UNIVERSIDADE BEIRA INTERIOR

Development and validation of a method for the determination of cocaine and its metabolites in hair using QuEChERS and GC-MS/MS

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(2º ciclo de estudos)

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Dedicatória

Dedico este meu trabalho a toda a minha família, amigos e namorada, os quais sempre acreditaram em mim desde o começo e sempre me apoiaram durante todo o processo de desenvolvimento do mesmo.

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Resumo

A cocaína (COC) é um dos estimulantes ilícitos mais consumidos a nível mundial, apresentando elevado potencial aditivo e graves efeitos toxicológicos. A análise de cabelo oferece vantagens únicas na toxicologia forense, permitindo documentar consumos crónicos e, em alguns casos, episódios agudos. Neste estudo, desenvolveu-se uma versão miniaturizada do método de extração reviQuEChERS (*Quick, Easy, Cheap, Rugged, and Safe*) para a determinação de COC e dos seus principais metabolitos — anidroecgonina metil éster (AEME), ecgonina metil éster (EME), cocaetileno (COET), benzoilecgonina (BEG) e norcocaína (NCOC) — em amostras de cabelo.

O procedimento consistiu na utilização de 3 mL de ácido fórmico a 5% em acetonitrilo (ACN) e 625 mg de NH_4HCO_2 como solvente e sal de partição, respetivamente, seguido de extração em fase sólida dispersiva (d-SPE) com 175 mg de $MgSO_4$ e 55 mg de amina secundária primária (PSA). As amostras foram analisadas por cromatografia gasosa acoplada à espetrometria de massas em tandem (GC-MS/MS).

Após otimização por design of experiments (DoE), o método foi validado de acordo com as diretrizes da *Society of Hair Testing (SOHT)*. O intervalo de trabalho estabelecido foi de 0,05–5 ng/mg para todos os analitos, exceto para a AEME (0,5–5 ng/mg). As recuperações variaram entre 20–29% (AEME), 24–27% (EME), 33–42% (COC), 43–77% (COET), 25–48% (BEG) e 27–37% (NCOC). O método foi aplicado a amostras reais, nas quais a COC foi detetada em quase todos os casos, frequentemente acompanhada de metabolitos em concentrações acima do limite inferior de quantificação (LLOQ).

O método µ-QuEChERS demonstrou ser uma estratégia sustentável, sensível e multi-analítica para a determinação de cocaína e metabolitos em cabelo, com elevado potencial de aplicação em toxicologia clínica e forense.

Palavras-chave

Cocaina;metabolitos;cabelo;análise forense;µ-QuEChERS.

Abstract

Cocaine (COC) is one of the most widely consumed illicit stimulants worldwide, with a high addictive potential and severe toxicological effects. Hair analysis offers unique advantages in forensic toxicology, as it allows the documentation of chronic use and, in some cases, acute exposure. In this study, a miniaturised version of the QuEChERS (*Quick, Easy, Cheap, Rugged, and Safe*) extraction method was developed for the determination of COC and its major metabolites — anhydroecgonine methyl ester (AEME), ecgonine methyl ester (EME), cocaethylene (COET), benzoylecgonine (BEG), and norcocaine (NCOC) — in hair samples.

The procedure involved 3 mL of formic acid (5%) in acetonitrile (ACN) and 625 mg of NH_4HCO_2 as solvent and partitioning salt, respectively, followed by a dispersive solid-phase extraction (d-SPE) step using 175 mg of $MgSO_4$ and 55 mg of primary secondary amine (PSA). Samples were analysed by gas chromatography coupled to tandem mass spectrometry (GC-MS/MS).

Following optimisation through design of experiments (DoE), the method was validated according to the guidelines of the *Society of Hair Testing (SOHT)*. The working range was set at 0.05–5 ng/mg for all analytes, except AEME (0.5–5 ng/mg). Recoveries ranged from 20–29% (AEME), 24–27% (EME), 33–42% (COC), 43–77% (COET), 25–48% (BEG), and 27–37% (NCOC). The method was successfully applied to authentic hair samples, in which COC was detected in almost all cases, often accompanied by metabolites at concentrations above the lowest limit of quantification (LLOQ).

The µ-QuEChERS method proved to be a sustainable, sensitive, and multi-analytical approach for the determination of cocaine and metabolites in hair, with strong potential for application in clinical and forensic toxicology.

Keywords

Cocaine;metabolites;hair;forensic analysis;µ-QuEChERS.

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ACN	Acetonitrile
AEME	Anhydroecgonine methyl ester
BEG	Benzoylecgonine
CNS	Central nervous system
COC	Cocaine
COET	Cocaethylene
CV	Coefficient of variance
DoE	Design of experiments
DP	Dermal papilla
d-SPE	Dispersive solid-phase extraction
EI	Electron impact
EME	Ecgonine methyl ester
EPRW	European Pesticide Residue Workshop
GAC	Green analytical chemistry
GC	Gas chromatography
GC-MS	Gas chromatography coupled with mass spectrometry
GC-MS/MS	Gas chromatography coupled with tandem mass spectrometry
GCB	Graphitised carbon black
HF	Hair follicle
HPLC-DAD	High-performance liquid chromatography coupled to diode-array detector
HS	Hair shaft
IS	Internal standard
L-THP	L-tetrahydropalamatine
LC-MS	Liquid chromatography coupled with mass spectrometry
LC-MS/MS	Liquid chromatography coupled with tandem mass spectrometry
LLE	Liquid-liquid extraction
LLOQ	Lowest limit of quantification
LOD	Limit of detection
LOQ	Limit of quantification
MRM	Multiple reaction monitoring
MS	Mass Spectrometry
MS-MS	Multidimensional mass spectrometry
MSTFA	*N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide
NCOC	Norcocaine

PSA	Primary secondary amine
QuEChERS	Quick, Easy, Cheap, Rugged, and Safe
RE	Relative error
SIM	Selected ion monitoring
SOHT	Society of Hair Testing
SPE	Solid phase extraction
TMCS	Trimethyl chlorosilane
VTA	Ventral tegmental area
µ-QuEChERS	Miniaturized-QuEChERS

Chapter 1 | Literature review

1. Introduction

1.1. Cocaine

1.1.1. Cocaine history

Cocaine (COC) is a natural substance, specifically a tropane alkaloid, found in the leaves of the *Erythroxylum coca* plant. This plant grows naturally in South America, Mexico, Indonesia, and the West Indies [1,2]. The use of coca leaves dates back to ancient civilizations, where they were employed for religious and ceremonial purposes. In these contexts, coca leaves were placed in the cheek and mixed with saliva, producing a local anesthetic effect that was even used during ritual trephinations [1]. The COC alkaloid was first isolated in Germany in the mid-1800s, and by 1884, it had been introduced as a clinical anesthetic. Only one year later, in 1885, reports of "crack" smoking began to appear in medical clinics in the Bahamas. In the following decades, advances in coca cultivation, refining techniques, and new forms of COC self-administration were documented [1,3].

Today, COC is considered the second most widely abused illicit substance worldwide, after cannabis, with an estimated 18 million users [2]. The concomitant use of COC and alcohol is common, since alcohol prolongs COC's effects through the formation of cocaethylene (COET). This metabolite has a half-life of approximately 150 minutes — about one hour longer than COC — and is also associated with increased cardiotoxic and hepatotoxic risks, while reducing some of the dysphoric effects linked to COC use [1]. Street COC samples currently show an average purity of around 40%. This low purity reflects the addition of various adulterants aimed at increasing bulk and, in some cases, enhancing the perceived potency of the drug. Such adulterants include sugars, talc, and cornstarch, but also alcohol and, occasionally, opioids such as heroin, which reflects patterns of polydrug use intended either to minimize negative effects or to intensify the "high" [1,4].

1.1.2. Cocaine addiction and mechanism of action

Addiction to COC is a chronic neurological disorder characterized by compulsive drug-seeking behaviors and high relapse rates during abstinence. COC acts on the central nervous system (CNS), increasing alertness, arousal, and excitement [2]. The addictive

potential of COC is mainly explained by its action on the brain's reward system, which regulates fundamental physiological activities such as sexual behavior, food and water intake, and motivation. This system comprises several brain structures with dopaminergic neurotransmission, including the mesolimbic pathway. Specifically, the mesolimbic dopaminergic pathway involves projections from the ventral tegmental area (VTA) to the nucleus accumbens, playing a central role in COC's rewarding effects. In contrast, the mesocortical pathway, projecting from the VTA to the prefrontal cortex, is more relevant during relapse episodes. Because VTA neurons often project to both targets, the mesolimbic and mesocortical systems overlap, and together are referred to as the mesocorticolimbic system [2,5].

