

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

--Manuscript Draft--

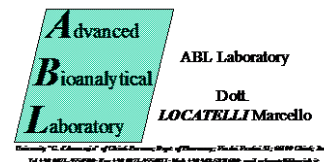
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Abstract:	Nowadays it is increasingly important from a pharmacological, toxicological, and clinical point of view to have rapid and reliable screening tests available for the analysis of numerous compounds in very short time. Often these procedures 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

	<p>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 x 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.</p>
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Dear Editor

please find enclosed the revised manuscript “**Fast LC–MS/MS screening method for the evaluation of drugs, illicit drugs, and other compounds in biological matrices**”, submitted to **Talanta Open**, as full article paper.

We thank the Reviewers for Their evaluations and for the suggestions that are all accepted and reported in the revised version. Below are reported our point-by-point response to Reviewers comments.

The revised version of the manuscript has been read and approved by all authors, who declare no conflict of interest. This research has not been disclosed or published and is not under consideration for publication elsewhere.

Sincerely,

Marcello Locatelli, Ph.D.

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Reviewer #1

The manuscript titled “Fast LC–MS/MS screening method for the evaluation of drugs, illicit drugs, and other compounds in biological matrices” presented by G.M. Merone and co-workers have reported a very interesting paper dealing with the analysis of illicit drugs and other compounds in biological matrices. The paper is clearly presented and well organized. The subject is interesting; however, minor revisions are needed to improve the quality of the manuscript before its publication in Talanta Open.

Dear Reviewer, thank you for your evaluation of the herein submitted paper.

- Line 37: Please, replace “precipitation of proteins” with “protein precipitation”.
We thank the reviewer for His/Her suggestion. The correction has been reported in the revised version.
- Line 49: Please, rephrase “Furthermore, real blood and urine samples were tested to confirm the correct performance of the screening” with “Furthermore, real samples (human blood and urine) were analyzed to confirm the correct performance of the screening”.
We thank the reviewer for His/Her suggestion. The sentence has been corrected in the revised version, as kindly suggested.
- Line 79-82: This sentence should be rephrased as “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”.
We thank the reviewer for His/Her suggestion. The sentence has been rephrased in the revised version, as kindly suggested.
- Line 108-109: Please remove “for their drug detection window, less invasive sample collection and reduced adulteration”.
We thank the reviewer for His/Her suggestion. The sentence has been removed in the revised version, as kindly suggested.
- Line 151: The post–mortem blood samples was collected from the femoral vein? Please, specify.
We thank the reviewer for His/Her observation. As correctly supposed, the blood collection has been carried out by venipuncture from the femoral vein. This information has been reported in the revised version.
- Line 169: Please, replace the sentence “Blood and post–mortem blood samples require a preliminary step of protein precipitation using a methanol solution containing deuterated internal standards...” with “For blood and post–mortem blood samples a preliminary step of protein precipitation was performed using MeOH with deuterated internal standards...”

We thank the reviewer for His/Her suggestion. The correction has been reported in the revised version.

Reviewer #2

The manuscript entitled "Fast LC-MS/MS screening method for the evaluation of drugs, illicit drugs, and other compounds in biological matrices" is an interesting work but not suitable for publication. The main problem is the lack of novelty. The very interesting experimental data of the manuscript have already been published by the authors previously (M. Locatelli, A. Tartaglia, H.I. Ulusoy, S. Ulusoy, F. Savini, S. Rossi, F. Santavenere, 405 G.M. Merone, E. Bassotti, C. D'Ovidio, E. Rosato, K.G. Furton, A. Kabir. *Fabric-Phase 406 Sorptive Membrane Array As a Noninvasive In Vivo Sampling Device For Human 407 Exposure To Different Compounds*. *Anal. Chem.* 93 (2021) 1957-1961. 408 <https://dx.doi.org/10.1021/acs.analchem.0c04663>).

In addition, a similar work has also already been published (Detection and identification of 700 drugs by multi-target screening with a 3200 Q TRAP® LC-MS/MS system and library searching, S. Dresen & N. Ferreirós & H. Gnann & R. Zimmermann & W. Weinmann, *Anal Bioanal Chem* (2010) 396:2425-2434 DOI 10.1007/s00216-010-3485-2).

