

# Optimizing screening cutoffs for drugs of abuse in hair using immunoassay for forensic applications

Arianna Giorgetti<sup>1,C,D</sup>, Jennifer P. Pascali<sup>1,E</sup>, Guido Pelletti<sup>1,A,F</sup>,  
Marco Garagnani<sup>1,A</sup>, Raffaella Roffi<sup>1,C</sup>, Marialuisa Grech<sup>2,E</sup>, Paolo Fais<sup>1,E</sup>

<sup>1</sup> Department of Medical and Surgical Sciences, Unit of Legal Medicine, University of Bologna, Italy

<sup>2</sup> Azienda Unità Sanitaria Locale di Bologna, Italy

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;  
D – writing the article; E – critical revision of the article; F – final approval of the article

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## Address for correspondence

Guido Pelletti  
E-mail: guidopelletti@gmail.com

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## Abstract

**Background.** In forensic toxicology, positive immunoassay (IA) test results do not hold forensic validity and need to be confirmed with mass spectrometry (MS). On the other hand, a negative result is a strong indication that the drug and/or the drug metabolites are not present in the sample and that confirmatory analyses are not necessary. Consequently, a negative IA result must have forensic validity since it can be admitted in court during a trial.

**Objectives.** Screening cutoffs for the analysis of hair samples using immunoassays (IAs) were retrospectively optimized based on the Society of Hair Testing (SoHT) confirmation cutoffs and the utility of the test for forensic applications was discussed.

**Materials and methods.** Hair samples taken from 150 patients with a history of drug addiction were analyzed with ILab 650, Werfen (Milan, Italy) using DRI<sup>®</sup> reagents. Confirmatory analyses were subsequently performed using the ACQUITY UPLC<sup>®</sup> System, Waters Corporation (Milford, USA). Screening cutoffs were retrospectively optimized using receiver operating characteristic (ROC) analysis.

**Results.** A total of 162 single positive results were obtained for confirmatory analysis (10 for amphetamines/methamphetamines, 11 for MDMA, 37 for cocaine, 40 for THC, 33 for methadone, and 31 for opiates). The optimized screening cutoffs were 0.27 IA ng/mg for amphetamines, 0.51 IA ng/mg for MDMA, 0.59 IA ng/mg for cocaine, 0.14 IA ng/mg for cannabinoids, 0.63 IA ng/mg for methadone, and 0.26 IA ng/mg for opiates. An area under the curve (AUC) greater than 0.95 was obtained with very high sensitivity and specificity for all drugs.

**Conclusions.** The presented screening method proved to be a useful technique on hair samples for the classes of drugs most commonly found in Italy and Europe and can be applied to forensic analysis.

**Key words:** forensic sciences, forensic toxicology, immunoassay

## Cite as

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## Background

The analysis of hair matrix is an elaborate and time-consuming multi-step process. The first step involves a wash to eliminate external contamination, followed by hair segmentation or pulverization and extraction (liquid/liquid, solid phase, solid phase micro-extraction). In most laboratories, the instrumental analysis is performed with immunoassay (IA) screening followed by chromatography (gas, liquid) coupled with mass spectrometry (MS).<sup>1</sup>

Immunoassays were the first technique used to detect drug use in hair and, since then, IA techniques have been widely used in forensic toxicology as screening tests.<sup>2,3</sup> However, positive results from an IA test do not hold forensic validity due to its risk of improper interpretation of test results and should be confirmed using a MS-based technique.<sup>4,5</sup> On the other hand, a negative result strongly suggests that neither the drug nor its metabolites are detected in the sample, making further confirmation analysis unnecessary.<sup>6</sup> Therefore, a negative result, since it is not followed by confirmation analysis, is often presented in court during a trial and can hold forensic validity.<sup>5</sup> To achieve this result, the cutoff used to distinguish negative and positive screening samples needs to be optimized to avoid false negative (FN) values (i.e., sensitivity close to 100%).<sup>7</sup>