In general, the COC action in the CNS involves blockade of dopamine, norepinephrine, and serotonin reuptake, making it a potent reuptake inhibitor of these neurotransmitters [1,2]. This mechanism accounts for many of its adverse effects, especially on the cardiovascular system. Elevated catecholamine levels can precipitate life-threatening arrhythmias, while the local anesthetic action of COC impairs impulse conduction, favoring re-entry ventricular arrhythmias. Chronic COC use can also lead to histological alterations in cardiac tissue, including fibrosis, myocarditis, and contraction band necrosis [6]. Furthermore, like other local anesthetics, COC blocks the fast sodium current in sensory neurons, slowing or disrupting neural transmission [7].

Treating COC addiction remains highly challenging, with withdrawal symptoms resembling those observed in alcohol and opioid dependence. Various pharmacological approaches have been evaluated, including tricyclic antidepressants, bromocriptine, carbamazepine, and fluoxetine. Tricyclic antidepressants may alleviate anhedonia, dysphoria, depression, and craving, partly through stabilization of adrenergic and dopaminergic receptors by inhibiting catecholamine reuptake [7]. Bromocriptine, a dopamine agonist, can attenuate withdrawal symptoms by counteracting dopamine depletion [7].

Recent advances have explored L-tetrahydropalmatine (L-THP), which interacts with D1, D2, and particularly D3 dopamine receptors. The D3 receptor is a promising target for relapse prevention, making L-THP a potential anti-addiction agent. However, its antagonism at clinically relevant doses remains uncertain, and sedative properties complicate its therapeutic use. Notably, pure D1/D2 antagonists have proven largely ineffective due to side effects such as sedation and anhedonia, and may even increase COC self-administration as patients attempt to overcome receptor blockade [8].

In addition to pharmacological strategies, motivational enhancement and meditation-based interventions—such as acceptance and commitment therapy, dialectical behavior therapy, mindfulness-based stress reduction, and transcendental meditation—have

shown promise, focusing on the psychological dimension of recovery [9]. Importantly, despite ongoing research, no pharmacological treatment has yet been formally approved for COC addiction, with psychosocial approaches remaining the cornerstone of therapy. Historically, COC hydrochloride was also used therapeutically for its potent local anesthetic and vasoconstrictor properties, particularly in otolaryngology during procedures such as bronchoscopy or nasal surgery. The recommended maximal safe doses in adults ranged from 200 to 400 mg [7].

1.1.3. Cocaine pharmacokinetics

The pharmacokinetics of COC vary depending on its chemical/physical form, the route of administration, and co-consumption with other substances such as alcohol. COC hydrochloride, commonly referred to as powder COC, and the free base alkaloid, known as “crack” (named after the crackling sound produced when heated), are the two main forms in which the drug is encountered [1,4,10].

Routes of administration depend on the physical form. COC hydrochloride is mainly insufflated (“snorted”) or administered intravenously, since it is water-soluble. Crack COC, on the other hand, is smoked using glass pipes. When smoked or injected, COC reaches the brain in 6–8 seconds and 10–60 seconds, respectively. Intranasal use produces effects within ~5 minutes, while oral/mucosal administration—less common—has a slower onset. The onset, peak, and duration of effects vary by route: smoking results in a peak within 3–5 minutes and a duration of 5–15 minutes; intranasal use peaks at 15–20 minutes with effects lasting 60–90 minutes [1,4].

Absorption is rapid, but bioavailability depends on the route. Smoked and intravenous COC achieve >90% bioavailability, intranasal ~80%, and oral only ~30%. Distribution to tissues is also rapid, with a volume of distribution between 1–3 L/kg. Approximately 90% of circulating COC binds to albumin and α1-acid glycoprotein. Highest concentrations are found in the brain, spleen, kidney, and lungs, followed by blood, heart, and muscle. COC elimination half-life ranges from 40–90 minutes, depending on the route [1,4].

COC is metabolized via three major pathways. The primary route, accounting for ~50% of the absorbed dose, is hydrolysis by hepatic carboxylesterase 1, producing benzoylecgonine (BEG), the main metabolite and biomarker in toxicological analysis. Hydrolysis by carboxylesterase 2 yields ecgonine methyl ester (EME) [1,4]. A minor pathway involves hepatic *N*-demethylation, generating norcocaine (NCOC), which accounts for ≤5% of metabolism. Two additional metabolites form under specific conditions: COET, produced via transesterification of COC with ethanol (also mediated by carboxylesterase 1), and anhydroecgonine methyl ester (AEME), formed when crack

COC is smoked (Fig. 1). Notably, AEME may also form artifactually during gas chromatography due to high injector temperatures [1,4].

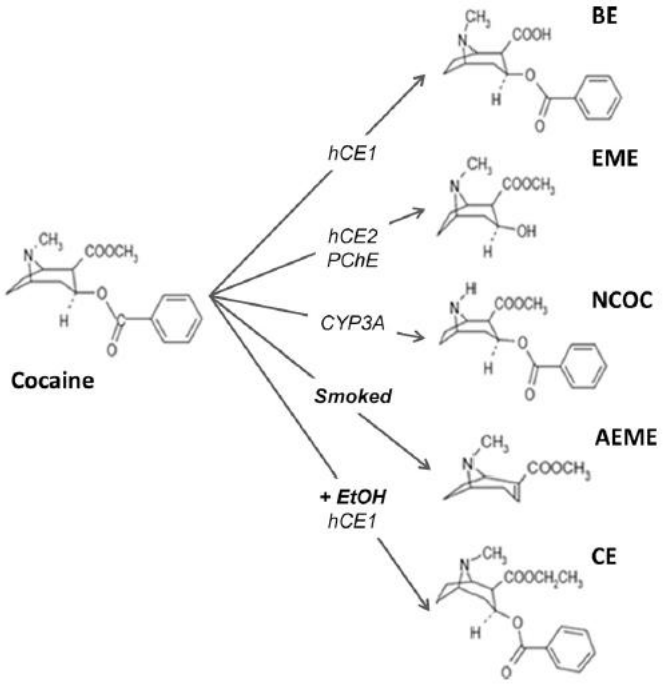
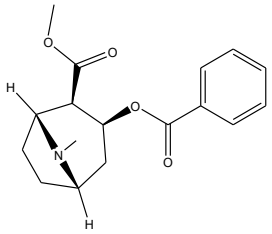


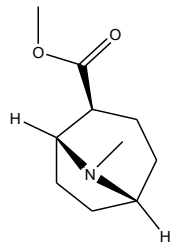
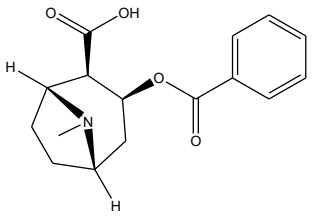
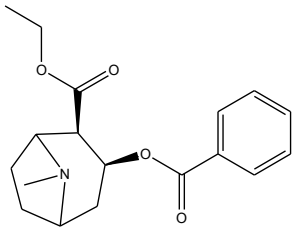
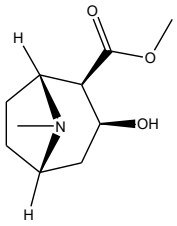
Figure 1 – Schematic representation of the major metabolic pathways of cocaine in humans, including the enzymes involved (CE = cocaethylene; BE = benzoylecgonine; hCE1 = carboxylesterase-1; hCE2 = carboxylesterase-2; PChE = plasma cholinesterase; EtOH = ethanol), adapted from Valente *et al.* [11]

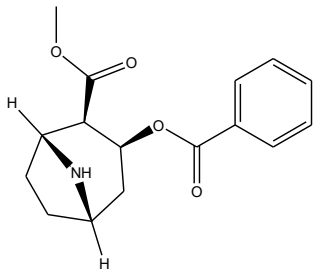
Excretion occurs mainly in urine, with EME and BEG representing 80–90% of metabolites. Only ~9.5–20% of COC is excreted unchanged, detectable in urine for up to 24–36 hours. Minor amounts (1–3%) of *N*-demethylated products are also eliminated. Fecal excretion represents a negligible pathway [1].

Table 1 presents the chemical structures and formulas of COC and the main metabolites, which were previously explained.

Table 1 – Chemical structures and formulas of cocaine and its metabolites.