Dear Reviewer, thank you for your evaluation of the herein submitted paper. However, the mentioned work "Fabric-Phase Sorptive Membrane Array as a Noninvasive In Vivo Sampling Device For Human Exposure To Different Compounds" published by Locatelli and co-workers in Analytical Chemistry in 2021 investigated the in vivo sampling of exhaled breath aerosol (EBA), and no biological matrices were involved in the previously study. Furthermore, the work focused on the in vivo application of an innovative extraction procedure rather than on the analysis of post-mortem biological samples, as reported in the submitted work.

Regarding the work published by Dresen and co-workers in 2010, "Detection and identification of 700 drugs by multi-target screening with a 3200 Q TRAP® LC-MS/MS system and library searching" (mentioned in the revised version of the manuscript and added as reference), in our work more attention has been paid to the "green" profile, without limiting analytical performance (the Green Analytical Procedure Index was included in the work). In this context, the sample preparation procedure reported in our study results greener and the reduction of waste deriving from the pre-analytical process was achieved. For blood and post-mortem blood samples preparation only a preliminary step of protein precipitation was performed using very low volume of MeOH. 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. In the work reported by Dresen and collaborators, the sample preparation phase involved liquid-liquid extraction, which is nowadays considered one of the least environmentally friendly sample preparation techniques as it uses large volumes of organic and toxic solvents. In fact, 1.5 mL of 1-chlorobutane were involved in extraction phase, highlighting the not quite green profile of the previous procedure reported.

In addition, in our submitted work the screening method was applied on to 100 blood and urine samples, as well as 50 post-mortem blood samples; in the work reported by Dresen and co-workers the authors have reported that "The method was applied to serum and urine samples of forensic case...". Among all the biofluids, whole blood is still nowadays one of the preferential and most informative biological fluids for both clinical and forensic investigations. However, analysis of serum or plasma could lead to loss of analytes in sample pretreatment steps, and consequently loss of important analytical information. For this reason, in recent years modern procedures have focused on whole blood analysis.

The submitted work presents another strong point: the method has involved two separate analyses in positive and negative ionization, without changing the instrumental parameters (polarity switching of the turbospray source) with an overall time of 18 min. The work reported by Dresen and collaborators works exclusively use positive ionization mode and for the analysis of compounds that can only be ionized in negative mode, a separate method should be used.

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

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

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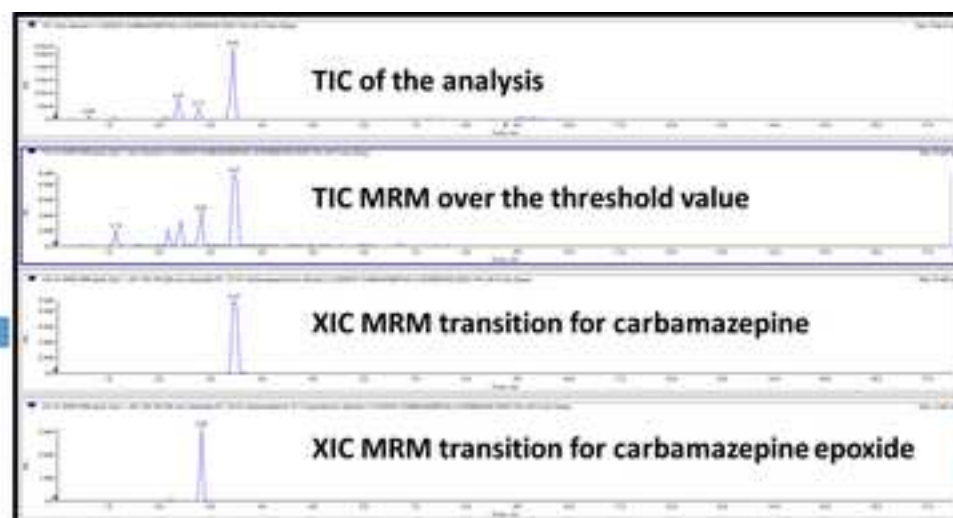
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Check to confirm
CARBAMAZEPINE