Most IA implementations are tailored for clinical contexts involving urine specimens, where concentrations and thresholds tend to be higher compared to levels in hair and blood samples. Consequently, the screening analysis of hair samples might lead to the omission of substances of forensic significance, and screening cutoffs must be tested and optimized concerning the cutoffs used for confirmation, which can vary according to the purpose of the analysis.<sup>8,9</sup> In forensics, reference cutoffs are those reported by the Society of Hair Testing (SoHT),<sup>10</sup> which enables the identification of drug use against external contamination or passive exposure.<sup>11</sup>

Prior research has indicated that IA technology was highly effective in assessing urine, serum,<sup>12</sup> and, more recently, whole blood taken from living subjects<sup>13</sup> or post-mortem.<sup>6</sup>

## Objectives

In the present paper, screening results using hair samples taken from patients in therapy for drug addiction obtained through DRI® IA (amphetamines, cannabinoids, cocaine, methadone, and opiates) were compared to the quantitative results of the toxicological analysis performed using ultra-performance liquid chromatography coupled to tandem MS (UPLC-MS/MS). Screening cutoffs were then retrospectively optimized based on SoHT<sup>14</sup> confirmation cutoffs.

## Materials and methods

### Samples

Head hair samples from 150 patients with a history of drug addiction and undergoing treatment at the substance abuse service were collected from January 2022 to March 2023 in the metropolitan area of Bologna, Italy. After performing the analysis for clinical purposes, an aliquot was taken and stored according to the 2022 SoHT Consensus on general recommendations for hair testing. For the purpose of this study, the samples were treated completely anonymously.<sup>10</sup> The samples consisted of head hair, ranging from 3 to 6 cm in length, and were collected by cutting as close to the skin as possible. The hair strands were maintained in alignment until analysis. Toxicological analyses were performed within 72 h of sampling. Two analyses were performed on each sample, an IA analysis using ILab 650 (Werfen, Milan, Italy) and a UPLC-MS/MS analysis using an ACQUITY UPLC® System (Waters Corporation, Milford, USA). The 1<sup>st</sup> IA test was utilized as the screening test to be optimized, and the 2<sup>nd</sup> test (UPLC-MS/MS) as the confirmatory test.

### Immunoassay analysis

Six drugs/classes were assessed: amphetamines/methamphetamines, MDMA, cocaine, cannabinoids, methadone, and opiates.

### Reagents

The following reagents were used: DRI® Amphetamines Assay, DRI® Ecstasy Assay, DRI® Cocaine Metabolite Assay, DRI® Cannabinoid Assay, DRI® Methadone Assay, and the DRI® Opiate Assay.<sup>15</sup> The hair assay cutoffs for the original IA application were opiates 0.2 ng/mg (REF W150135; anti-morphine antibodies), methadone 0.2 ng/mg (REF W150596; anti-methadone antibodies), cocaine metabolite 0.2 ng/mg (REF W150055; anti-benzoylcegonine antibodies), amphetamines 0.2 ng/mg (REF W150017; anti-amphetamine and anti-methamphetamine antibodies), MDMA (ecstasy) 0.2 ng/mg (REF W15100075; anti-MDMA antibodies), and cannabinoids 0.1 ng/mg (REF W150185; anti-THCCOOH antibodies).

### Sample preparation

Sample preparation followed the manufacturer's suggestions, as follows: an aliquot of 33 mg of hair matrix finely chopped in 2–3-mm-long fragments were placed in a glass tube with a screw cap. To exclude the possibility of positivity due to external contamination, 1 mL of SLV-VMA-T washing solution (Comedical, Trento, Italy; REF SSSLVT000050) was added to the tube with the sample, gently mixed for about 30 s, and then the solution was

removed. After washing, 400  $\mu$ L of VMA-T (Comedical; REF SSVMAT001007) extraction reagent was added to the test tube and incubated for 1 h at 100°C. After cooling at room temperature, the sample was centrifuged for 5 min at 3,000 g. Immunoassay analysis was performed using an iLab-650 Clinical Chemistry System (Werfen).

