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Fast LC–MS/MS screening method for the evaluation of drugs, illicit drugs, and other compounds in biological matrices

G.M. Merone [a,$], A. Tartaglia [b,$], S. Rossi [c], F. Santavenere [c], E. Bassotti [d], C. D'Ovidio [e], E. Rosato [b], U. de Grazia [f], M. Locatelli [b,*], P. Del Boccio [b,g], F. Savini [c]

[a] Department of Neuroscience, Imaging and Clinical Sciences, University of Chieti–Pescara "G. d'Annunzio", Chieti 66100, Italy
[b] Department of Pharmacy, University of Chieti–Pescara "G. d'Annunzio", Via dei Vestini 31, Chieti 66100, Italy
[c] Pharmatoxicology Laboratory, Hospital "Santo Spirito", Via Fonte Romana 8, Pescara 65124, Italy
[d] R&D Department Eureka Lab Division, Chiaravalle, Italy
[e] Department of Medicine and Aging Sciences, Section of Legal Medicine, University of Chieti–Pescara "G. d'Annunzio", Chieti 66100, Italy
[f] Fondazione IRCCS Istituto Neurologico Carlo Besta, Laboratory of Neurological Biochemistry and Neuropharmacology, Via Celoria 11, Milan 20133, Italy
[g] Center for Advanced Studies and Technology (CAST), University of Chieti–Pescara "G. d'Annunzio", Via Luigi Polacchi 11, Chieti, Chieti 66100, Italy

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ABSTRACT

Nowadays it is increasingly important from a pharmacological, toxicological, and clinical point of view to have rapid and reliable screening tests available for the analysis of numerous compounds in very short time. Often these procedures involve innovative and *eco–friendly* extraction and purification techniques, but it is necessary to apply preliminary steps such as the protein precipitation (plasma or whole blood) or enzymatic hydrolysis, to obtain a quantitative dosage also of the metabolites (urine). In this work a rapid screening procedure in liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) for the qualitative evaluation of 739 compounds in biological samples (blood, post–mortem blood, and urine) has been reported. The method also considers the deuterated internal standards (d9–methadone and d3–monohydroxycarbazepine) to monitor the performances of the screening (check of the fragmentation process and retention times). The procedure involves two separate analyses in positive and negative ionization and a chromatographic run of 18 min, without modifying the instrumental parameters (except the ionization polarity of the turbospray source). The chromatographic separation was carried out using a Restek Allure PFP Propyl (5 μm, 60Å, 50 × 2.1 mm) column in gradient elution mode. The instrument works in Multiple Reaction Monitoring (MRM) mode on 697 specific transitions for the compounds subject to screening. Furthermore, real samples (human blood and urine) were analyzed to confirm the correct performance of the screening.

1. Introduction

In the last decade, the consumption of illicit substances and above all the widespread diffusion of the so–called New Psychoactive Substances (NPSs) continue to cause concern. On the other hand, the consumption of licit substances, such as alcohol and tobacco, has registered a decrease. These are just some of the results published in the latest report of the European School Survey Project on Alcohol and other Drugs (ESPAD), which has involved more than thirty European countries and about one hundred thousand students, who in 2016 participated in the survey by answering an anonymous questionnaire [1]. This study provided trends (within 49 European countries) on the commonly used substances and moreover, having been conducted for about 20 years, it allowed to observe changes over time. As in previous years, the most widely used illegal drug was cannabis (such as hashish and delta–9–tetrahydrocannabinol), followed by synthetic cannabis (called "spice", which is part of the NPSs), cocaine, stimulants (amphetamines and ecstasy), and hallucinogens (LSD and hallucinogenic mushrooms), whereas heroin is the least common. The production and diffusion of NPSs started decades ago, with the aim of creating pharmacologically active compounds that could mimic the effects of psychotropic drugs (opioids, cannabinoids, psychostimulants, classic hallucinogens, anesthetics and sedatives/hypnotics). The widely diffusion of these NPSs takes place mainly on Internet, through the so–called *dark net*, and those

* Corresponding author.
E-mail address: marcello.locatelli@unich.it (M. Locatelli).
$ These authors contributed equally

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most circulating include tryptamines, synthetic cathinones, synthetic cannabinoids, plant substances, piperazines, phenethylamines, phencyclidine–like substances, aminoindanes and other substances [2]. At the same time, the number of cases of severe and fatal poisonings associated with NPSs consumption has also increased. One of the major limitations still present today in the context of NPSs is related to the fact that appropriate confirmation methods have not yet been developed and validated. This could lead to the negativity of the test even if the subject has taken illicit substances. The competent bodies must therefore always be updated on new substances (on the market or not), on the procedures to be applied, but above all they must have effective, fast and sensitive methods of analysis.