Chemical Structure	Formula
 Cocaine (COC)	$C_{17}H_{21}NO_4$

 Anhydroecgonine methyl ester (AEME)	C10H13NO2
 Benzoylecgonine (BEG)	C16H19NO4
 Cocaethylene (COET)	C18H23NO4
 Ecgonine methyl ester (EME)	C10H17NO3

 Norcocaine (NCOC)	C16H19NO4

2. Hair

2.1. Hair cycle

The hair coat is mainly composed of proteins (65–95%, predominantly keratin), water (15–35%), lipids (1–9%), and trace minerals (<1%). Its primary role is thermoregulation in mammals, and it undergoes continuous renewal through a cyclical process [12,13]. The hair cycle comprises three phases: anagen (growth), catagen (regression), and telogen (resting). The anagen phase is the longest and determines hair length, as it is the period of active hair shaft (HS) formation. Catagen corresponds to a short transitional stage marked by apoptosis and the cessation of cellular differentiation. Telogen is the resting stage, during which there is minimal biological activity in the follicle and hair shedding occurs [13]. The hair follicle (HF) is the structure responsible for this cyclic activity. It is a specialized mini-organ originating from ectodermal–mesodermal interactions and located within the dermis [14]. During anagen, proliferation of secondary hair bud cells near the dermal papilla (DP) initiates active growth. Histologically, HFs in anagen appear elongated and oriented at an angle that allows hair to lie flat on the skin. At this stage, stromal cells have a rapid cycle of ~18 hours [12,15]. Catagen begins with the cessation of HS growth, progressive decline in proliferative capacity, and activation of apoptotic pathways, leading to rapid follicular regression [15]. Telogen, in contrast, is characterized by minimal HF activity, dormancy of the follicle, and detachment of the HS [13,15].

2.2. Hair in toxicology

The incorporation of drugs of abuse and their metabolites into hair is a complex process influenced by multiple biological, physicochemical, and environmental factors. It is

generally accepted that substances may be integrated into the HS through three main pathways: (i) via the bloodstream supplying the HF during the anagen phase of the hair cycle, (ii) through diffusion from sweat and sebum after the HS has emerged, and (iii) by external environmental contamination. These mechanisms are not mutually exclusive and contribute in varying proportions depending on the specific substance, hair type, and exposure conditions [12,13,16].

i. Incorporation via the bloodstream

During the anagen phase, the HF exhibits high proliferative activity and is richly vascularised by the DP. Drugs present in plasma, following systemic distribution, reach the follicular matrix through this blood supply. Here, keratinocytes undergo rapid cell division, and melanocytes are metabolically active. Drugs and metabolites can diffuse into these cells and become permanently incorporated into the keratin structure of the growing HS [12].

- Lipophilicity: more lipophilic compounds cross cell membranes more efficiently and are incorporated at higher levels.
- Affinity for melanin: basic drugs (e.g. COC, amphetamines, …) show strong binding to melanin in the hair cortex, explaining the higher concentrations typically detected in dark hair compared to lighter hair.
- Protein binding: functional groups enable interaction with keratin proteins, further stabilising drug incorporation.

Thus, the bloodstream is considered the primary incorporation route and provides the closest correlation with systemic drug intake.

ii. Incorporation via sweat and sebum

Once the HS emerges from the skin surface, it remains in direct contact with sweat (from eccrine glands) and sebum (from sebaceous glands). Drugs and metabolites excreted via these secretions may diffuse passively into the outer layers of the HS, particularly the cuticle [17].

- This mechanism is relevant for compounds that are repeatedly consumed, as sweat and sebum can act as a continuous endogenous source of exposure.
- Increased sweating or sebaceous activity can significantly enhance drug incorporation through this pathway.

iii. External contamination (exogenous)

Because hair is exposed to the environment, it can adsorb drugs directly from the atmosphere. Smoke from substances such as cannabis, crack COC, heroin, or methamphetamine may deposit onto the hair surface, with particles or vapour penetrating the cuticle to some extent. Direct contact with drug powders or solutions can

also result in contamination, producing potential false-positive results if adequate decontamination steps are not performed [18].

- This mechanism is particularly important in forensic contexts and underscores the need for strict interpretative criteria.
- To distinguish between environmental exposure and actual consumption, analysis of specific metabolites is essential (e.g. BEG for COC, 11-nor-tetrahydrocannabinol (THC)-COOH for cannabis).

Several parameters influence the extent and mode of drug incorporation into hair:

- Individual characteristics: hair colour, type (straight or curly), melanin content, and growth rate (~1 cm/month on average).
- Physicochemical properties of the substance: pKa, lipophilicity, molecular weight, and solubility.
- Cosmetic treatments: bleaching, dyeing, straightening, or heat exposure can degrade incorporated drugs or produce artefactual metabolites (e.g. formation of AEME from COC).
- Duration of exposure: prolonged or repeated consumption leads to more uniform and persistent incorporation along the HS.

Understanding the mechanisms of incorporation is critical for interpreting analytical results in both forensic and clinical toxicology [12,17]:

- It explains why different hair segments correspond to distinct timeframes of drug use.
- It accounts for interindividual variability, such as higher sensitivity in dark hair.
- It highlights the importance of including washing steps and metabolite analysis to confirm active consumption.
- It helps to identify potential analytical artefacts and the limitations of hair testing.

Hair has gained increasing attention in forensic and clinical toxicology due to its non-invasive collection, long detection window, and stability after death, in contrast with other biological matrices [12]. Sampling is typically performed at the back of the head (*vertex posterior*), where growth variability is minimal (Fig. 2). Although pubic, axillary, or body hair may also be used, interpretation is more complex [12]. Assuming an average growth rate of 1 cm per month, each centimeter of hair represents approximately one month of exposure history, with distal segments reflecting older consumption [19].

Despite these advantages, hair analysis presents limitations. It cannot establish acute impairment or prove that an individual was under the influence at a specific time. Moreover, while it can indicate frequency and severity of exposure, it cannot determine the degree of addiction [19].

Hair testing has wide applications: postmortem toxicology, drug-facilitated crimes, divorce and child custody cases, monitoring in detoxification programs, and insurance investigations. In postmortem contexts, blood and urine are preferred matrices for reflecting short-term exposure. However, in cases of advanced decomposition, hair—particularly the segment closest to the root—may provide evidence of exposure within one to two days before death [19]. Neonatal testing is another relevant application, as drug incorporation in neonatal hair can reveal maternal consumption. Hair testing may also be used in victims of crime, where drug use at the time of the incident could affect testimony reliability [20].

Overall, hair is a reliable matrix for documenting chronic drug use. It is stable, easily transported, and less susceptible to adulteration compared to urine or blood.

In addition, several aspects are particularly relevant for the interpretation of hair analysis in toxicology. Segmental hair analysis allows the reconstruction of drug-use history over time, making it possible to distinguish between continuous, chronic intake and single or sporadic exposures. Another factor influencing drug concentrations is ethnic variability, especially differences in hair pigmentation. Dark, melanin-rich hair tends to incorporate higher levels of basic drugs such as COC and amphetamines, which can lead to interindividual variability and must be considered in forensic contexts. Furthermore, drugs incorporated into the HS are highly stable, remaining detectable for months or even years, which justifies the use of hair as a retrospective biomarker of exposure. Finally, the detection of specific metabolites (e.g. BEG for COC, 11-nor-THC-COOH for cannabis), as said before, is essential to differentiate true drug consumption from environmental contamination, providing stronger forensic evidence.

Nevertheless, it is prone to false-positive results due to environmental contamination, especially from smoked drugs. To minimize this risk, the Society of Hair Testing (SOHT) recommends standardized washing steps to remove superficial contaminants and ensure that detected drugs are incorporated within the HS [16,21]. Various washing protocols exist depending on the analytical technique. Typically, ~50 mg of hair is required per analysis [16,22]. For example, in one study on opiates and COC, hair was washed sequentially (5 min each) with deionized water, petroleum benzine, and dichloromethane. After drying, the sample was cut (~1 cm segments), weighed (~50 mg), and extracted with 4 mL of methanol under ultrasonication for 5 h at 50 °C. Following evaporation and reconstitution in phosphate buffer (pH 6.0), solid-phase extraction (SPE) was performed. Finally, the extract was derivatized with 70 µL of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA), pyridine (30 µL), and iso-octane (100 µL) for 15 min at 90 °C before gas chromatography coupled with mass spectrometry (GC-MS) analysis [23].

Beyond the analytical procedures, the interpretation of hair results requires strict criteria to avoid misclassification. According to the SOHT, the detection of COC in hair alone is not sufficient to establish active use. To confirm consumption, at least one specific metabolite must also be identified, most commonly BEG or NCOC. The presence of AEME can further support cocaine intake by smoking. To minimise the risk of false positives due to external contamination, SOHT has proposed cut-off concentrations, generally set at 0.5 ng/mg for COC and 0.05 ng/mg for metabolites (EME, COET, BEG, and NCOC), which should be applied as interpretative criteria in forensic and clinical toxicology. AEME does not have a proposed cut-off value by SOHT.