### Calibrators and control samples

Calibration was performed using a 5-point calibration curve (0 included) with “CAL VMA-T calibrators Drugs of abuse and medicaments in hair” (Comedical; REF SSCALT000008)<sup>16</sup> as follows: amphetamines (0–0.50–1.00–2.00–4.00 ng/mg), ecstasy (0–0.45–0.90–1.80–3.60 ng/mg), cocaine (0–0.45–0.90–1.80–3.60 ng/mg), cannabinoids (0–0.12–0.24–0.48–0.96 ng/mg), opiates (0–0.40–0.80–1.60–3.20 ng/mg), and methadone (0–0.80–1.60–3.20–6.40 ng/mg). Quality control (QC) samples for each drug were performed using TricoCheck® (Comedical; REF SSVMAT001007), as follows: amphetamines 0.85 ng/mg, ecstasy 1.25 ng/mg, cocaine 1.05 ng/mg, cannabinoids 0.45 ng/mg, methadone 1.68 ng/mg, and opiates 1.10 ng/mg.

### Cross-reactions among classes of drugs

Concerning possible cross-reactions, positive and negative lists for each test are provided by the manufacturer in their performance guides.<sup>15</sup> Cross-reactivity for each class of drugs was also assessed internally by adding QC concentrations (2 for each drug) to blank blood samples. Given that we did not detect any cross-reactions among the various drug classes included in the study, samples testing positive for more than one drug were individually considered for data analysis for each specific drug. When expressing the IA values for drugs of abuse, it is not advisable to use “ng/mg” since IA tests provide semi-quantitative results. Therefore, when referencing IA tests, “ng/mg” should be interpreted as “ng/mg IA units”.

### UPLC-MS/MS analysis

A minimum quantity of 25 mg was needed to perform the analysis. For extraction, 0.3 mL of a mixture of methanol/water (1:1, v/v) with 0.1% formic acid was added to the hair sample and incubated at 45°C overnight. For analysis, after centrifugation, 2  $\mu$ L were injected into the UPLC-MS/MS system. We utilized an ACQUITY UPLC® System (Waters Corporation) equipped with a C18 column (2.1 $\times$ 150 mm, 1.8  $\mu$ m; Waters Corporation). Mobile phase A consisted of a 5 mM ammonium formate aqueous solution, while mobile phase B was composed of acetonitrile, with both phases containing 0.1% formic acid.

Analytes were eluted at a flow rate of 0.4 mL/min. The elution gradient started at 13% B, isocratic for 0.5 a min; from 0.5 to 10.0 min, it was increased to 50% B, from

10.0 to 12.5 min, it was increased to 95% B, and from 12.5 to 15 min it was decreased to 13% B.

The column temperature was maintained at 50°C. The method was internally validated for a range of analytes, including morphine, codeine, dihydrocodeine, 6-monoacetylmorphine, heroin, tramadol, methadone, MDMA, MDEA (3,4-methylenedioxy-N-ethylamphetamine), MDA (3,4-methylenedioxyamphetamine), methamphetamine, amphetamine, cocaine, benzoylecgonine, cocaethylene, and THC, with a linearity range of 0.1–1 ng/mg, in line with the cutoff for identifying drug users proposed by SoHT.<sup>14</sup> The method was fully validated according to European Medicines Agency (EMA) guidelines,<sup>17</sup> by assessing selectivity, linearity, accuracy, precision, limit of quantification (LOQ), limit of detection (LOD), matrix effect, and recovery. The first point on the calibration curve was considered the lower limit of quantification (LLOQ).  $R^2$  values exceeded 0.99 for all tested molecules. All parameters tested were within acceptable limits.

### Study design and statistical analyses

For identifying positive cases, the cutoffs proposed by the SoHT were used.<sup>14</sup> Table 1 reports the class of drugs of the IAs and the corresponding confirmation parameters. All 150 samples were analyzed using IAs as the screening test, and with UPLC-MS/MS as the confirmatory test.

