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Evaluation of artificial drug incorporation into hair for production of quality control samples

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Hair analysis is a thoroughly used tool in the field of forensic toxicology. Despite its imminent growth, there are still some points of concern surrounding the use of this matrix, such as the lack of reference material for internal quality control (IQC). Currently, the most widely used approach when developing a hair test is to spike the hair solutions with the compound of interest. However, when doing so, it is not possible to evaluate its actual reproducibility and accuracy. As an alternative, IQC could be produced artificially by the laboratories and research groups themselves. The aim of this work was to evaluate the phenomenon of artificial incorporation of drugs into hair to produce in-house IQC. For this purpose, different substances were considered, consisting of amphetamine, MDMA, cocaine, diazepam, and morphine. Results have rather distinct incorporation rates for each analyte under study. Morphine has shown to be the least incorporated analyte with incorporation rates ranging from 0.08 to up to 0.25% and diazepam showing high incorporation rates of up to 3.75%. Certain parameters such as incubation time, agitation process, sample homogenization and sample washing have played a role in the way that analytes incorporate into the hair matrix not to mention the inherent chemical profiles of each drug. Overall, by perceiving the incorporation profile of each analyte it is then possible to produce in-house IQC, with different concentrations. By doing so, researchers will then have access to properly fortified samples as a reliable tool for evaluating their own methodology.

Keywords: Fortified hair samples; material reference; quality control; hair analysis and alternative matrices.

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Introduction

Taking a look into the history of hair analysis in forensic toxicology, it is believed that the first ever case published in English, reporting the use of hair for poison determination, dates back to 1858 (1). To this day, hair analysis have been thoroughly employed and by 2015 it was considered the third most fundamental biological matrix in drug testing (2).

The main three aspects contributing to hair being a matrix of choice over blood and urine, on certain occasions are: its substantially longer window of detection, allowing for retrospective analysis, the possibility to establish a detailed timeline given its growth rate and its enduring characteristics. Such features allow researchers to investigate chronic and past consumption of individuals, to pin point a specific time frame and to test this matrix for centuries after its growth, without the need for any major storage conditions (3).

Hair is nowadays consistently used in forensic toxicology and its applications include *postmortem* toxicology, drug-facilitated crimes, divorce and child custody proceedings as well as follow-up of detoxification programs (2). In fact, hair analysis can be used in a wide range of areas across science disciplines. For instance, in the veterinary field, as it has previously been used for testing mineral intake of horses in Arizona (4) as well as in a clinical setting, where hair analysis were a key tool to determine endogenous cortisol levels for advancing patient care (5).

Recently, a study published by Pragst et al. has used hair to test more than 140 families with drug consuming parents. The authors have found that 95% of family drug hair tests were positive for one to five drugs with the highest occurrence being cocaine (79.7%) followed by THC (50.2%) (6).

Regardless of its purpose, hair analysis, comparable to other biological matrices, must comply with the quality assurance required for all testing laboratories wishing to provide scientific evidence for decision-making (e.g., positive, or negative) based on analytical data (7). Therefore, laboratories wishing to perform toxicological analysis should be accredited with ISO/IEC 17025 as part of their quality control and moreover, to participate in external proficiency testing to demonstrate the ability to perform quantitative analysis, mainly on cut-off levels (8).

Accordingly, reference material has gained increasing attention not only as external proficiency tests, but also for method development and validation, estimation of measurement uncertainty, and as an internal quality control (IQC) since spiked samples do not mimic the real condition (9-11).

Although spiked samples are still widely used, mainly for method development, they do not allow for the truthful evaluation of the extraction steps or even the influence of

hair length. Unlike liquid matrices, such as urine or blood, there is no incorporation of analytes into the hair matrix when employing the spiking procedure. For that reason, a validation process containing simply spiked controls is not entirely capable of assessing reproducibility and accuracy of the method (9,11).

In fact, this issue has been seen during the first proficiency and interlaboratory tests performed for drug abuse on hair samples, presenting high scatter of quantitative results (12,13).

That is why using authentic hair samples would be considered a representative specimen for method development and validation. However, the limited amount of sample and its homogeneity complexity, contribute to its scarceness. Additionally, as a commercially-available quality control, its use is impractical due to its significant high cost (9). Therefore, this study proposed the use of in-house artificial production of internal quality specimens, also called fortified samples. The aim of the present study was to evaluate the phenomenon of artificial incorporation of different drug classes into hair in order to produce internal quality controls.