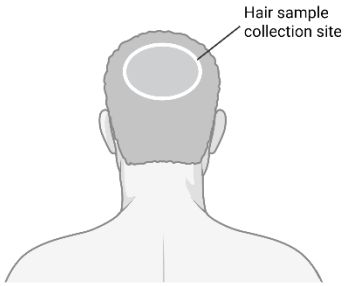


Figure 2 – Schematic representation of the *vertex posterior*

A particular challenge is the metabolite AEME, which forms when COC is smoked (crack or basuco). Both forms undergo thermal degradation, with conversion of COC to AEME reaching 50–80% between 255–420 °C and >80% above 650 °C [24]. However, AEME may also appear artifactually in gas chromatography (GC) due to high injector temperatures. In addition, cosmetic treatments such as hair straightening can alter COC and AEME concentrations. Restrepo *et al.* [10] reported a 56.9% decrease in COC after thermal hair treatment, sometimes reducing levels below the SOHT cut-off, producing false negatives. Simultaneously, AEME concentrations increased by 20.8–626.7% (median 110.3%), indicating that cosmetic procedures can significantly affect hair toxicology results [10].

3. QuEChERS

3.1. QuEChERS method and green analytical chemistry

Given that the QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) technique will be applied in the experimental part of this dissertation, a brief introduction to its theoretical basis, workflow, advantages, and applications in clinical and forensic toxicology is presented below.

The QuEChERS method is a simple and versatile sample preparation technique that combines a partitioning step with a subsequent clean-up using dispersive solid-phase extraction (d-SPE). First introduced in 2002 at the European Pesticide Residue Workshop (EPRW) in Rome, and published by Anastassiades *et al.* [25], the original QuEChERS approach was developed for the extraction of pesticide residues — ranging from non-polar to very polar compounds — from fruits and vegetables [26]. Since its introduction, QuEChERS has gained wide acceptance due to its numerous advantages, including compliance with principles of "green chemistry" [27], operational simplicity, flexibility in terms of sample size, and rapid execution [28]. Owing to these benefits, its application has quickly expanded beyond pesticide analysis to include other analytes, such as antidepressants, amines, and environmental pollutants, as well as to different matrices, including biological fluids [29–31] [26].

The QuEChERS procedure is based on a salting-out extraction, in which the addition of salts promotes partitioning between an aqueous and an organic phase, followed by a d-SPE clean-up step. In its original form, a 10 g homogenised sample was extracted with 10 mL of acetonitrile (ACN), vigorously shaken, and partitioned with magnesium sulphate ($MgSO_4$) and sodium chloride (NaCl) to enhance phase separation. After centrifugation, the upper layer was subjected to d-SPE using porous sorbents such as primary secondary amine (PSA), combined with $MgSO_4$, to remove matrix interferences (Fig. 3). The resulting extract could then be analysed, for example, by GC-MS [26,32].

Over the years, several modifications and miniaturised adaptations of QuEChERS have been developed to improve selectivity and broaden its applicability. As reviewed by Rivai *et al.* [33], these variations can be tailored to the analyte and matrix under investigation. For instance, Dybowski *et al.* [34], applied the method to whole blood samples for the determination of Δ^9-THC using $MgSO_4$ and NaCl, whereas Campêlo *et al.* [35], adapted the AOAC 2007.1 version of QuEChERS with sodium acetate (NaOAc) and $MgSO_4$ for the determination of antidepressants in post-mortem blood samples [33].

Other studies highlight the diversity of reagents that can be employed in QuEChERS. Zhang *et al.* [36], for example, emphasised the effectiveness of ACN as extraction solvent, discouraging the use of ACN/methanol mixtures, as ACN alone provides satisfactory recoveries. pH control can be achieved with acetic acid (1–5% v/v) or formic acid (1–5% v/v), added to ACN in the extraction step, as described by Pelixo *et al.* [37]. For partitioning, alternatives to $MgSO_4$/NaCl such as $MgSO_4$/sodium sulphate (Na_2SO_4) or ammonium formate (NH_4HCO_2) can be used. In the clean-up step, PSA remains the most widely employed sorbent, as in the original protocol, but NH_2 and octadecyl silica (C18) are also reported as effective alternatives [36,37].

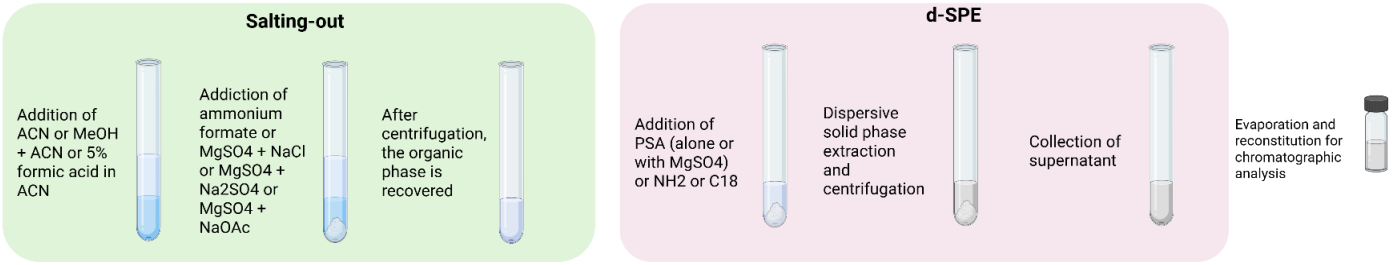


Figure 3 – Schematic representation of the main steps involved in the QuEChERS clean-up technique

Miniaturised adaptations of the QuEChERS method have been developed not only to make the procedure faster and more cost-effective but also to enhance its environmental sustainability. These adaptations often reduce sample and solvent volumes while maintaining acceptable recoveries, precision, and accuracy. Such approaches are particularly relevant for matrices that naturally yield low volumes of extract, such as fruit by-products or certain biological samples. For instance, Mateus *et al.* [38], applied a miniaturised QuEChERS protocol for the determination of 105 pesticide residues. Because fruit by-products typically yield low extract volumes (<10%), the standard method requiring 10 g of sample was not feasible. In their adaptation, only 100 mg of sample and 1 mL of ACN were used, followed by vortex mixing and an ultrasound bath. Partitioning was achieved with a mixture of $MgSO_4$, NaCl, sodium citrate, and disodium hydrogen citrate sesquihydrate, and the extract was cleaned up using PSA and $MgSO_4$ before analysis. Similarly, Yamasaki *et al.* [39] investigated pesticide residues in fruits and vegetables using three approaches: one conventional (15 g sample) and two miniaturised protocols (2 g and 0.5 g sample). The smaller-scale extractions used

proportionally reduced amounts of ACN (with 1% acetic acid) and salts ($MgSO_4$ and NaOAc). Interestingly, these protocols did not include a d-SPE step, highlighting that some studies labelled as "QuEChERS" may in fact omit essential components of the original method, limiting their comparability.

In toxicology, QuEChERS has been successfully applied not only to classical biological matrices such as blood, plasma, and urine, but also to alternative specimens including hair, nails, oral fluid, and breast milk. Kim *et al.* [39] demonstrated its suitability for hair and nail samples, achieving excellent intra- and inter-day precision (0.3–12.3%) and accuracy (93.3–105%) for nicotine metabolites. Similarly, Júnior *et al.* [40] applied the method to oral fluid for the detection of psychoactive drugs, reporting limits of quantification (LOQs) of 0.1–1.5 ng/mL, limits of detection (LODs) of 0.04–0.5 ng/mL, recoveries of 80–120%, and acceptable precision (<20%). Di Trana *et al.* [41] further showed that oral fluid often yielded better recoveries and lower LODs for hexahydrocannabinol compared with blood or urine, although blank oral fluid matrices presented higher baseline noise. Breast milk has also been explored: Pajewska-Szmyt *et al.* [42] successfully applied QuEChERS to detect polychlorinated biphenyls, with recoveries above 96% and favourable precision and reproducibility values.

In this study, hair was used as the principal matrix, and the application of QuEChERS to hair samples remains relatively scarce. A study by Pelixo *et al.* [37] on ketamine and norketamine demonstrated the feasibility of this approach, as favourable LOD and LOQ values were achieved (0.01 and 0.05 ng/mg for ketamine, respectively, and 0.8 ng/mg for both limits in the case of norketamine). Reported recoveries ranged from 47–76% for ketamine and 14–27% for norketamine, which were considered satisfactory. Despite these promising results, reviews have highlighted an important caveat: many studies claim to employ QuEChERS but apply only part of the procedure, frequently omitting the d-SPE clean-up step [43]. Such inconsistencies complicate cross-study comparisons and emphasise the need to clearly distinguish between the complete QuEChERS method and partial adaptations.