To optimize the screening cutoffs, the result of the IA test was semi-quantitatively expressed in terms of “ng/mg IA units”; the results of the confirmatory analysis were considered positive when the SoHT criteria were respected, considering the main analyte and, when necessary, metabolites. As an example, for a positive cocaine result, the SoHT document assumed that “The presence of benzoylecgonine, norcocaine, cocaethylene, hydroxylcocaines, or hydroxy-benzoylecgonine must be considered

Table 1. Confirmation cutoffs for each class of drugs

Immunoassay test (IA)	Confirmation (LC-MS/MS)	Confirmation cutoff [ng/mg]
Amphetamine	amphetamine methamphetamine	0.20
MDMA	MDMA	0.20
Cocaine	cocaine <sup>1</sup>	0.50
Cannabinoids	THC	0.05
Methadone	methadone	0.20
Opiates	morphine codeine dihydrocodeine 6-monoacetylmorphine heroin tramadol	0.20

<sup>1</sup> with the presence of metabolites (benzoylecgonine, norcocaine, cocaethylene, hydroxyl-cocaines or hydroxy-benzoylecgonine); IA – immunoassay; LC-MS/MS – ultra-performance liquid chromatography coupled to tandem mass spectrometry; MDMA – 3,4-methyl-enedioxy-methamphetamine; THC – tetrahydrocannabinol.

to confirm use". Then, the result is considered "positive" at confirmation analysis only when cocaine was found above the cutoff, with the presence of a metabolite.

Receiver operating characteristic (ROC) curves were built using GraphPad Prism 9.5.1 software (GraphPad Software, San Diego, USA), which computes them from raw data. To this scope, screening results testing negative by UPLC/MSMS were inserted as "negative", while screening results corresponding to a positive result by UPLC/MSMS were inserted as "positive". Due to the cross-reactivity of the IA amphetamines test to methamphetamine, for the optimization of the amphetamines IA test, the sample was considered positive on confirmation analysis when the presence of amphetamine or methamphetamine above the confirmation cutoff was assessed. A sample was considered positive for cocaine when the presence of cocaine above the confirmation cutoff and metabolites were assessed, as requested by SoHT guidelines. A sample was considered positive for opiates when a result was positive for morphine, codeine, dihydrocodeine, 6-monoacetylmorphine, heroin, or tramadol above the confirmation cutoffs. A sample was considered positive for MDMA, cannabinoids and methadone when the presence of MDMA, THC and methadone, respectively, were encountered.

The ability of a test to discriminate between positive and negative samples is provided as the area under the ROC curve (area under the curve (AUC)) calculated with a standard error (SE) and 95% confidence interval (95% CI), as well as a p-value. The software automatically tabulates and plots the sensitivity and specificity of the test using each value in the data table as a possible cutoff value. A likelihood ratio is additionally calculated. Screening cutoff values were retrospectively optimized using contingency tables and assessed through ROC analysis. The SE of the area is calculated using the equation from Hanley and McNeil.<sup>18</sup>

We determined the optimal cutoff by summing the sensitivity and specificity. Sensitivity, calculated as  $TP/(TP+FN)$ , and specificity, calculated as  $TN/(TN+FP)$ , were determined using the numbers of true positives (TP), true negatives (TN), false positives (FP), and false negatives (FN). In cases where the sensitivity at the optimized cutoffs was less than 1, we retrospectively calculated the cutoff with a sensitivity equal to 1, referred to as the "highest sensitivity cutoff" (HS cutoff). Since the samples were processed anonymously for the purposes of this study, which did not involve the collection of any personal data, obtaining ethical approval was deemed unnecessary.

## Results

Overall, 150 hair samples were analyzed. Since there was no cross-reactivity detected among various drug classes, multiple drugs found in the same sample were individually considered for statistical analysis. A total of 162 single

positive results were obtained during confirmatory analysis (10 for amphetamine/methamphetamine, 11 for MDMA, 37 for cocaine, 40 for THC, 33 for methadone, and 31 for opiates). Optimized screening cutoffs for IA test using ROC analysis were as follows:

- 0.27 IA ng/mg for amphetamines;
- 0.51 IA ng/mg for MDMA;
- 0.59 IA ng/mg for cocaine;
- 0.14 IA ng/mg for cannabinoids;
- 0.63 IA ng/mg for methadone;
- 0.26 IA ng/mg for opiates.

For the compounds that did not reach 100% sensitivity at the optimized cutoff, HS-cutoffs were calculated, as follows: 0.21 IA ng/mg for amphetamines (specificity: 86.4%), 0.24 IA ng/mg for cocaine (specificity: 82.0%) and 0.03 IA ng/mg for cannabinoids (specificity: 66.4%). The ROC curves and a graphical representation of plots of positive and negative results at the optimized cutoffs are presented in Fig. 1 and Fig. 2.