Moreover, the misuse of prescription drugs has become a global health problem in the last few years. Several drugs, such as benzodiazepines (BDZs), are not only used for clinical purposes, but they have reached the *black market* as new psychoactive substances, also due to their easy availability, and are used for recreational purposes [3]. In fact, although BZDs are used by 10–20% of the population for the treatment of anxiety and insomnia, their physical and psychological dependence can lead to abuse, resulting in intoxication or overdose. BDZs, at high doses, can cause loss of consciousness and memory and for these reasons play a significant role in road accidents and in various crimes, including sexual assaults, homicides, and suicide [4]. Another class either of prescription drugs that are misused alone or in combination with alcohol or other drugs of abuse in a variety of toxicological and forensic case are antiepileptic drugs (AEDs). Furthermore, the new generation of AEDs have been recently associated with an increased *off–label* prescription in non–epilepsy disorders, mostly in patients with psychiatric diseases, leading to the increasing risk of self–poisoning and suicide. As a result, several newer AEDs have been classified as controlled substances in the United States [5]. Given the gravity of these crimes, their identification and analysis, especially in the forensic field, is now increasing.

The fast detection and identification of these substances have activated a global analytical challenge, and it is increasingly important from a pharmacological and toxicological point of view to have rapid and reliable screening tests available in order to process many samples in a short time (screening) and in case of positivity, continue with the confirmation analyses. This challenge is particularly important, since detection and screening tools find their application in different contexts, allowing not only the monitoring of drug abuse, but also the analysis of seized products, post–mortem case analyses, etc. Furthermore, the analytical challenge also involves the variety and complexity of the biological matrices to be investigated, with particular attention to non–conventional matrices (hair, oral fluids, dried blood spot), which have gained great interest in recent years. Moreover, the greatest challenge for analytical chemists is also related to the development of fast and robust methods, with minimal sample manipulation to avoid target analytes loss [6, 7]. In this context, several innovative methodologies have been developed such as the molecular imprinted polymer–solid phase extraction (MIP–SPE) [8], fabric phase sorptive extraction (FPSE) [9–14], the Biofluids sampler (or BFS) [15], or even "dilute and shoot" procedures [16]. Additionally, the use of highly hyphenated instrument configurations (such as LC–MS/MS), allowed to meet this need for sensitivity and selectivity [17]. However, it is often necessary to apply preliminary techniques such as protein precipitation (for plasma or whole blood analysis) and the implementation of enzymatic hydrolysis procedures to obtain a quantitative dosage also of the conjugated metabolites (on urine).

The aim of this project, following our research on illicit drugs [18], was the development of a rapid screening procedure in LC–MS/MS for the qualitative evaluation of 739 bioactive compounds in biological samples (blood, post–mortem blood and urine) by applying simple and classic procedures such as enzymatic hydrolysis (for urine samples) and protein precipitation (for blood). In this work particular attention has been paid to the *green* profile of the procedure, mostly in sample preparation steps, without limiting analytical performance. The sample preparation phase results in a *green* phase that minimizes sample handling, reducing waste resulting from this pre-analytical process. For blood and post–mortem blood samples protein precipitation was performed using low volume of MeOH, whereas urine samples have been subjected to enzymatic reaction using only 100 μL of the solution containing the enzyme and once hydrolysed, the sample was diluted with an aqueous solution containing the deuterated internal standards. The novelty of this work is therefore also the totally environmentally friendly procedure, which does not include preliminary phases that use large volumes of organic and toxic solvents, such as the liquid-liquid extraction (LLE) present in other similar works reported in the literature [17].

In this method, deuterated internal standards were used to monitor the correct performance of the screening. The herein reported method has involved two separate analyses in positive and negative ionization, without changing the instrumental parameters (except the ionization polarity of the turbospray source) with an overall time of 18 min. This method represents a powerful tool for the monitoring of high number of compounds thanks to the instrumental configuration and above all to the protocol of acquisition of the instrumental signal. In fact, this protocol envisages proceeding with the conventional analysis and only if the signal exceeds a certain threshold value, then the mass spectrometer automatically begins to monitor the specific MRM transition and the entire fragmentation spectrum to obtain a comparable data with the mass spectra present in the database. Following the "match" process, the correct identification of the compound is then provided following the retention time and the mass fragmentation spectrum (MS/MS).

2. Materials and methods

2.1. Standards and solvents

All the solvents and chemicals were supplied in the kit purchased from Eureka srl Lab Division (code SC9000). The kit also contains the mobile phases for LC–MS/MS analyses (M1: H_2O + 2 mM ammonium formiate + 0.2% formic acid; M2: Acetonitrile + 2 mM ammonium formiate + 0.2% formic acid), and the reagents used for the protein precipitation and enzymatic digestion (with glucuronidase) ready to use. Restek Allure PFPP (5 μm 60Å – 50 × 2.1 mm) column was used in this method. The full list of analytes included in the screening was reported in our previous work [19].