This technique is considered a green approach, as it is one of the most promising, user-friendly, and high-throughput extraction procedures. It requires reduced amounts of sample, solvents, and laboratory glassware, aligning with the principles of green chemistry originally presented by Anastas and Warner in 2000 [44]. In 2013, these principles were adapted by Gałuszka *et al.* [27] to address the needs of analytical chemistry, since only four of the original twelve principles could be directly applied [26,27].

These four were: prevention of waste (principle 1), safer solvents and auxiliaries (principle 5), design for energy efficiency (principle 6), and reduction of derivatisation (principle 8).

Building on this, Gałuszka *et al.* [27] reformulated the framework into 12 principles of Green Analytical Chemistry (GAC), which are [27]:

1. Direct analytical techniques should be applied to avoid unnecessary sample treatment.
2. Minimal sample size and minimal number of samples should be used.
3. *In situ* measurements should be performed whenever possible.
4. Integration of analytical processes and operations saves energy and reduces reagent consumption.
5. Automated and miniaturised methods should be selected.
6. Derivatisation should be avoided.
7. The generation of large volumes of analytical waste should be avoided, and appropriate waste management must be ensured.
8. Multi-analyte or multi-parameter methods are preferred over single-analyte methods.
9. Energy consumption should be minimised.
10. Reagents obtained from renewable sources should be prioritised.
11. Toxic reagents should be eliminated or replaced.
12. Operator safety should be maximised.

To facilitate their application, the authors proposed the mnemonic “SIGNIFICANCE”, which summarises the 12 principles of GAC [27]:

S – Select direct analytical techniques
I – Integrate analytical processes and operations
G – Generate as little waste as possible and manage it properly
N – Never waste energy
I – Implement automation and miniaturisation
F – Favour reagents from renewable sources
I – Increase operator safety
C – Carry out in situ measurements
A – Avoid derivatisation
N – Note that sample size and number should be minimal
C – Choose multi-analyte or multi-parameter methods
E – Eliminate or replace toxic reagentes

4. Gas chromatography coupled with tandem mass spectrometry

Since this dissertation employed a chromatographic method—specifically gas chromatography coupled with tandem mass spectrometry (GC-MS/MS)—an introduction to the technique is provided here, followed by an overview of GC-MS and GC-MS/MS, their components, differences, and respective advantages and limitations.

Chromatography is based on the principle that different molecules in a mixture applied onto a stationary phase will separate from one another under the influence of a mobile phase. Separation occurs due to differences in molecular properties such as adsorption, partition, affinity, or molecular weight [45].

In gas chromatography (GC), the stationary phase is typically a column containing a liquid phase absorbed onto the surface of an inert solid. The carrier gas (commonly helium or nitrogen) constitutes the mobile phase, into which the vaporised sample is introduced. GC is a versatile, highly sensitive, and rapid technique that enables the separation of small amounts of analytes (Fig. 4) [45].

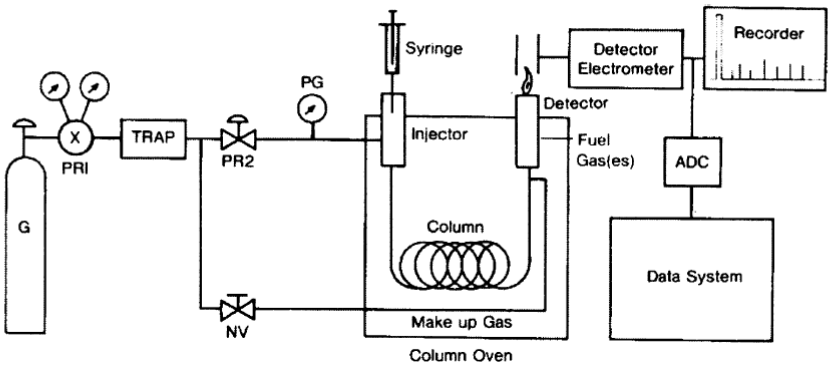


Figure 4 – Schematic diagram of a basic gas chromatograph, adapted from Bartle *et al.* [46]

One of the most widely applied analytical techniques for complex mixtures is GC coupled with mass spectrometry (GC-MS). This approach combines the separation capacity of chromatography with the sensitivity and accuracy of mass spectrometry (MS). However, only compounds that are sufficiently volatile can be analysed by GC-MS. When a tandem mass spectrometer is employed, the technique is referred to as GC-MS/MS. Compared with GC-MS, GC-MS/MS provides significantly greater selectivity without a substantial loss of sensitivity [47].

According to *Fundamentals of Analytical Chemistry* by Skoog et al. [48], the ideal detector for GC should demonstrate:

1. Adequate sensitivity (10^{-8}–10^{-5} g of solute/s);

2. Good stability and reproducibility;
3. A linear response to solutes across several orders of magnitude;
4. A broad temperature range (ambient to ≥400 °C);
5. A short, flow-independent response time;
6. High reliability and ease of use, tolerating inexperienced operators;
7. Uniform response to all solutes, or alternatively predictable selectivity for specific classes;
8. Non-destructive analysis of the sample.

Modern detectors include flame ionisation, thermal conductivity, electron capture, thermionic, and mass spectrometers. In this work, the detector employed was a mass spectrometer.

The most common ionisation source in GC-MS is electron impact (EI), in which molecules are bombarded with high-energy electrons, producing positive and negative ions as well as neutral fragments. This high degree of fragmentation is particularly useful for compound identification. Chemical ionisation is also used in conjunction with GC-MS, although less frequently.

Tandem mass spectrometry (MS/MS) involves the transfer of ions from one mass analyser into a second for further ionisation and separation prior to detection. In a triple quadrupole instrument, the first quadrupole acts as in conventional GC-MS, the second quadrupole serves as a collision cell to generate product ions, and the third quadrupole separates the resulting ions before detection via an electron multiplier (Fig. 5) [49].

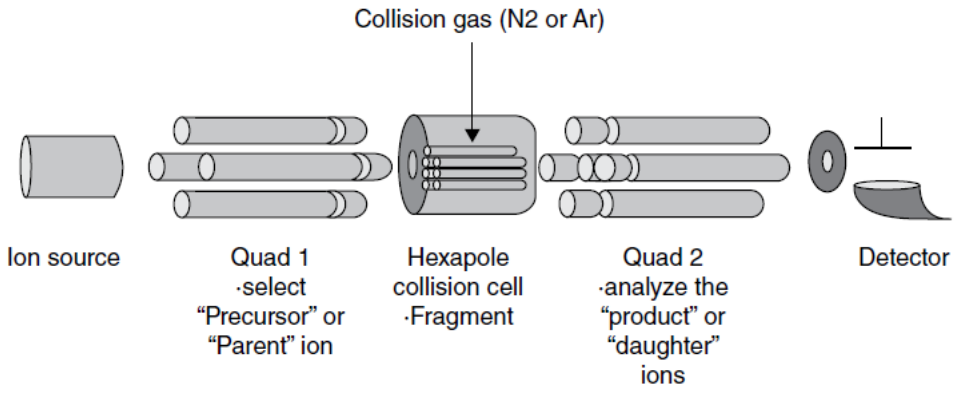


Figure 5 – Schematic diagram of a triple quadrupole mass spectrometer, adapted by McNair *et al.* [49]

- The main advantages of the triple quadrupole usually used in GC-MSMS over the GC-MS technique include:
- In qualitative analysis, the first quadrupole can be scanned, a daughter ion selected, re-ionised in the second quadrupole, and then scanned in the third.

- In selected ion monitoring (SIM), additional selectivity can be achieved by re-ionising the selected ions in the second quadrupole before analysis in the third.
- When both the first and third quadrupoles are operated in SIM mode, sensitivity improvements of one to two orders of magnitude can be achieved compared to traditional SIM.

GC-MS/MS is particularly advantageous when:

- High chemical noise complicates spectra in SIM mode;
- Characteristic ions co-elute with isobaric impurities;
- Additional structural information is required for unknown compounds;
- SIM fragmentograms require confirmatory data;
- Greater sensitivity and specificity are necessary [50].

Different acquisition modes are employed:

- Full-scan, where the first analyser records all mass spectra of ionised analytes;
- Selected Ion Monitoring (SIM), where only selected ions characteristic of target analytes are monitored, offering higher sensitivity but reduced scope;
- Multiple Reaction Monitoring (MRM), which enhances sensitivity and specificity by monitoring selected precursor/product ion transitions, and is widely used for semi- and fully quantitative applications [50].