An AUC greater than 0.95 was obtained for all drugs. Table 2 shows the sensitivity and specificity for all optimized cutoffs.

## Discussion

In this study, the cutoffs obtained through IA-based screening were optimized with respect to forensic interpretative cutoffs.<sup>14</sup> In the routine of hair analysis, the optimized screening cutoff and the confirmation cutoff should not be confused.<sup>11</sup> For IA screening, the use of cutoffs is an attempt to limit the number of FP cases, thus limiting the number of confirmation analyses performed, which are expensive and time-consuming, whilst not missing TP results. Immunoassays, although simple to perform, are prone to interferences that can cause FN or FP. On the other hand, the use of confirmation cutoffs comes from the consensus document of the SoHT, which produced cutoff values for a range of analytes to differentiate between deliberate drug consumption and the possibility of incidental exposure or endogenous production.<sup>14</sup> These are not analytical cutoffs, and analyte concentrations below these values may indicate deliberate administration.<sup>11</sup> The proposed approach ensures that the screening test is optimized not only with the analytical result of the laboratory, often identified with the LLOQ, but with the interpretative cutoff.

Moreover, the screening results should be interpreted in semi-quantitative terms (expressed as IA ng/mg units), as the numerical result cannot be directly compared to the confirmation cutoff. For instance, concerning cocaine, the test has a higher affinity for benzoylecgonine, while the interpretative SoHT cutoff requires the presence of cocaine above a certain value, coupled with the presence of metabolites. Consequently, we may encounter higher concentrations in the screening results that cannot be equated to those of the confirmation cutoff.