2.2. Sample collection and storage

For the sample collection and storage, the current regulations, and guidelines for investigation on samples taken from living person were observed [20]. Informed consent and ethical approval were not necessary, due the samples were leftover from routine clinical therapeutic drug monitoring (all samples were anonymized). The post–mortem blood samples were collected from autopsies performed following suspicious deaths related to the abuse of certain substances. In particular, the blood was collected by venipuncture from the femoral vein. In this case, the analysis fell within the preliminary requirements authorized by the magistrate. In fact, the magistrate grants the authorization for the collection and analysis aimed at ascertaining the hypothesis of a crime with a specific assignment. The use of these samples is relevant to the subject matter of the judicial investigation and not for further research purposes. Therefore, are not bound by the favourable opinion of the Ethics Committee and/or the consent of family members or legal representatives. The judicial authority with specific assignment authorized post-mortem samples collection and analysis. As such, do not require the approval of protocols by the Ethics Committee and the signature of informed consent.

The biological samples, taken through sterile containers (urine), and count tubes (blood and post–mortem blood), followed the current legal

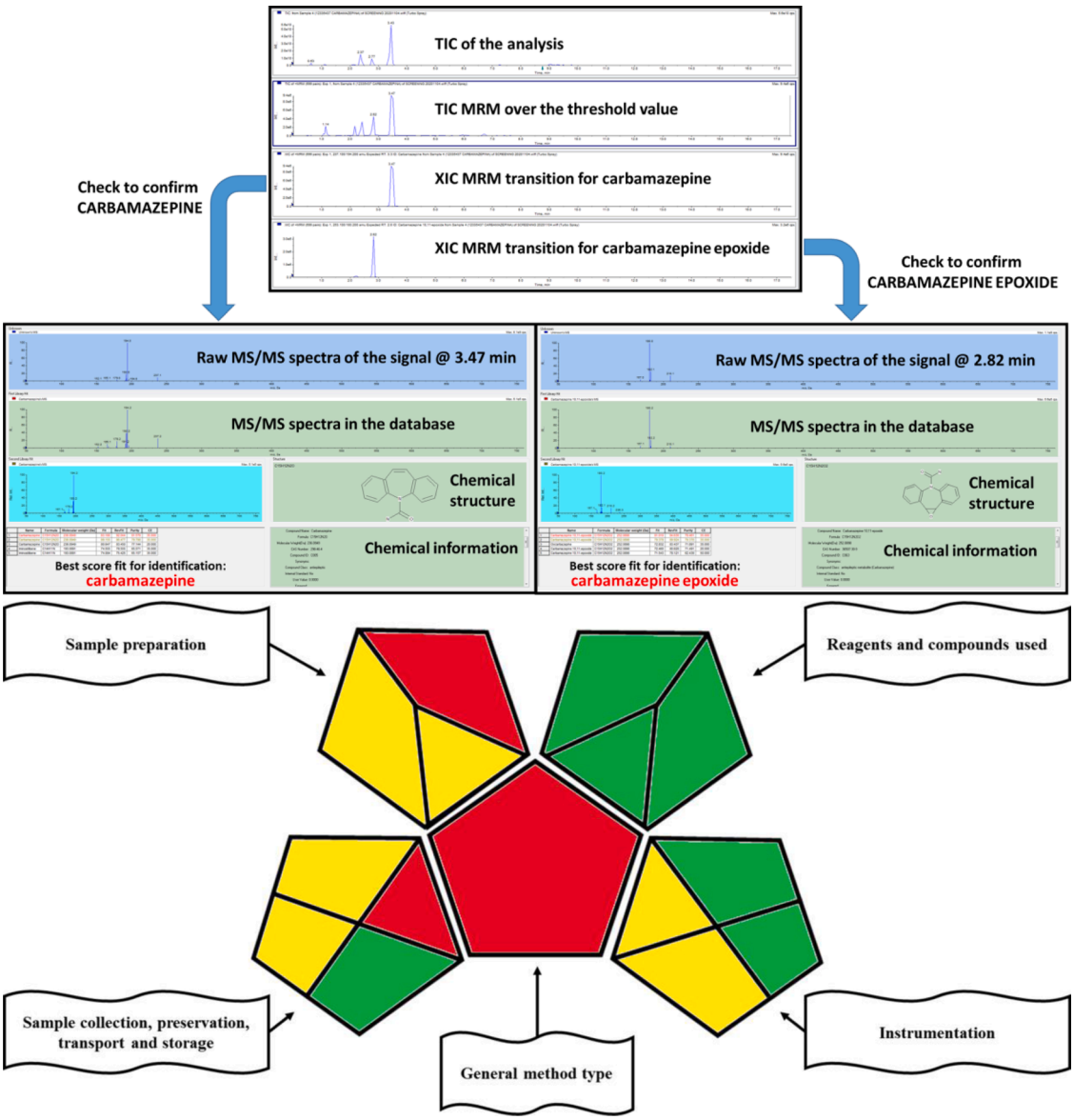

Fig. 1. Analysis on a real sample positive to carbamazepine and carbamazepine epoxide.

supervision procedures, and were stored at –20°C until the analysis.