Materials and Methods

Chemical and reagents

Methanol and acetonitrile HPLC grade were purchased from Merck (Darmstadt, Germany). Water was purified using a Milli-Q system (Millipore, Billerica, MA, USA). Formic acid (98–100% grade) was purchased from Merck (Darmstadt, Germany), ammonium formate was obtained from Fluka (≥ 97% purity; Buchs, Switzerland) and dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (> 99%; St. Louis, USA). All reference standards were ≥ 98% purity. Water (18 MΩ) was purified using a Milli-Q purification system (Millipore Corp., Bedford, MA, USA).

Standards and solutions

The analytical standards of cocaine, amphetamine, MDMA, morphine and diazepam at a concentration of 1.0 $mg.mL^{-1}$ were obtained from Cerilliant Analytical Reference Standards (Round Rock, TX, USA). Internal standards (cocaine-d3, diazepam-d5, MDMA-d5 and morphine-d3) at a concentration of 100 $\mu g.mL^{-1}$ were obtained from Cerilliant Analytical Reference Standards (Round Rock, TX, USA). Work solutions of the analytes and IS were diluted at concentrations of 10 $\mu g.mL^{-1}$ and 1 $\mu g.mL^{-1}$ using methanol for amphetamine, MDMA, morphine and diazepam and acetonitrile for cocaine Stock solutions were appropriately refrigerated (2 - 8 °C), when not in use. According to the manufacturer's certificate of analysis, the purity of all analytes is above 99%.

Instrumentation

The LC system consisted of an Acquity UPLC (Waters, USA) which consisted of a degasser, a binary pump and an autosampler coupled to a Quattro Premier XE mass spectrometer (Micromass, UK). The chromatographic separation was achieved on an Acquity UPLC BEH C18 column. The chromatographic conditions were evaluated in order to obtain a satisfactory chromatographic separation for all compounds. The mobile phase consisted of 1 mM ammonium formate in water (eluent A) and acetonitrile (eluent B) both supplemented with 0.1% formic acid at 400 $\mu L.\ mL^{-1}$ flow rate and 45 ºC. The total run time was 7.0 min, including re-equilibration at the initial conditions. The gradient was programmed as follows: 0 – 3 min, 10 – 45 % B; 3 – 3.8 min, 45 – 80 % B; 3.8 – 4.0, 80 - 10 %; 4.0 – 7.0, 10 % B. The mass spectrometer was operated in positive mode (ESI+; $[M+H]^+$), as follows: desolvation gas, 1100 $L.h^{-1}$; cone gas, 200 $L.h^{-1}$; desolvation temperature, 450 ºC; source temperature, 120 ºC; capillary voltage, 1000 V. The MRM transitions for all analytes and their respective internal standards are listed in Table 1. For data evaluation, MassLynx® software was used to obtain peak areas. Statistical analysis was performed on Microsoft® Excel.

Table 1. MRM transitions and experimental conditions employed in the developed UPLC-MS/MS method.

Analyte	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (V)
Amphetamine	136.03	119.01	19
		90.92	14
Cocaine	303.96	181.81	21
		104.68	32
Diazepam	285.14	193.12	32
		153.99	27
MDMA	193.97	162.91	14
		104.93	24
Morphine	286.19	201.10	55
		165.05	25
Cocaine-d3	307.20	189.91	21
		104.76	32
Diazepam-d5	290.20	226.96	32
		197.88	27
MDMA-d5	199.10	164.98	14
		135.28	24
Morphine-d3	289.19	201.12	55
		165.10	25

Preparation of fortified hair samples

The fortified hair samples/artificial internal quality controls were prepared according to the recommendations from the National Institute of Standards and Technology (NIST) with adaptations to reach cutoff concentrations (12). Aliquots of drug-free human brunette hair (50 mg), from a single individual volunteer were first washed with water and a mild detergent, followed by 2.0 mL of dichloromethane for 15 min at 37 °C (8). Once dried, the samples were incubated in a solution of water-DMSO (1:1), in triplicate, each spiked with cocaine (200 ng.mg^{-1}), amphetamine (120 ng.mg^{-1}), MDMA (200 ng.mg^{-1}), morphine (120 ng.mg^{-1}) and diazepam (80 ng.mg^{-1}), separately. The incubation was performed for three, six, nine and 12 days.