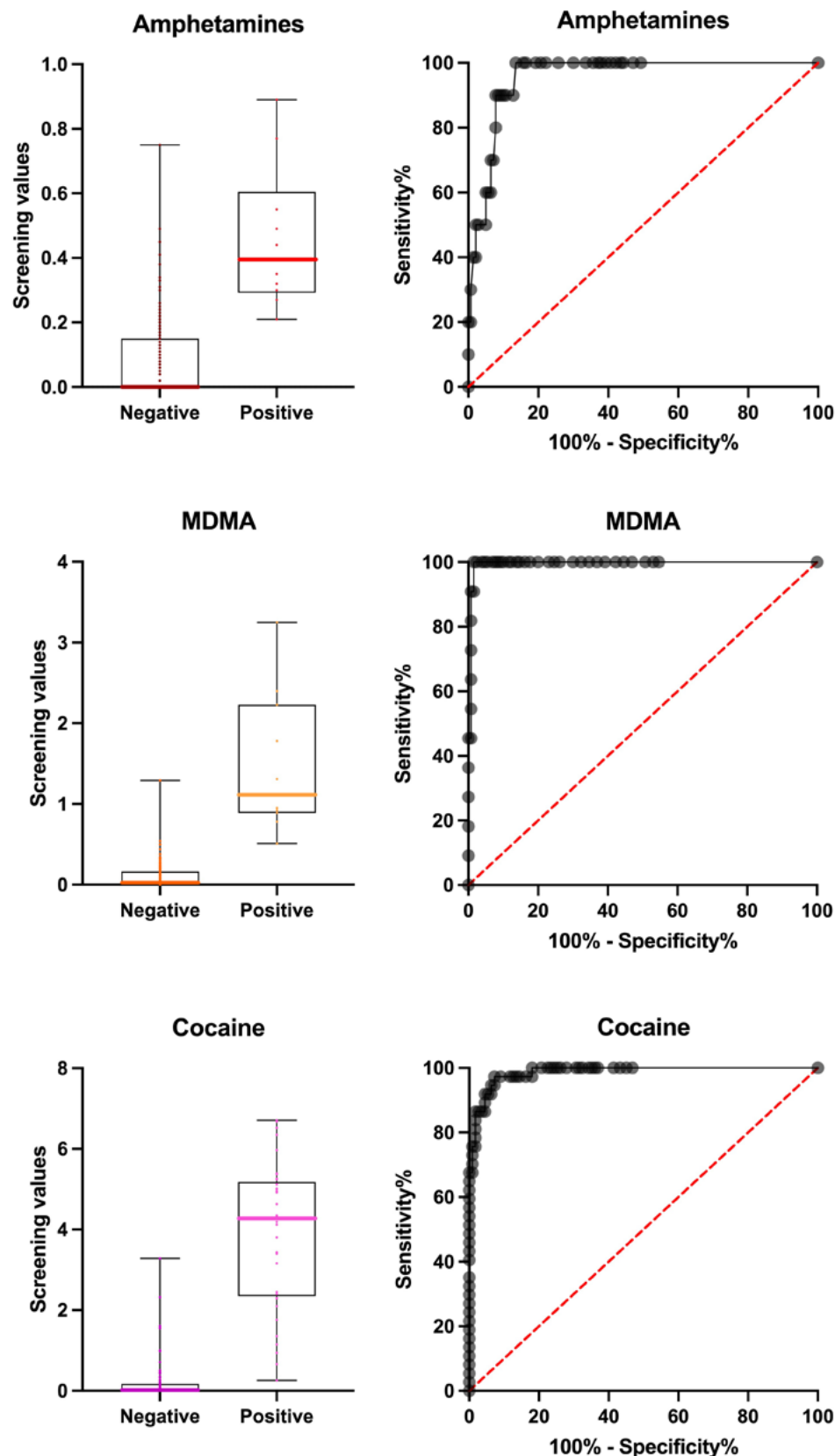


Fig. 1. Plots (left) and receiver operating characteristic (ROC) curves (right) for amphetamines, MDMA and cocaine. The plots depict 1<sup>st</sup> quartile (Q1) and 3<sup>rd</sup> quartile (Q3) (represented by black thin lines) and the median (represented as the height of the columns)

All semi-quantitative optimized cutoffs showed satisfying results in terms of sensitivity and specificity, with a value of sensitivity greater than 90% and a very good AUC for all compounds. The AUC is a reliable parameter that considers both sensitivity and specificity and, hence,

directly measures the diagnostic power of the test. These AUC results indicate the reliability of the optimized cut-offs for all the molecules tested.<sup>9</sup> Some laboratories that perform hair testing during their daily routines report positive identifications to analytical limits routinely (i.e.,