2.3. Sample preparation

2.3.1. Blood and post–mortem blood samples

For blood and post–mortem blood samples a preliminary step of protein precipitation was performed using MeOH with deuterated internal standards (300 µL). After vortexing, the sample was centrifuged for 10 min at 10.000 × *g*. The supernatant was then diluted with an aqueous solution containing 0.1% formic acid to improve the ionization efficiency (1:1, *v:v*). Once diluted, 30 µL of the sample were injected in LC–MS/MS apparatus.

2.3.2. Urine samples

Urine samples have been subjected to enzymatic reaction, to hydrolyse any metabolites present. The hydrolysis reaction uses 500 µL of urine sample, and 100 µL of the solution containing the enzyme (glucuronidase, ≥100000 U/mL) were added. The obtained solution was then placed in incubation for 3 hours at 60 °C. Once hydrolysed, the sample was diluted with an aqueous solution containing the deuterated internal standards (1:6, *v:v*), and after vortexing, 30 µL have been injected for LC–MS/MS analysis.

2.4. LC–MS/MS instrumentation and parameters

The hyphenated instrumentation (ABSciex API 4500 QTrap plus Shimadzu Nexera X2 LC system composed by SIL–30AC autosampler, LC–30AD pump, and CTO–20AC column oven) were present in the accredited, according to UNI CEI EN ISO/IEC 17025:2018, Pharmatoxicology Laboratory — Hospital "*Santo Spirito*" (ACCREDIA, laboratory n. 2274 ASLPE, accreditation n. 1822L). The LC–MS/MS method optimized for screening analysis results in a rapid elution gradient and a change in flow rate during the chromatographic run. The initial conditions are 90% (M1) and 10% (M2). The chromatographic column, Restek Allure PFPP (5 µm, 60Å, 50 × 2.1 mm), thermostated at 40°C (±1°C) was used [19]. The ion source mass spectrometer parameters were as follow: Turbo Spray with curtain gas at 30, ion spray voltage 5400 V, temperature 550 °C, ion source gas 1 at 55 and ion source gas 2 at 60. Additionally, was used the MRM scan type, with declustering potential set at 60 and entrance potential at 10.

Using these parameters, the signal acquisition mode was then set. In this screening method, and given the high number of compounds, it was

decided to exploit the response potential deriving from the QTrap configuration and management software. Analyses are conducted in MRM (Multiple Reaction Monitoring) mode on 697 specific transitions and only when the signal exceeds the threshold value of 8000 cps (count per second, set according to the signal–to–noise ratio (*S/N*) obtained by injecting a mixture of chemical standards at the cut-off concentration level required by law (especially for illicit drugs, about 300 ng/mL) the "*Enhanced Product Ion*" (or EPI) mode is activated from which an increase in the fragmentation process is obtained and a spectrum of MS/MS that is compared with the library to obtain confirmation of the presence of the compound and its correct identification [19]. It should be noted that the threshold value was also chosen to avoid having the fragmentation of all the molecular ions (value too low) or never get to fragmentation (value too high). The analyses, keeping the instrumental parameters unaltered, were carried out both in positive ionization mode and in negative. All the samples tested positive following this screening method were then subjected to confirmation analysis, to obtain confirmation of the positivity and the quantity of substance present.

3. Results and discussion

3.1. LC-MS/MS screening method

The NPSs phenomenon is still a growing problem over the world. Identifying these compounds in biological samples has become a challenge for both clinical and forensic laboratories. The methods used in these analyses should be fast, simple, sensitive, specific, cheap, and capable of detecting a large number of compounds, belonging to different chemical classes. Screening methods using LC–MS/MS have believed to be the best solution, as they can be easily modified and expanded for new compounds. Rapid sample preparation was also required, so that LC–MS/MS methods can replace immunoassay tests that are still missing for most of NPS classes. In this screening protocol, the procedure applied in the preliminary phase does not differ from other procedures already applied for the determination of glucuronated metabolites (for urinalysis) [21]. Similarly, the procedure applied in the case of blood and post–mortem blood analyses involves a simple step of protein precipitation with an organic solvent (methanol) which already contains the deuterated internal standards.