After each time-point, the hair samples were removed and washed with methanol. The aliquots of methanol were then analysed in the same method to confirm no drug presence out of hair prior to investigation. Once dried, the hair was then incubated overnight at 55 °C with 2 mL of methanol and the internal standards (cocaine-d3, diazepam-d5, MDMA-d5 and morphine-d3; 2 ng.mg^{-1}). After incubation, the methanol was transferred into a new clean tube, dried at 50 °C under N2 stream and finally re-constituted with 50 µL of mobile phase A (1 mM of ammonium formate in water with 0.1% formic acid) and 3 µL were injected into the UPLC-ESI-MS/MS system.

The methodology used to analyze the incorporated samples was validated in-house according to the recommended international parameters. The following guidelines have been followed: Scientific Working Group for Forensic Toxicology - SWGTOX (14) and Society of Hair Testing – SoHT (8).

Results and Discussion

Analytical validation

The obtained LoQ values were 0.5 ng.mg^{-1}for cocaine, 0.2 ng.mg^{-1} for amphetamine, MDMA and morphine and 0.05 ng.mg^{-1} for diazepam. The calibration curves were linear over the specified range as follows: 0.5-20 ng.mg^{-1} for cocaine; 0.2-10 ng.mg^{-1} for amphetamine, MDMA and morphine; 0.05-10 ng.mg^{-1} for diazepam. Coefficients of determination were in the range of 0.997-0.999. Precision and accuracy were tested for all analytes according to quality control (QC) levels, in six replicates. The results are summarized in Table 2. The relative standard deviation (RSD%) for precision ranged from 1.0 to 10,7%. The values found for accuracy were between 87.4 and 116.1%, in accordance with the following international guidelines. The acceptance criteria were 20% for precision (relative standard deviation: RSD) and for accuracy bias in the low concentrations and 15% in the medium and higher concentrations. Figure 1 shows the chromatogram obtained by UPLC-ESI-MS/MS from a hair sample spiked with cocaine, amphetamine, MDMA, morphine and diazepam submitted to the developed method.

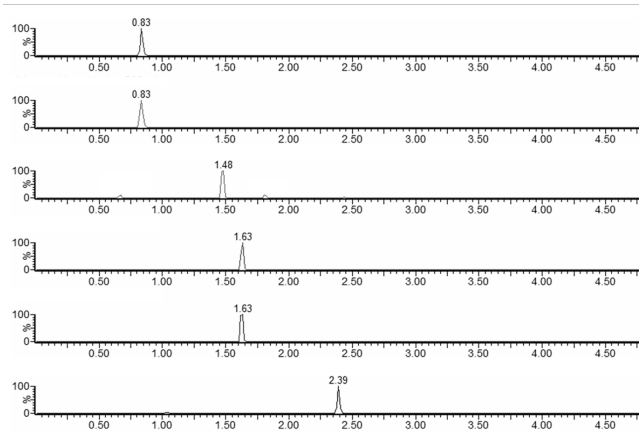

Figure 1. Chromatogram obtained by UPLC-ESI-MS/MS from a hair sample spiked at 1 ng.mg^{-1} with cocaine, amphetamine, MDMA, morphine and diazepam submitted to the developed method.

Table 2: Results of method precision and accuracy for amphetamine, cocaine, diazepam, MDMA and morphine in hair samples.

Analyte	*QC level*	*Precision (RSD%)*	*Accuracy (%)*
Amphetamine	QC1	6.7	94.4
	QC2	6.4	99.0
	QC3	5.0	101.2
Cocaine	QC1	1.6	90.5
	QC2	1.0	104.9
	QC3	1.5	96.5
Diazepam	QC1	7.3	116.1
	QC2	6.8	95.0
	QC3	9.9	95.0
MDMA	QC1	6.0	87.4
	QC2	6.7	91.1
	QC3	3.8	92.0
Morphine	QC1	10.7	96.3
	QC2	6.8	99.3
	QC3	8.1	95.0

Incorporation measurement

For quality assurance, SoHT recommends that the quality control samples should be prepared from ‘‘authentic’’ hair samples collected from known drug users. Nonetheless, the same guide does recognize the problematic issues involving homogeneity and lack of availability of old case samples. For this reason, suppliers of quality control material, using both authentic and

fortified drug-free hair, are presented on the society website (8).

