26	366	–	0.50	3.8	0.31	285	0.88	237	5.9	–
15b Sildigra	0.21	307	–	0.50	3.4	–	282	0.57	238	10	–
15c Sildigra	0.27	327	–	0.40	4.6	–	322	1.2	127	8.4	–
15d Sildigra	0.15	190	–	0.20	1.8	0.11	–	1.2	134	5.1	–

Conflict of interest

Author declare no conflict of interest.

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