Sample handling was reduced to a minimum thanks to the instrument configuration used: exploiting the potential of the acquisition applied in MS/MS it was possible to increase the qualitative analytical information, obtaining a series of important advantages. These range from increased selectivity (two MRM transitions, increasing the number of identification points, or IP), to increased sensitivity (EPI fragmentation process involves an accumulation of fragment ions in the last sector of the MS by increasing the signal–to–noise ratio), up to the increase of analytical information (MS/MS spectra acquired automatically are processed using the Information Dependent Acquisition (IDA), the Dynamic Background Subtraction (DBS), and the Dynamic Fill Time (DFT)) [22]. Another important advantage was the immediate comparison of the MS/MS spectra acquired with the database in order to obtain a correct identification of the compound. In particular, the reported screening procedure allowed the verification and identification of 739 compounds (699 compounds in positive ionization mode and 40 in negative mode), thanks to the minimal sample manipulation that avoided losing of analytical information during the overall process. Furthermore, the procedure was simple to apply and has allowed the development of a kit and a method that can be easily transferred to other LC-MS/MS configurations.

3.2. Real samples analysis

In general, the analysis and identification of drugs of abuse both in conventional and unconventional matrices include two different analytical phases: a preliminary screening, which aims to maximize

Table 1
Real samples results and comparison with confirmatory procedure for blood samples (n = 100).

		Proposed LC-MS/MS screening method																					
		Lorazepam			Diazepam			Clonazepam			Clobazam			Lamotrigine			Oxcarbazepine			Carbamazepine			Primidone		
		Positive	Negative	Total	Positive	Negative	Total	Positive	Negative	Total	Positive	Negative	Total	Positive	Negative	Total	Positive	Negative	Total	Positive	Negative	Total	Positive	Negative	Total
Confirmatory LC-MS/MS	Positive	15	0	15	11	0	11	14	0	14	10	0	10	13	0	13	14	0	14	15	0	15	8	0	8
	Negative	0	50	50	0	50	50	0	50	50	0	50	50	0	50	50	0	50	50	0	50	50	0	50	50
	Total	15	50	65	11	50	61	14	50	64	10	50	60	13	50	63	14	50	64	15	50	65	8	50	58

Table 2
Real samples results and comparison with confirmatory procedure for urine samples (n = 100).

		Proposed LC-MS/MS screening method														
		Cocaine			Opiates			Methamphetamine			THC			Amphetamine/MDMA		
		Positive	Negative	Total	Positive	Negative	Total	Positive	Negative	Total	Positive	Negative	Total	Positive	Negative	Total
Confirmatory ILabTauruS	Positive	32	0	32	23	1	24	48	0	48	43	0	43	8	0	8
	Negative	0	30	30	0	30	30	0	30	30	0	30	30	0	30	30
	Total	32	30	62	23	31	54	48	30	78	43	30	73	8	30	38

THC: delta–9–tetrahydrocannabinol; MDMA: Methylenedioxymethamphetamine

Table 3
Real samples results and comparison with confirmatory procedure for post–mortem blood samples (n = 50).

		Proposed LC-MS/MS screening method														
		Cocaine			Opiates			Methamphetamine			THC			Amphetamine/MDMA		
		Positive	Negative	Total	Positive	Negative	Total	Positive	Negative	Total	Positive	Negative	Total	Positive	Negative	Total
Confirmatory LC-MS/MS	Positive	21	0	21	12	0	12	14	0	14	26	0	26	2	0	2
	Negative	0	15	15	0	15	15	0	15	15	0	15	15	0	15	15
	Total	21	15	36	12	15	27	14	15	29	26	15	41	2	15	17