Due to scarce reference material, one of the major challenges for hair analysis performing laboratories is to obtain satisfactory results in proficiency tests, using only spiked samples for both method development and validation. Therefore, fortified hair samples produced in-house should be considered as an accessible internal quality control, once it provides an analytical challenge as real drug users' hair would. Currently, the incorporation of drugs into hair using DMSO is not yet fully understood. Nonetheless, it was possible to observe that all tested analytes showed a different pattern of incorporation over time. The results obtained are shown in Table 3, including the average concentrations for each substance following the 4 assays.

Table 3. Spiked and detected concentrations in produced quality controls (QC) hair samples.

Analytes	QC Spiked ($ng.mg^{-1}$)	Days			
		3rd day	6th day	9th day	12nd day
	Concentration ($ng.mg^{-1}$)				
Amphetamine	120	0.2	0.5	0.5	0.6
Cocaine	200	0.3	0.8	1.2	1.5
Diazepam	80	0.5	0.5	2.5	3.0
MDMA	200	0.2	0.7	0.7	0.8
Morphine	120	0.1	0.2	0.3	0.3

For the amphetamine group, the highest concentrations obtained were of 0.6 and 0.8 $ng.mg^{-1}$ for amphetamine and MDMA, respectively Although these compounds showed different incorporation rates, of 0.17 up to 0.5% for amphetamine and 0.10 up to 0.4% for MDMA, it was noted that both have similar patterns of incorporation as no increase of concentration has been observed from the sixth to the ninth day. The CV% among triplicates were < 12% for amphetamine and much higher for MDMA, with a value of 36%.

For cocaine, the incorporation rate was progressive over the course of days, ranging from 0.15 to 0.75% with coefficients of variation (CV%) < 25% among the triplicates. The highest concentration obtained was of 1.5 ng/mg meaning three times greater than its cut-off value.

The highest incorporation was found for diazepam, 3 ng/mg. The rate remained constant for six days, reaching a value of 0.57%. From the ninth to 12th day, it was possible to observe a high increase in the incorporation rate reaching values of 3.1 and 3.75%, respectively. For this analyte, CV% were less than 16%.

Lower rates were obtained for morphine, ranging from 0.08 to 0.25%, given that the incorporation rate of 0.25% has been reached on the ninth day. The highest concentration verified was 0.3 $ng.mg^{-1}$. The CV% were < 27% among triplicates. The figures 2, 3, 4 and 5 present the different incorporation rates obtained for all analytes over time.

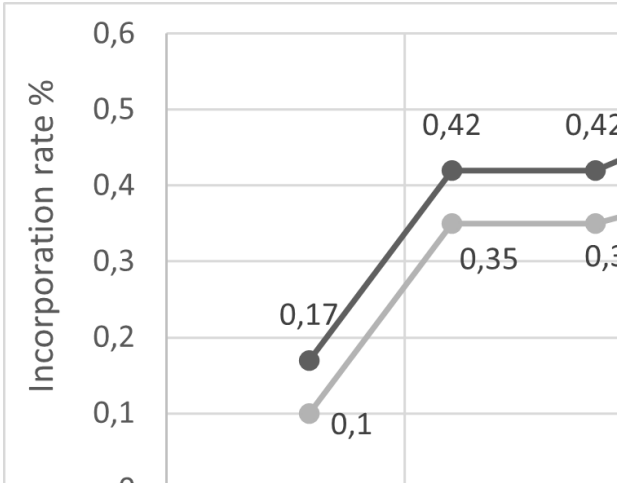

Figure 2. Incorporation rates (%) according to incubation days for amphetamine and MDMA.