The SIM acquisition method is more commonly applied in single quadrupole mass spectrometers. In this approach, the first analyser (MS1) transmits only a restricted number of preselected ions characteristic of the target analyte, thereby enhancing sensitivity for specific compounds within a complex mixture [50].
Another widely used acquisition mode is Multiple Reaction Monitoring (MRM), which enables improved sensitivity by effectively removing background noise associated with non-target compounds. Owing to its ability to provide high selectivity and quantitative accuracy, MRM has gained increasing popularity and is now routinely applied for both semi-quantitative and fully quantitative analyses [51].
Despite its clear analytical benefits, GC-MS/MS presents several disadvantages compared with conventional GC-MS. These include high capital costs, the need for highly trained operators, and significantly greater system maintenance requirements [49].
According to McNair *et al.* [49], the general advantages and disadvantages of GC itself may be summarised as follows:
Advantages:

- Rapid analysis, typically within minutes;

- High efficiency, providing excellent resolution;
- Requires only small sample volumes (µL).

Disadvantages:

- Restricted to volatile compounds;
- Limited applicability for thermally labile analytes;
- Requires coupling with mass spectrometry for unambiguous peak identification.

When selecting GC-MS/MS as the analytical technique for this work, several considerations were taken into account. In comparison with liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), which is often preferred for non-volatile or thermally labile compounds, GC-MS/MS offers excellent separation efficiency and sensitivity for analytes that are sufficiently volatile and thermally stable. The reliability of this technique also depends on the use of appropriate sample preparation methods (e.g., QuEChERS, SPE, or liquid-liquid extraction (LLE)), and in some cases, derivatization may be required to improve volatility and thermal stability. Among acquisition modes, MRM is regarded as the gold standard for quantitative analysis in complex matrices, since it allows simultaneous monitoring of quantifier and qualifier ions, thereby improving selectivity and confidence in results. Ultimately, the choice of SIM or MRM must be supported by full method validation, including evaluation of LOD, LOQ, linearity, accuracy, precision, selectivity, and robustness. Despite its advantages, GC-MS/MS is limited to volatile and thermally stable analytes, which explains why LC-MS/MS is often preferred in forensic and pharmaceutical toxicology. Nevertheless, in the context of this study, GC-MS/MS provided the optimal balance of sensitivity, selectivity, and robustness for the compounds under investigation.

Chapter 2 | Aims

The main objective of this dissertation is to develop and validate a miniaturised extraction and clean-up method based on QuEChERS (µ-QuEChERS) for the determination of COC and its major metabolites (BEG, EME, NCOC, COET, and AEME) in hair samples, using GC-MS/MS.

The specific objectives are to:

1. Optimise the µ-QuEChERS method conditions through a design of experiments (DoE), evaluating the effect of solvent volume, partitioning salts, and sorbents used in the d-SPE step.

2. Assess the validation parameters of the method (selectivity, linearity, limits of detection and quantification, precision, and accuracy), in accordance with international recommendations for forensic toxicology.
3. Apply the developed method to authentic hair samples obtained from real-case scenarios, in order to confirm its applicability in clinical and forensic toxicology.
4. Compare the performance of the proposed method with conventional extraction approaches, highlighting the advantages of μ-QuEChERS in terms of speed, simplicity, cost-effectiveness, and compliance with green chemistry principles.
5. Demonstrate the potential scalability and implementation of the method in routine toxicology laboratories, and explore its applicability to other drugs of abuse in hair.

Chapter 3 | Experimental procedure

1. Materials and methods

1.1. Reagents and Standards

The analytical standards for AEME, EME, COC, COET, BEG, and NCOC, along with the deuterated IS EME-d3, COC-d3, COET-d3, and BEG-d3, were obtained from Sigma-Aldrich (Sintra, Portugal). Methanol, dichloromethane, and ACN were acquired from Fisher Scientific (Loughborough, UK). Potassium dihydrogen phosphate and dipotassium phosphate were purchased from Enzymatic (Santo Antão do Tojal, Portugal). Formic acid and ammonium acetate (NH_4OAc) were sourced from Sigma-Aldrich (Sintra, Portugal), while the PSA, $MgSO_4$, NaOAc, and NH_4HCO_2 were obtained from Laborspirit (Sintra, Portugal). Deionized water was supplied by a Milli-Q system (Millipore, Billerica, MA, USA).

The standard stock solutions had a concentration of 100 μg/mL. Working solutions were prepared by diluting the standard stock solutions to concentrations of 100 ng/mL, 1 μg/mL, and 10 μg/mL in methanol, while the internal standard (IS) solution was prepared by diluting the 100 μg/mL stock solution to 2.5 μg/mL.

MSTFA and trimethyl chlorosilane (TMCS) were purchased from Macherey-Nagel (Düren, Germany), while the microwave applied for the derivatization was acquired from Samsung (Lisbon, Portugal).

1.2. Hair samples

For method optimisation and validation, blank hair samples were required and were obtained from staff members of RISE-Health (Covilhã, Portugal) who had no prior exposure to COC. Authentic hair samples were collected from individuals identified as drug users during an electronic music festival that took place in Portugal from 22 to 29 July 2022. The collection process complied with the principles of the Declaration of Helsinki and received approval from the Ethics Committee of Universidade da Beira Interior (approval code CE-UBI-Pj-2022–04).

All hair samples were collected from the posterior vertex region, cut as close to the scalp as possible using sterilised scissors. Afterwards, they were placed in paper envelopes, stored at room temperature, shielded from sunlight, and appropriately coded, ensuring no personal identification of the donors.

1.3. Chromatographic and mass spectrometry conditions

Chromatographic analyses were carried out using an Agilent Technologies HP 7890A gas chromatograph coupled to an Agilent Technologies 7000B triple quadrupole mass spectrometer (QqQ MS, Waldbronn, Germany). Separation was achieved on a 30 m length and 0.25 mm internal diameter capillary column with a 0.25 μm film thickness, coated with (5 %-phenyl)-methylpolysiloxane (Agilent J&W, Santa Clara, CA, USA). The oven temperature began at 90 °C (held for 2 min), followed by a ramp of 20 °C/min up to 300 °C, where it was held for 3 min, giving a total run time of 15.50 min.

The injector was maintained at 240 °C, and 2 μL of sample was introduced in splitless mode, using helium as the carrier gas at a flow rate of 0.8 mL/min. The ion source and detector were kept at 230 °C and 280 °C, respectively. In the collision cell, helium and nitrogen were supplied at 1.5 and 2.5 mL/min, respectively. Electron ionisation was applied at 70 eV with a filament current of 35 μA. Data acquisition and processing were performed with MassHunter Workstation software (version B.02.01, Agilent Technologies).

The transitions, retention times, collision energies (of both transitions), and dwell times are detailed in Table 2.

Table 2 – MS detection parameters

Analyte	Retention time (min)	Quantitative transition (m/z)	Qualitative transition (m/z)	Collision energy (eV)	Dwell time (μs)

AEME	7.13	151.5 – 92.0	180.5 – 152.1	20 (15)	50
EME	8.19	271.5 – 83.1	181.3 – 82.0	5 (10)	50
EME-d3	8.19	274.5 – 86.1	-	10	50
COC	11.65	182.5 – 82.2	182.5 – 150.1	10 (5)	50
COC-d3	11.65	184.1 – 85.0	-	10	50
COET	11.88	196.5 – 82.0	196.5 – 150.2	10 (5)	50
COET-d3	11.88	199.1 – 199.1	-	5	50
BEG	11.90	238.8 – 82.2	238.8 – 122.2	20 (20)	50
BEG-d3	11.90	241.9 – 85.1	-	20	50
NCOC	11.98	178.1 – 105.1	178.1 – 135.1	15 (10)	50

* ( ) collision energy used for the qualitative transition.

1.4. Sample preparation

The preparation of samples began with a decontamination step, in which the hair was sequentially washed for 15 minutes each with dichloromethane, deionised water, and methanol, using a tube roller mixer (70 rpm). After every wash, the solvent was discarded and the hair dried with paper. Once the final wash was complete, the samples were left to air-dry overnight. For authentic samples, the last wash was stored for further analysis. On the following day, the dried hair was cut into small fragments, and 50 mg was weighed into glass tubes. Each portion was then extracted with 2 mL of methanol. The mixture was vortexed to ensure homogenisation and subsequently incubated overnight at 65 °C. The supernatant was collected into a clean glass tube, to which 15 µL of IS (2.5 µg/mL) was added. The solvent was evaporated under a nitrogen stream, and the resulting residue was reconstituted in 1 mL of 0.1 M phosphate buffer (pH 6.25) before proceeding to the clean-up step.