Check to confirm
CARBAMAZEPINE EPOXIDE

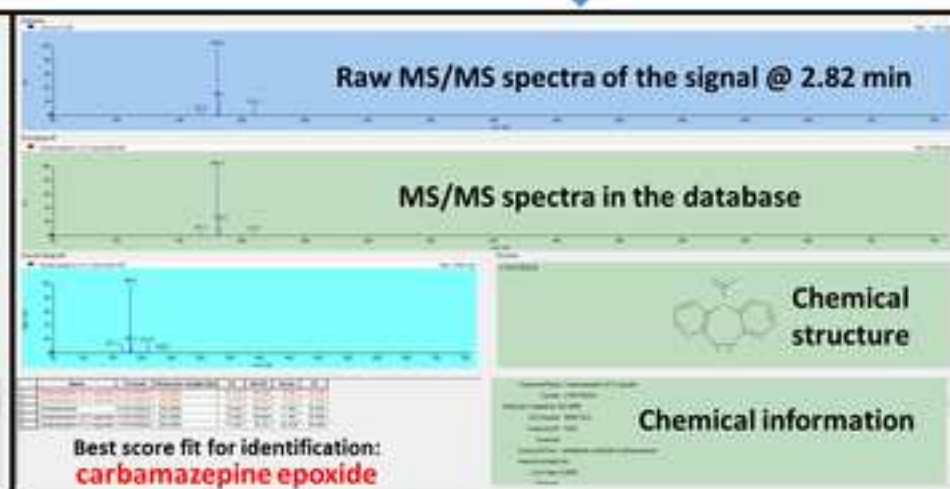
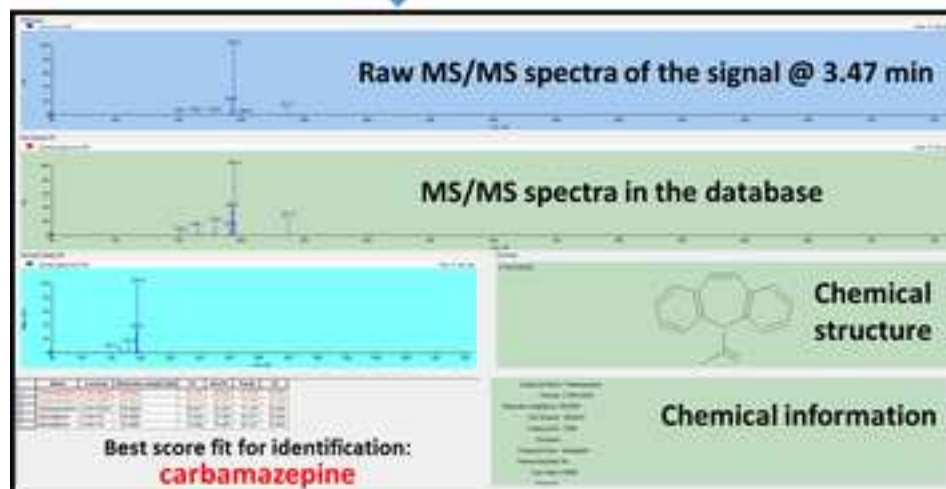