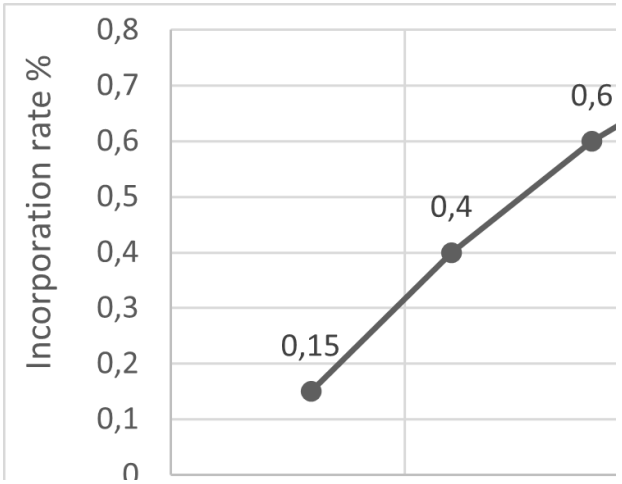

Figure 3. Incorporation rates (%) according to incubation days for cocaine.

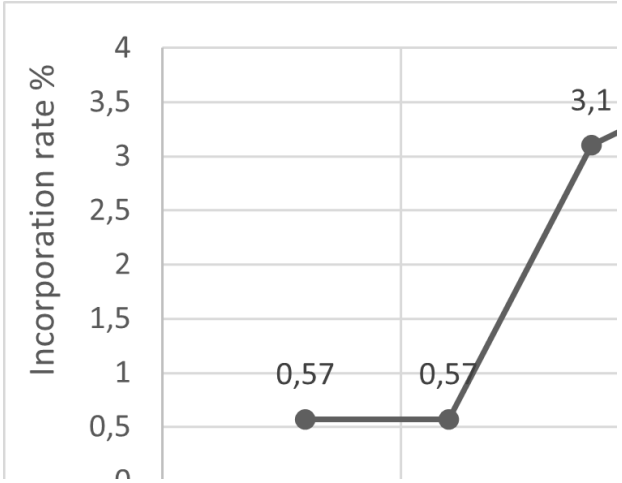

Figure 4. Incorporation rates (%) according to incubation days for diazepam.

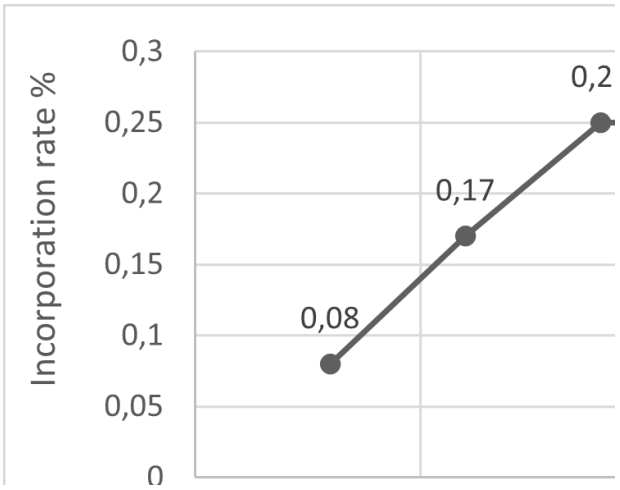

Figure 5. Incorporation rates (%) according to incubation days for morphine.

Comparing the results obtained with previously NIST experiments, it is possible to observe that the influence of incubation time is significant for increased incorporation rates. NIST tests were performed between 16.5 to 19 days, reaching an average of 2.9% of rate. In addition to time, the agitation process can also influence the way analytes incorporate into the matrix. Possibly, the lack of agitation in this study decreased the surface contact between matrix and analyte and consequently impaired the homogenization of the samples. Thus, we strongly recommended the evaluation of this parameter during the fortification process, in order to achieve higher incorporation rates and lower %CV% among the replicates.

Another aspect which has played a role in this procedure was the samples washing. SoHT accepts organic solvents, such as dichloromethane or acetone, for the washing process once they only remove superficial contamination. On the other hand, methanol should be avoided due to also its extraction capability (8). Despite following the NIST protocol, methanol could, indeed, be the cause for relatively lower incorporation rates and should be replaced for this step.

The mechanisms involved in the incorporation of the substance into the hair or the factors that influence this process are not fully understood (15). However, the physicochemical properties of substances as well as the physiological characteristics of each individual influence the incorporation mechanism. Considering the physicochemical properties, lipophilic substances have greater affinity to penetrate hair cells, in addition to the pH difference between the blood and the hair bulb promotes a greater incorporation of basic substances when compared to acidic substances. It should also be pointed out that melanin provides a greater incorporation of basic substances (3,16).