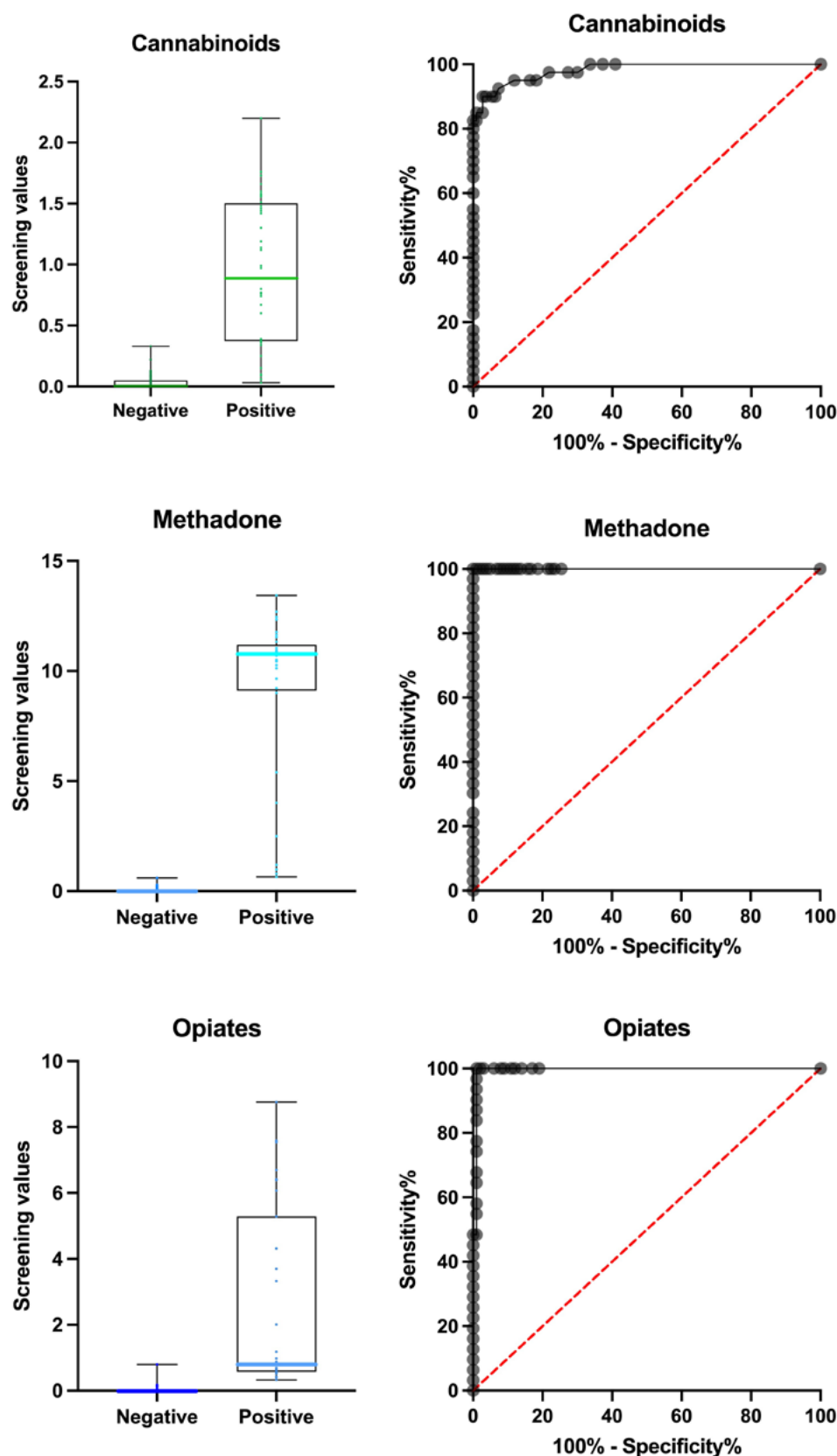


Fig. 2. Plots and receiver operating characteristic (ROC) curves for cannabinoids, methadone and opiates. The plots depict 1<sup>st</sup> quartile (Q1) and 3<sup>rd</sup> quartile (Q3) (represented by black thin lines) and the median (represented as the height of the columns)

LOQ or LOD), risking giving a false impression of past drug use. Other laboratories use either the SoHT or “in-house” cutoff values and may report the same analytical result as “negative” or “not detected”, thus risking not

supplying valuable information in the context of a particular case.<sup>11</sup>

The results of this study showed that the proposed screening technique at the optimized cutoffs exhibits

**Table 2.** Results obtained for each class of drugs. For each class of drugs, optimized cutoffs along with main statistical results are reported

Optimized cutoff	Amphetamines	MDMA	Cocaine	Cannabinoids	Methadone	Opiates
AUC	0.956	0.995	0.986	0.981	1.000	0.994
SE	0.018	0.005	0.007	0.010	<0.001	0.005
95% CI	0.920–0.991	0.986–1.000	0.973–1.000	0.961–1.000	(0.999–1.000)*	0.985–1.000
p <sup>H</sup>	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
O cutoff	0.270	0.510	0.590	0.140	0.630	0.260
Sensitivity (%)	90.000	100.000	97.300	90.000	100.000	100.000
95% CI	59.600–99.500	74.100–100.000	86.200–99.900	77.000–96.000	89.600–100.000	89.000–100.000
Specificity (%)	92.100	98.500	92.800	97.300	100.000	99.000
95% CI	86.500–95.600	94.600–99.700	86.400–96.300	92.300–99.300	96.400–100.000	94.600–100.000
HS cutoff	0.210	–	0.240	0.030	–	–
Sensitivity (%)	100.000	–	100.000	100.000	–	–
95% CI	72.300–100.000	–	90.600–100.000	91.200–100.000	–	–
Specificity (%)	86.400	–	82.000	66.400	–	–
95% CI	79.800–91.100	–	73.800–88.000	57.100–74.500	–	–