THC: delta–9–tetrahydrocannabinol; MDMA: Methylenedioxymethamphetamine

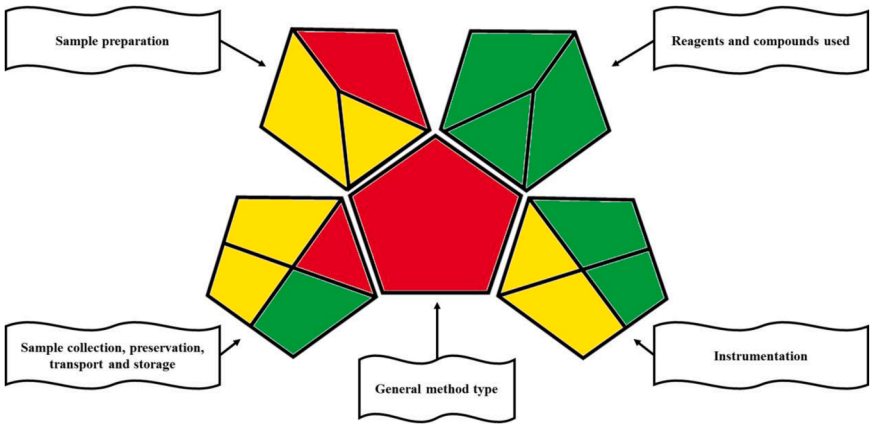

Fig. 2. GAPI pictogram for the herein reported LC–MS/MS screening procedure

diagnostic sensitivity and identify all presumed positives (even at the cost of including "false positives"), and a second confirmation phase which aims to maximize diagnostic specificity and identify, among the supposed positives, the "true positives". This latter analysis is the only one to have a medico–legal value. Thus, the samples were all analysed with the immunoenzymatically screening methods already in use and accredited (ILabTauruS). This screening method was tested by on to 100 blood and urine samples, as well as 50 post–mortem blood samples; all the samples were also analysed with confirmation test, to check the obtained screening results. In **Figure 1** was shown the chromatogram related to real sample positive to carbamazepine and carbamazepine epoxide. In this figure, was reported the Total Ion Current (TIC) of the analysis, the TIC related to all MRM transitions that exceeded the set threshold value of 8000 cps, and the 2 Extracted Ion Chromatogram (XIC) related to the MRM transition of carbamazepine and carbamazepine epoxide. In particular, the screening procedure then foresees to compare the MS/MS spectrum of the sample (raw MS/MS) with the spectrum stored in the database (MS/MS database). Following a matching process, the system provides the chemical structure, information and the best–fit score in order to obtain the correct identification of the compound. In *supplementary materials S.1* were shown other results obtained from real samples that have given outcome of positivity towards other chemicals considered in this work.

From the **Tables 1–3** is possible to see how this procedure was able to meet the needs of a screening method using an LC-MS/MS instrument, allowing chemicals to be identified in an extremely short time (18 minutes per analysis) for a high number of molecules (739). In particular, the comparison gives 100% correct identification of the positives and the type of substance. From the screening analyses herein reported, and subsequently confirmed by the analyses, "polyconsumers" were also identified, i.e. subjects who have used several types of bioactive compounds that this method was able to discriminate and identify correctly.

3.3. Green analytical procedure index (GAPI) evaluation

In recent years, more attention has been paid to the development of procedures with low environmental impact, without limiting analytical performance. In this context, Green Analytical Chemistry (GAC), is inserted through the use of eco-compatible solvents and maximum reduction of waste deriving from the pre-analytical process. In particular, since 2018 a very important evolution was observed for the *green* characterization of the analytical procedures. In fact, up to that date, it was often difficult to justify (or not) whether a method could be *green* or not. Since 2018, Płotka-Wasylka has reported an interesting article on the green analytical procedure index (GAPI) [23], which allows to visually and immediately highlighting how a laboratory procedure impact on environmental. By following the rules of this approach and critically evaluating the individual elements of the entire procedure (solvents, energy consumption of the instrumentation, quantity of waste, etc.), it is possible to develop a pictogram that visually gives an idea of the *green* profile. As shown in **Figure 2**, the procedure reported here shows a quite good *green* profile based on the GAPI index. In the *supplementary materials S.2* has been reported the table (and the pictogram explained) where the "color codes" used and the specifications relating to the method were showed.

Certainly, the *green* profile could be improved through the future development of *on–line* sampling procedures, the possibility of reducing the manipulation of the sample both in the collection and *clean–up* phase (rather than derivatizing, trying to directly insert the relative MRM transitions metabolites present in the urine). In this way, the yellow and red areas relating to sampling and sample preparation could be converted into green, further reducing the environmental impact of the method.

Conclusion

The herein reported method has allowed the indentification of an extremely high number of xenobiotics in a single chromatographic run, by exploiting the potential for signal acquisition given by the hyphenated instrumental configuration. The method was also applicable without particular problems, as it has been shown to have 100% correct identification and qualification of the substance (diagnostic efficiency), following the comparison of the results obtained with the immunoassays tests (already accredited and in use) and the confirmation analyzes by LC–MS/MS.

Another great advantage of the present method was represented by the fact that it can be applied not only on matrices such as blood and urine, but also on cadaveric blood and on other unconventional and alternative matrices (saliva, vitreous humor, keratin matrix, etc.). As previously mentioned, the *green* profile, also characterized by the GAPI pictogram, represents a further strength of the method reported. During the sample preparation phase, the volume of the sample and the solvents used, as well as the handling of the sample were reduced to a minimum, fully adhering to the principles of Green Analytical Chemistry (GAC). The procedure described represents a high-throughput method that allows processing many samples on a very large number of xenobiotics with an absolutely reduced time expenditure.

Supplementary material

Merone et al - Supplementary - TalantaOpen.docx

Declaration of Competing Interest

E.B. is employee at Eureka Lab Division. The other authors declare that does not exist any economic interest or any conflict of interest.