In fact, understanding the incorporation profile of each analyte and the main variables during the fortification process, laboratories could improve these conditions, including initial standard addition concentration, according to their needs.

Overall, the current study was able to propose a rather appropriate alternative for producing internal quality control samples focusing on obtaining more reliable quantification results using artificial incorporated samples.

Conclusions

Overall, it was possible to produce internal quality control, with different concentrations, by controlling variables such as time, agitation and washing procedure, according to the interests of each laboratory. By making use of the proposed alternative, laboratories and world-wide research groups can have access to their in-house personalized fortified samples as an overall more representative tool for evaluating their own methodology.

Conflict of interest

The authors declare no conflicts of interest.

References

1. Casper JL. Praktisches Handbuch der Gerichtlichen Medizin. A. Hirschwald, Berlin, 1858.
2. Jurado C. Forensic Applications of Hair Analysis. In: Kintz P., Salomone A., Vicenti M. Hair Analysis in Clinical and Forensic Toxicology. Academic Press, 2015 p. 241-273.
3. Pragst F, Balikova MA. State of the art in hair analysis for detection of drug and alcohol abuse. Clin Chim Acta. 2006; 370:17-49.
4. Wells LA, LeRoy R, Ralston SL. Mineral intake and hair analysis of horses in Arizona. J Equine Vet Sci. 1990; 10:412-416.
5. Greff MJE, Levine JM, Abuzgaia AM, Elzagallaai AA, Rieder MJ, Van Uum SHM. Hair cortisol analysis: An update on methodological considerations and clinical applications. Clin Biochem. 2019; 63:1-9.
6. Pragst F, Krumbiegel F, Thurmann D, Westendorf L, Methling M, Niebel A, et al. Hair analysis of more than 140 families with drug consuming parents. Comparison between hair results from adults and their children. Forensic Sci Int. 2019; 297:161-170.
7. Eller SCWS, Flaiban LM, Paranhos BAPB, Da Costa, JL, Lourenço FR, Yonamine M. Analysis of 11-nor-9-carboxy-Δ9-tetrahydrocannabinol in urine samples by hollow fiber-liquid phase microextraction and gas chromatography–mass spectrometry in consideration measurement uncertainty. Forensic Toxicol. 2014; 32:282-291.

8. Cooper GAA, Kronstrand R, Kintz P. Society of Hair Testing guidelines for drug testing in hair. Forensic Sci Int. 2012; 218:20-24.
9. Turfus SC, Beyer J, Gerostamoulos D, Drummer OH. A comparison of the performance of quality controls prepared from spiked, fortified and authentic hair for ethyl glucuronide analysis. Forensic Sci Int. 2013; 232:60-66.
10. Lee S, Park Y, Han E, Choe S, Lim M, Chung H. Preparation and application of a fortified hair reference material for the determination of methamphetamine and amphetamine. Forensic Sci Int. 2008; 178:207-212.
11. Lee S, Miyaguchi H, Han E, Kim E, Park Y, Choi H, et al. Homogeneity and stability of a candidate certified reference material for the determination of methamphetamine and amphetamine in hair. J Pharm Biomed Anal. 2010; 53: 1037-1041.
12. Welch M.J., Sniegoski L.T., Tai S. Two new standard reference materials for the determination of drugs of abuse in human hair. Anal Bioanal Chem. 2003; 376:1205-1211.
13. Jurado C, Sachs H. Proficiency test for the analysis of hair for drugs of abuse, organized by the Society of Hair Testing. Forensic Sci Int. 2003; 133:175-178.
14. Scientific Working Group for Forensic Toxicology (SWGTOX). Standard practices for method validation in forensic toxicology. J Anal Toxicol. 2013; 37:452-474.
15. Kintz P. Value of hair analysis in postmortem toxicology. Forensic Sci Int. 2004; 142(2-3):127-134.
16. Vogliardi S, Tucci M, Stocchero G, Ferrara SD, Favretto D. Sample preparation methods for determination of drugs of abuse in hair samples: A review. Anal Chim Acta. 2015; 857:1-27.