AUC – area under the curve; O cutoff – optimized cutoff; HS cutoffs – cutoffs with highest sensitivity, which correspond to optimized cutoff for MDMA, methadone and opiates. \*Due to the property of AUC ( $\leq 1.000$ ), symmetrical interval (0.999–1.001) was truncated on the right to (0.999–1.000); p<sup>H</sup> – level of statistical significance of the Hanley–McNeil method; 95% CI – 95% confidence interval; SE – standard error; HS – high sensitivity.

a very high sensitivity for all drugs, avoiding the FN rate with an acceptable level of specificity even using HS-cut-offs namely cutoffs with a sensitivity of 100%. Depending on the analysis's purpose, each laboratory may opt to use either the optimized cutoffs or the HS-cut-offs. For example, in fields such as analyses for driving license re-granting or other forensic areas where FN is not accepted, the HS-cutoff may see a broader application. Conversely, in other fields with a high volume of analyses and patient assessments guided by clinical and anamnestic parameters, laboratories might choose the optimized cutoff for cost-effectiveness reasons, involving closer short-term monitoring of select patients.

Contrary to what was previously observed in the blood,<sup>6,13</sup> hair tests are reliable for the screening of amphetamines, as the biogenic amines, mainly responsible for FP results in blood, are not present in the hair matrix. The use of the term “cutoff” instead of “LLOQ” was deliberate, specifically to denote a threshold where the laboratory can make a decision based on its cost-effectiveness requirements regarding whether to confirm the sample. It extends beyond being solely an analytical result, as it would be in the case of LLOQ.

In recent years, numerous forensic toxicology laboratories have shifted from immunoenzymatic techniques to multi-targeted methods utilizing MS-based approaches, allowing for the simultaneous detection of a wider range of molecules.<sup>19,20</sup> However, the importance of retaining an immunoenzymatic technique resides in having a distinct method for confirmation. This proves particularly advantageous for laboratories that may lack either 2 MS-based techniques or 2 divergent methods for conducting both screening and confirmation analyses. Such

laboratories could find benefit in employing immuno-enzymatic techniques, especially for analyses with high sample volumes.

Forensic toxicology plays a primary role in the research within the field of biomedical-legal sciences,<sup>21,22</sup> and in recent years, it has mainly focused on MS-based techniques, which have high sensitivity and specificity. We believe that the robustness of the preliminary experiment could pave the way for further validation studies on IA tests, encompassing a larger number of samples and other drugs of forensic interest.

## Limitations

The study sample consisted of hair samples taken from a specific population, namely patients with a drug use disorder, primarily undergoing maintenance therapy with methadone. Consequently, the results related to methadone may be overestimated due to elevated methadone levels in the samples, which are much higher than the confirmatory cutoff. This could stem from methadone maintenance therapy in positive study participants and the absence of patients with irregular methadone intake in our study population. To address this limitation, screening cutoffs should be refined using a larger sample size and diverse populations of forensic relevance. This method was optimized for the drugs of abuse most commonly found in drivers in our country,<sup>22</sup> but other substances that can influence the ability to drive, such as prescription drugs,<sup>23</sup> were not included. Therefore, in the future, the screening panel should be extended to common prescription drugs (benzodiazepines and medical opioids). Regarding the sample size, a power

analysis was not applied, but the samples received during a specific time period were analyzed according to the casework requirements. This has been specified in the limitations of the study.

## Conclusions

Compared to routine urine or blood drug testing, IAs for hair analysis are not commonly employed due to the low concentrations required for this purpose. The study's strengths lie in the optimization of IA cutoffs with interpretative values, a substantial sample size, and the achievement of a high level of sensitivity and specificity at the optimized cutoff points, along with a very high AUC during ROC analysis. In conclusion, this screening method has demonstrated its utility when applied to hair samples for the most commonly detected drug classes in Italy and Europe. It can find application in both forensic and clinical analyses.

## Data availability


The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Consent for publication


Not applicable.


## ORCID iDs

Arianna Giorgetti  <https://orcid.org/0000-0002-0441-9787>

Jennifer P. Pascali  <https://orcid.org/0000-0002-1363-3400>

Guido Pelletti  <https://orcid.org/0000-0003-3263-1758>

Marco Garagnani  <https://orcid.org/0000-0002-4320-2556>

Paolo Fais  <https://orcid.org/0000-0002-2270-9956>

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