Figure 2

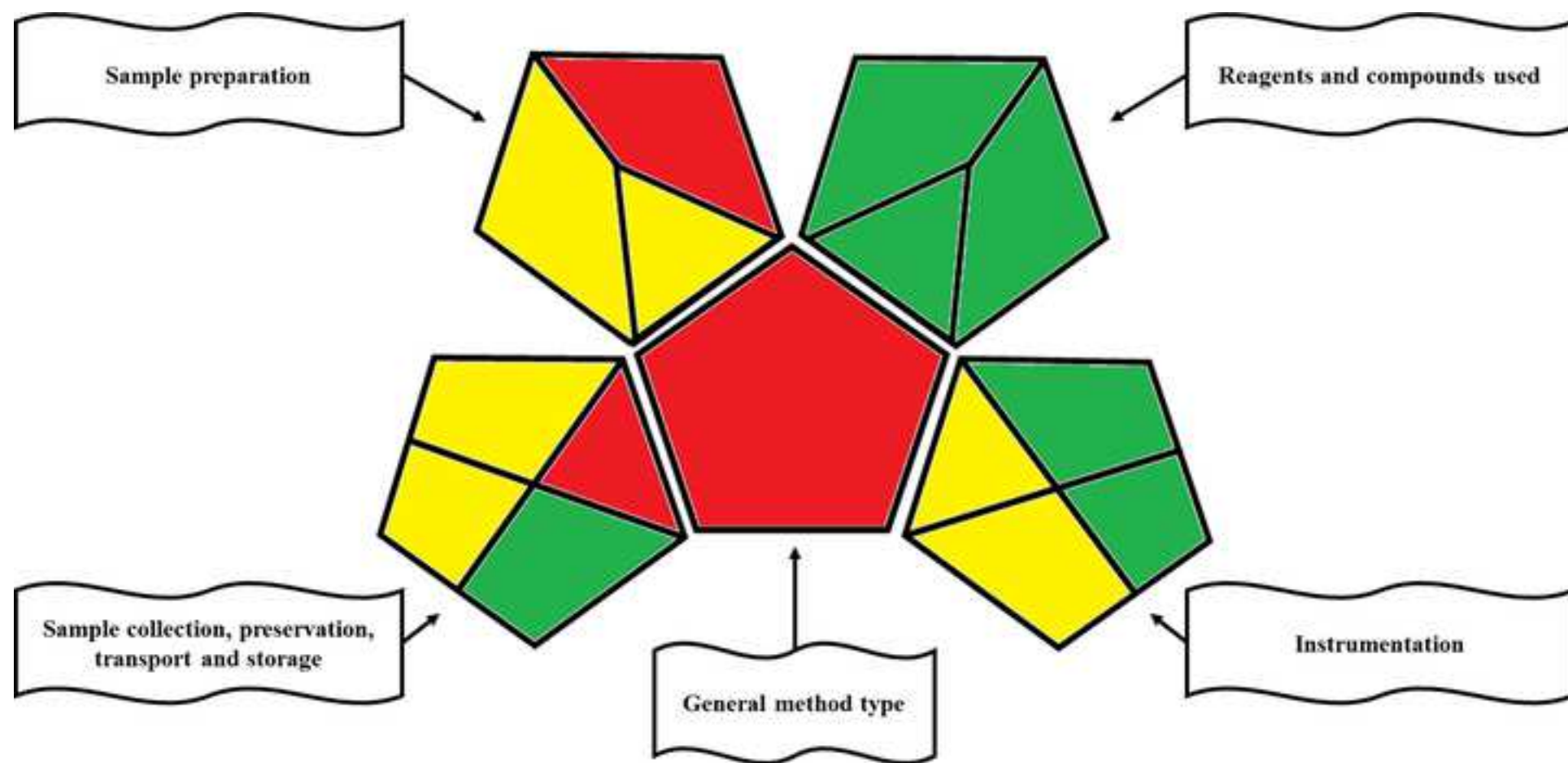


Table 1. Real samples results and comparison with confirmatory procedure for blood samples (n = 100).

		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

1. Fast LC–MS/MS screening method for compounds in biological matrices
2. Blood, urine, and post mortem blood analyses
3. Bioactive compounds, drugs and illicit drugs fast screening analyses
4. Innovative configurations to maximize the instrumental performances
5. Green Analytical Procedure Index (GAPI) evaluation





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Supplementary Material

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

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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 μ m, 60Å, 50 x 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.

Keywords: LC–MS/MS; illicit drugs; toxicological/forensic application; post–mortem analysis; screening method; biological matrices.

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 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 very low volume of MeOH, whereas urine samples have been subjected to enzymatic reaction using only 100 μ L 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 pre-preparation 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: H₂O + 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 μ m 60Å – 50 x 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 supervision procedures and were then 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 $10.000 \times g$. 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 thus obtained was 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) was 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 has 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, Pharmacotoxicology 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, were 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 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, by way of example, 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 large quantity 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.

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

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

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

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

3.3 Green Analytical Procedure Index (GAPI) evaluation

In recent years, more attention has been paid to the development of procedures that can have an environmental impact as low as possible, without limiting analytical performance. In this context we speak of *green analytical chemistry*, of eco-friendly solvents, of reduction of waste deriving from the pre-analytical process. In particular, it is in 2018 that a very important evolution is observed for the characterization of the procedures in this sense. In fact, up to that date, it was often difficult to justify (or not) whether a method could be *green* or not. Since 2018, Plotka-Wasyłka has reported an interesting article on the green analytical procedure index (GAPI) [23], which allows to visually and immediately highlighting how much a laboratory procedure can be with reduced environmental impact. 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 how much the device/procedure can or it can't be green. 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.