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Supplementary materials

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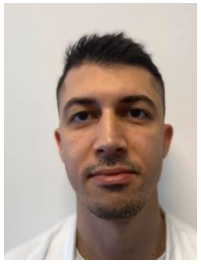
Giuseppe Maria Merone: Pharmatoxicology Laboratory - "*Santo Spirito*" Hospital, Via Fonte Romana 8, Pescara 65124, Italy. Giuseppe Maria Merone is a Medical Doctor at the Pharmacotoxicology and Analytical Quality Laboratory at "*Santo Spirito*" Hospital, Pescara, Italy. In 2021 he obtained PhD in Medical Biotechnology with research thesis titled "Innovative methodological aspects in the determination of xenobiotics through mass spectrometry". His research activity is focus on dosage of different pharmacological classes, toxicological screening, and analysis of drugs of abuse in high performance liquid chromatography coupled with mass spectrometry (LC-MS/MS) in different biological matrices (blood, urine, oral fluid, hair, cadaveric samples) .

Angela Tartaglia: Department of Pharmacy, University of Chieti–Pescara "G. d'Annunzio", Via dei Vestini 31, Chieti 66100, Italy; Angela Tartaglia obtained her master's degree in Pharmacy in 2018 at the "*G. d'Annunzio*" University of Chieti (Italy), with research thesis titled "FPSE-HPLC-PDA method for the quantification of anticancer drugs in human whole blood, plasma and urine". From March 2018 to June 2018 she joined at Aristotle University of Thessaloniki, School of Chemistry, Laboratory of Analytical Chemistry for additional research on microextraction procedures and method validation focused on food products. She is currently holding a PhD in Biomolecular and Pharmaceutical Science, having defended a thesis entitled "Analytical chemistry and health: innovative approaches for the determination of biologically active compounds in complex matrices". Her research activity is focused on the development and validation of chromatographic methods (according to international guidelines, ICH) for the qualitative and quantitative determination of biologically active molecules in complex matrices such as biological fluids, cosmetic formulations, food and environmental samples. Moreover, the research regards the optimization of new protocol for sample preparation, with particular attention on innovative green (micro)extraction, mostly fabric phase sorptive extraction (FPSE) .

Sandra Rossi: Pharmatoxicology Laboratory - Hospital "Santo Spirito", Via Fonte Romana 8, Pescara 65124, Italy. Sandra Rossi is a laboratory technician coordinator at the Pharmacotoxicology and Analytical Quality Laboratory at "Santo Spirito" Hospital, Pescara, Italy Her research activity is focus on dosage of different pharmacological classes, toxicological screening, and analysis of drugs of abuse in high performance liquid chromatography coupled with mass spectrometry (LC-MS/MS) in different biological matrices (blood, urine, oral fluid, hair, cadaveric samples) .



Francesco Santavenere: Pharmatoxicology Laboratory - Hospital "Santo Spirito", Via Fonte Romana 8, Pescara 65124, Italy. Francesco Santavenere is a laboratory technician at the Pharmacotoxicology and Analytical Quality Laboratory at "*Santo Spirito*" Hospital, Pescara, Italy. His research activity is focus on dosage of different pharmacological classes, toxicological screening, and analysis of drugs of abuse in high performance liquid chromatography coupled with mass spectrometry (LC-MS / MS) in different biological matrices (blood, urine, oral fluid, hair, cadaveric samples) .

Elisa Bassotti: R&D Department Eureka Lab Division, Chiaravalle, Italy. Elisa Bassotti is an Analytical Chemist with experience in the development of chromatographic methods with UV / Vis and Fluorescence detectors. In the last few years, her research activity was focused in mass spectrometry coupled with UHPLC, particularly the clinical and pharmacotoxicological field .

Cristian D'Ovidio: Department of Medicine and Aging Sciences, Section of Legal Medicine, University of Chieti–Pescara "G. d'Annunzio", Chieti 66100, Italy; Prof. Cristian D'Ovidio obtained his master's degree in Medicine and Surgery in March 1999 from the University "G. d'Annunzio" of Chieti (Italy). He obtained his Specialization in Forensic Medicine in October 2003. He currently is Associate Professor of forensic medicine at the "G. d'Annunzio" University. He is a member of the "Società Italiana di Medicina Legale e delle Assicurazioni" (S.I.M.L.A.) and was elected to the National Board of the Italian Group of Forensic Pathologists (G.I.P.F.). Prof. D'Ovidio carries out medical-legal research in various sectors of the discipline such as medical professional liability, clinical governance and risk management, forensic dentistry, medical ethics and deontology, criminology and especially forensic pathology and autopsy techniques, also applied to histo-pathological and immunohistochemical sciences. In recent years, he has expanded the application fields of his research lines by participating in research groups in the field of forensic genetics, forensic toxicology, forensic pharmacology and applied analytical chemistry, finally in the study of the phenomena of inflammatory processes. These lines of research are documented by numerous scientific articles and by participation, as speaker, in National and International conferences and congresses .