The final µ-QuEChERS procedure (Fig. 6) was established as follows: the reconstituted sample in 1 mL of phosphate buffer (pH 6.25) was combined with 625 mg of NH_4HCO_2 and 3 mL of 5% formic acid in ACN. The mixture was vortexed for 10 s and centrifuged at 3500 rpm for 2 min. The resulting supernatant was transferred to a new tube, to which 175 mg of $MgSO_4$ and 55 mg of PSA were added. The vortexing and centrifugation steps were repeated under the same conditions, and the supernatant was collected once more before being evaporated under a nitrogen stream. Finally, the residue was reconstituted with 50 µL of MSTFA + 5% TMCS derivatising agent using a microwave (2 min at 800W) and injected into the gas chromatographic system.

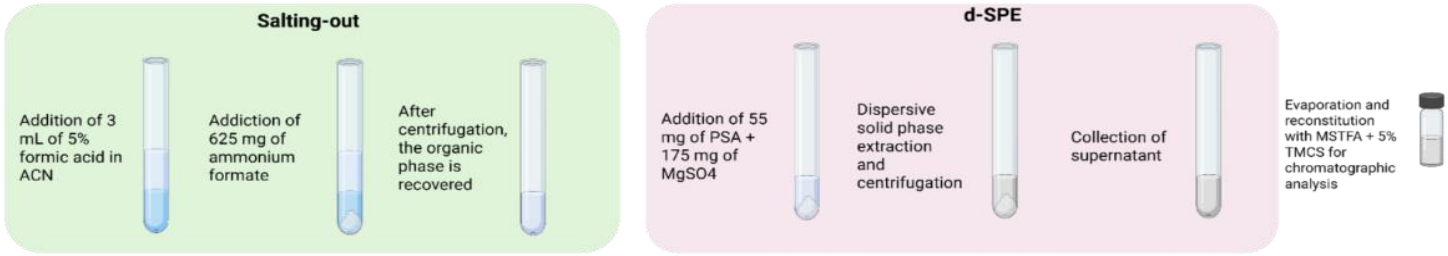


Figure 6 – Schematic representation of the final µ-QuEChERS sample preparation procedure

1.5. µ-QuEChERS clean-up method optimization

A DoE approach was then applied in Minitab version 17 to fine-tune the amounts of salts and solvents used. The variables considered included solvent volume (5% formic acid in ACN), salting-out salt quantity (NH_4HCO_2), d-SPE salt quantity ($MgSO_4$), and d-SPE sorbent quantity (PSA).

2. Results and discussion

2.1. µ-QuEChERS clean-up method development and optimization

The QuEChERS method relies on salt dispersion for extraction (salting-out effect) and on clean-up processes to isolate a wide range of analytes from various complex matrices [26]. Therefore, developing this technique requires a systematic process, starting with

assessing each component's ability to remove interferents, then selecting the most suitable materials. Lastly, optimizing component amounts is crucial to enhance extraction efficiency.

2.1.1. µ-QuEChERS partition components selection

A QuEChERS method previously validated and developed in the laboratory for ketamine [37] was adapted and used since it had already been successfully used for the determination of drugs on hair samples. The latter was based on the AOAC 2007.01 official method for pesticides [52].

The method was applied following the complete hair preparation described in section 1.4 and served as the baseline for developing the clean-up steps of hair extracts fortified with COC and all its metabolites of study. Modifications to the previous method were necessary to adjust the protocol for COC and its metabolites determination in hair using GC-MS/MS. Key differences from the AOAC official method included the sample amount, salt quantities in the salting-out step, adjustments to d-SPE salt quantities, the use of vortex mixing, and centrifugation parameters.

To guide the optimisation of the µ-QuEChERS method to determine COC and its metabolites, a literature review was conducted to identify appropriate partitioning components [26,53,54], integrated with detailed analysis of the previous method developed in the laboratory [37]. The literature review was structured in two stages: (1) assessment of the clean-up efficiency of different salts during the salting-out step, and (2) assessment of the same property for various salting-out solvents. Since the approach applied to COC was consistent with that of the earlier laboratory method [37], the same partitioning components were ultimately selected. A detailed explanation of the component selection is provided in the following section.

The selection of salts was initially guided by their ability to promote efficient phase separation with ACN, which had been pre-selected as the organic solvent due to its favorable separation from the aqueous phase [25]. Based on the literature, $MgSO_4$, NaOAc, and NH_4OAc were tested individually, as they are commonly employed in QuEChERS protocols. NH_4HCO_2 was also included owing to its high volatility, which reduces analytical interferences and enhances chromatographic performance [36]. The partitioning efficiency of these salts was experimentally evaluated in the laboratory, as described in [37]. Three sets of experiments were carried out with the following salt combinations: (1) 0.4 g $MgSO_4$ with 0.1 g NaOAc, (2) 0.5 g NH_4OAc, and (3) 0.5 g NH_4HCO_2 [37]. Each experiment was performed in triplicate, and the average abundances obtained were statistically assessed using Friedman's test in SPSS Statistics, a non-parametric pairwise comparison method. Among the tested salts, NH_4HCO_2

demonstrated superior performance due to its volatility, which minimised equipment contamination, enhanced analyte recovery, and produced higher relative abundances. Conversely, $MgSO_4$ was found to deposit within the GC liner, impairing analysis because of its low volatility [26,37]. For these reasons, NH_4HCO_2 was selected as the partitioning salt for the extraction of COC and its metabolites, consistent with its effectiveness in the extraction of other drugs of abuse in previously developed methods [37].

A solvent optimisation study had also been carried out previously in the laboratory. ACN was chosen as the primary candidate owing to its well-documented compatibility with aqueous matrices. Other solvents, including ethyl acetate, dichloromethane, and hexane, were excluded to enhance the environmental sustainability of the method. Since acidification is a common strategy to improve the extraction of alkaline analytes such as COC and its metabolites [26], three solvent systems were evaluated following the same approach as for the salts: (1) ACN, (2) ACN with 1% acetic acid, and (3) ACN with 5% formic acid. Among these, ACN with 5% formic acid was identified as the most effective and selected as the extraction solvent [37].

In the previous study, the d-SPE step considered PSA, C18, and graphitised carbon black (GCB), based on literature reports. GCB is mainly used for samples with high pigment content, such as carotenoids and chlorophyll, whereas C18 is effective for lipid-rich matrices. PSA was selected for its broad-spectrum capacity, efficiently removing sugars, lipids, organic acids, fatty acids, and certain pigments [26]. Although $MgSO_4$ is associated with low volatility, its ability to reduce water content made it a valuable addition to the d-SPE step, particularly since smaller amounts are required compared to the salting-out stage [25]. Consequently, PSA and $MgSO_4$ were chosen for d-SPE, consistent with the previously developed in-house method [37], and were considered effective for the extraction of COC and its metabolites.

2.1.2. µ-QuEChERS optimisation with DoE factorial design

Once the solvents and reagents were selected, the quantities of each constituent were optimised. To achieve this, a DoE approach was employed, using a 2-level full factorial design. The parameters and their respective low and high limits were established based on previous literature (Table 3).

Table 3 – Parameters and limits for the factorial design.

Factor	Low	High
5% formic acid in ACN volume (mL)	1	5
NH4HCO2 amount (g)	0.250	1
MgSO4 amount (g)	0.05	0.3

PSA amount (g)	0.01	0.1

The experiments, designed by the software to assess the interaction of multiple factors, were executed accordingly, with the respective responses recorded. Analysis of the Pareto chart (Fig. 7) revealed no significant variation in the clean-up procedure, for all analytes, except for AEME, that presented a significant response for the 5% formic acid in ACN (represented by "ACN") factor. By observing the main effects plot, all the factors, except for the 5% formic acid in ACN (represented by "ACN") factor showed a non-significant variance when the amount of reagent used was low or high. On the other hand, regarding the "ACN" factor, it was possible to verify that a greater amount used resulted in a better the response (Fig. 8). The interaction plot further indicated that almost all factors significantly influenced each other's, except for the interaction between NH_4HCO_2 (represented by "formiato") and $MgSO_4$, within the limits established for the DoE (Fig. 9). The Pareto charts, main effect plots, and interaction plots obtained for COC and all the other metabolites can been observed in the end, in the Annexes.