Figure 2. GAPI pictogram for the herein reported LC-MS/MS screening procedure.

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.

4. Conclusion

The herein reported method allows identifying 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.

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Conflict of 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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Figure and Table captions:

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

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

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

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

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

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

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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 ~~of proteins~~ (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 x 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 ~~samples~~) were ~~tested~~analyzed to confirm the correct performance of the screening.

Keywords: LC–MS/MS; illicit drugs; toxicological/forensic application; post–mortem analysis; screening method; biological matrices.

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 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. ~~This implies that the~~ The competent bodies ~~involved in the control on the territory~~ 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 ~~tools available, 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 ~~for their drug detection window, less invasive sample collection and reduced adulteration.~~ 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 [\[17,18\]](#), 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

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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 very 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 pre-preparation 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: H₂O + 2 mM ammonium formate + 0.2% formic acid; M2: Acetonitrile + 2 mM ammonium formate + 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 x 2.1 mm) column was used in this method. The full list of analytes included in the screening was reported in our previous work [~~48~~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 [1920]. 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 supervision procedures and were then stored at -20°C until the analysis.

2.3 Sample preparation

2.3.1 Blood and post-mortem blood samples

~~Blood~~For blood and post-mortem blood samples (~~100 μL~~) require a preliminary step of protein precipitation was performed using ~~a methanol solution containing~~MeOH with deuterated internal standards (300 μL). After vortexing, the sample was centrifuged for 10 min at $10.000 \times g$. 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 thus obtained was 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, $\geq 100000 \text{ U/mL}$) was added. The obtained solution was

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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 has 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 [4819]. 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, were 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 [4819]. 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 been 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) [2021]. 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)) [2122]. 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 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, by way of example, 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 large quantity 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.

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

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

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

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

3.3 Green Analytical Procedure Index (GAPI) evaluation

In recent years, more attention has been paid to the development of procedures that can have an environmental impact as low as possible, without limiting analytical performance. In this context we speak of *green analytical chemistry*, of eco-friendly solvents, of reduction of waste deriving from the pre-analytical process. In particular, it is in 2018 that a very important evolution is observed for the characterization of the procedures in this sense. 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-Wasyłka has reported an interesting article on the green analytical procedure index (GAPI) [2223], which allows to visually and immediately highlighting how much a laboratory procedure can be with reduced environmental impact. 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 how much the device/procedure can or it can't be green. 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.

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

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.

4. Conclusion

The herein reported method allows identifying 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.).

~~Another important element, following its characterization using~~ As previously mentioned, the green profile, also characterized by the GAPI pictogram, ~~the procedure proposed here turned out to represent 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 be quite green; a minimum, fully~~ adhering to the principles of Green Analytical Chemistry (GAC), ~~as well as being~~. The procedure described represents a high-throughput method that allows ~~you to process~~ processing many samples on a very ~~high~~ large number of xenobiotics ~~and with an absolutely reduced time expenditure of time.~~

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Conflict of 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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Figure and Table captions:




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

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

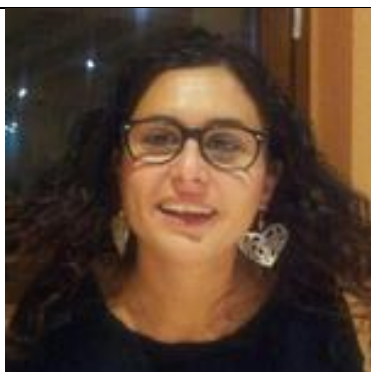
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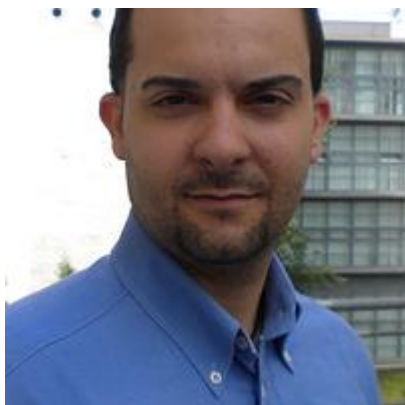
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(<https://www.cast.unich.it/facilities/proteomics-and-metabolomics>).

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