Enrica Rosato: Department of Medicine and Aging Sciences, Section of Legal Medicine, University of Chieti–Pescara "G. d'Annunzio", Chieti 66100, Italy; Enrica Rosato obtained her master's degree in Medicine and Surgery in October 2012 from the University "G. d'Annunzio" of Chieti (Italy) with thesis in Legal Medicine. From March 2013 enabled to exercise the medical profession with registration in the professional order of doctors of Pescara (Italy). From August 2013 to September 2018 she attended the specialization school in Forensic Medicine at the University of Rome "Tor Vergata" aggregated with the University "G. d'Annunzio" of Chieti-Pescara (Italy). She obtained her Specialization in Forensic Medicine in September 2018 with thesis in Legal Medicine. From June 2019 to July 2019, she participated at the 2019 SUMMER ANATOMY COURSE APPLICATION - DEPARTMENT OF CELL BIOLOGY, New York University School of Medicine. Since December 2020 to now, she is a PhD candidate at University "G. d'Annunzio" of Chieti (Italy), Department of Pharmacy Section of Analytical and Bioanalytical Chemistry. Her research activities are focused on the study of Post-mortem Interval with different application. In particular, she is focused on the development and validation of chromatographic methods (according to international guidelines, ICH) for the qualitative and quantitative determination of biologically active molecules in complex matrices obtained from cadavers such as biological fluid (eg serum, plasma, urine, whole blood, cerebrospinal fluid, bile, gastric content, vitreous humor), keratinized matrices (eg hair) and organ samples prelevated during autopsies .

Ugo de Grazia: Fondazione IRCCS Istituto Neurologico Carlo Besta, Laboratory of Neurological Biochemistry and Neuropharmacology, Via Celoria 11, 20133 Milan, Italy. Senior Researcher in the Laboratory of Neurological Biochemistry and Neuropharmacology, Fondazione IRCCS Istituto Neurologico Carlo Besta, Milan, Italy. His main area of research is therapeutic drug monitoring with particular interest in anticonvulsant medicines and benzodiazepines. In addition, in collaboration with other groups, I conduct research in the field of drugs of abuse and forensic pharmacology. His main area of expertise is chromatography with particular reference to LC-MS/MS. I also have a background in molecular biology .

Marcello Locatelli: Department of Pharmacy, University of Chieti–Pescara "G. d'Annunzio", Via dei Vestini 31, Chieti 66100, Italy; Prof. Marcello Locatelli earned his degree in Chemistry from the University of Bologna, Department of Chemistry "G. Ciamician" with a thesis on the "Development of an analytical methodology for the analysis and identification of protein adducts by mass spectrometry". Earned his PhD from the University of Bologna, Department of Chemistry "G. Ciamician" with a thesis on the "Combined analytical methods of mass spectrometry for the study of impurities in drugs and metabolites of biomolecules". Actually, he is Associate Professor in Analytical Chemistry at the University "G. d'Annunzio" of Chieti-Pescara, Department of Pharmacy. The research activity is devoted to the development and validation of chromatographic methods for the qualitative and quantitative determination of biologically active molecules in complex matrices from human and animal, cosmetics, foods, and environmental. It provides, to the study of all processes related to pre-analytical stages such as sampling, extraction and purification, separation, enrichment, even the application of conventional and hyphenated analytical methods for the accurate, sensitive and selective determination of biologically active molecules. Recently, particular attention is focused on innovative (micro)extraction procedures like MEPS, FPSE, MIP, DLLME, and SULLE. In the development of the method are tested also predictive models and chemometric both for the optimization of extraction protocols and for final data processing. Particular attention is given to the new instrument configurations for the quantitative analysis in complex matrix .

Fabio Savini: Pharmatoxicology Laboratory - Hospital "Santo Spirito", Via Fonte Romana 8, Pescara 65124, Italy. Dr. Fabio Savini is currently the Head of the Pharmacotoxicology and Analytical Quality Laboratory at "Santo Spirito" Hospital, Pescara, Italy. In 2010 he obtained PhD in Biomedical and Cytomorphological Sciences. His scientific experience has been mostly focused on dosage of different pharmacological classes, toxicological screening, and analysis of drugs of abuse in high performance liquid chromatography coupled with mass spectrometry (LC-MS/MS) in different biological matrices (blood, urine, oral fluid, hair, cadaveric samples) .

Piero Del Boccio: Department of Pharmacy, University of Chieti–Pescara "G. d'Annunzio", Via dei Vestini 31, Chieti 66100, Italy; Center for Advanced Studies and Technology (CAST), University of Chieti–Pescara "G. d'Annunzio", Via Luigi Polacchi 11, Chieti 66100 Chieti, Italy. Prof. Piero Del Boccio is currently the Head Researcher of the Proteomics and Metabolomics core Facility at Centre of Advanced Study and Technology (CAST) University "G. D'Annunzio", Chieti, Italy (https://www.cast.unich.it/facilities/proteomics-and-metabolomics). His scientific experience has been mostly focused on the identification and characterization of novel molecular biomarkers in biological fluids and in purified extracellular vesicles in different field of biomedical research, through mass spectrometry-based "omics" approaches .