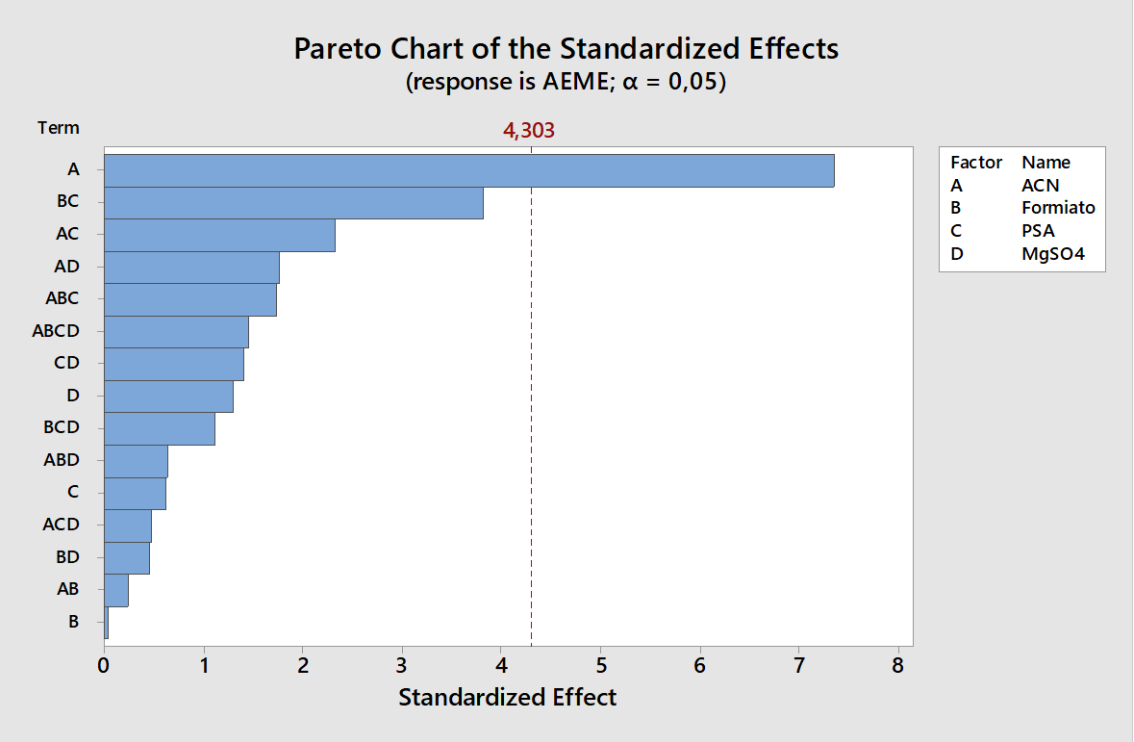


Figure 7 – Pareto chart of the standardized effects for AEME

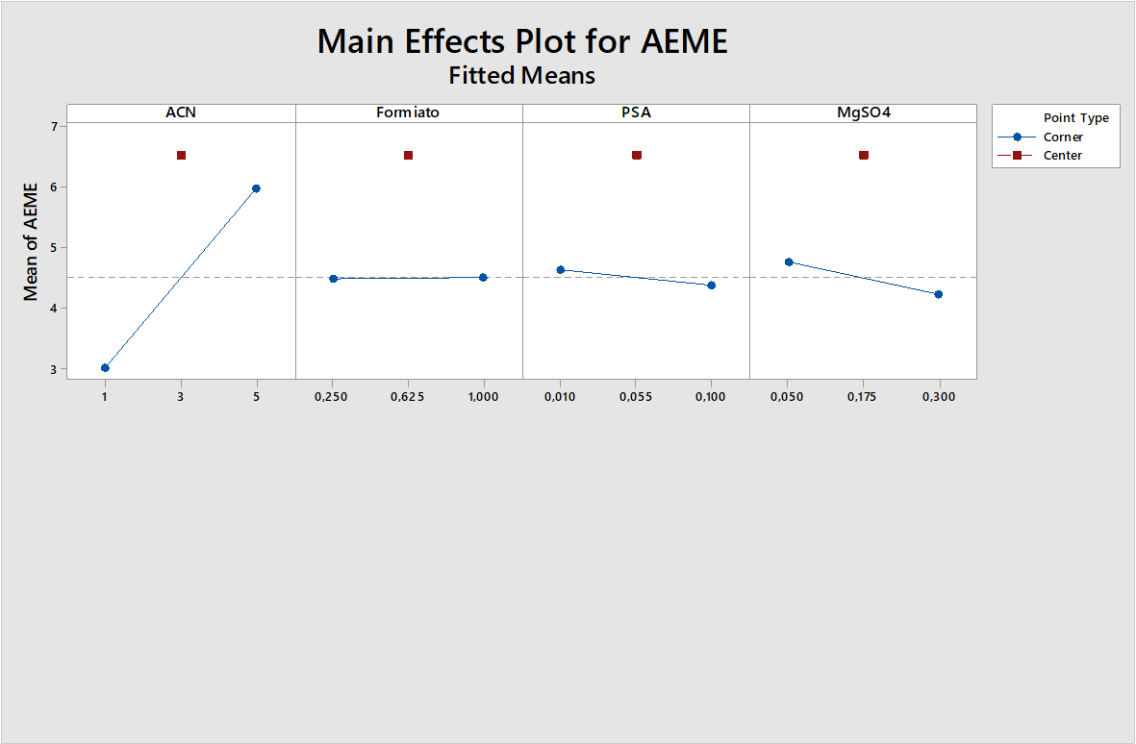


Figure 8 – Main effects plot for AEME

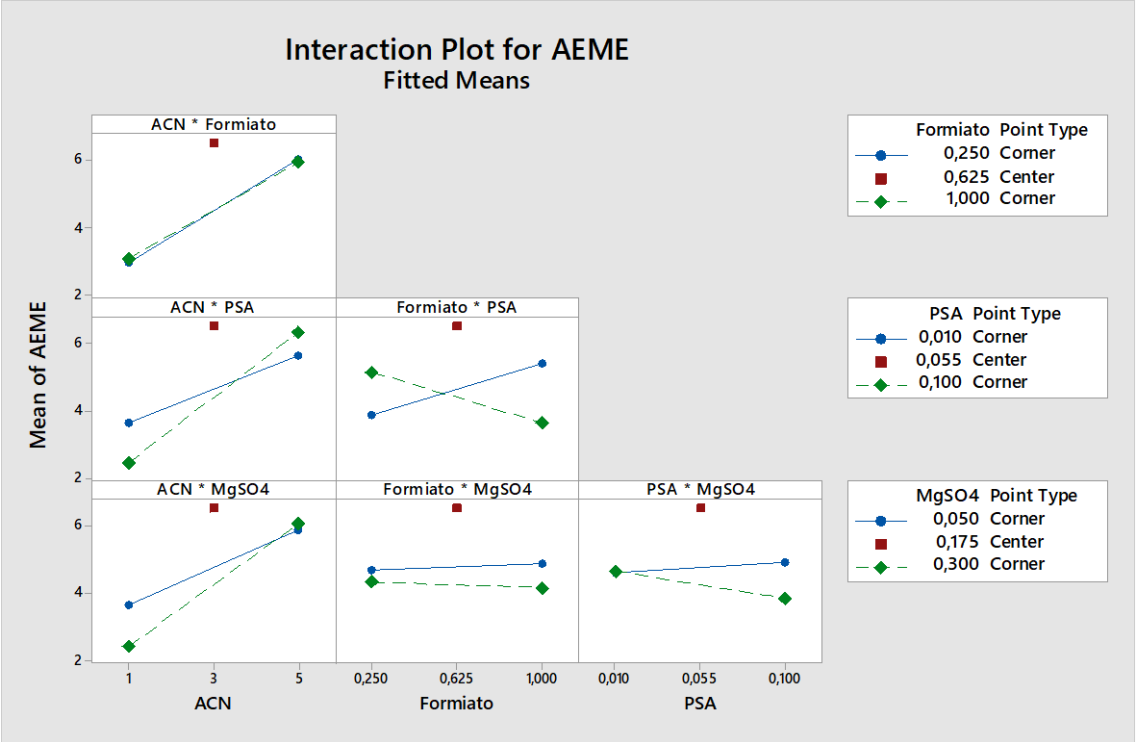


Figure 9 – Interaction plot for AEME

It is important to highlight that the analysis of the AEME Pareto chart, main effect, and interaction plots revealed the best protocol by using 5 mL of 5% formic acid in ACN, and the minimum evaluated amounts of the other reagents since they didn't produce

significant effect when their amounts increased. But with the analysis of all the Pareto charts, main effect plot, and interaction plot for all the other analytes, it was concluded that the best configuration would be using the quantities described in section 1.4.
This way, the final μ-QuEChERS optimized protocol involved adding 625 mg of NH_4HCO_2 and 3 mL of 5% formic acid in ACN to the reconstituted sample, followed by agitation in a vortex mixer and centrifugation at 3500 rpm for 2 min. After the centrifugation, the organic layer was collected, and afterwards, 175 mg of $MgSO_4$ and 55 mg of PSA were added and subsequently agitated with a vortex mixer and centrifuged. The supernatant was then evaporated under a nitrogen stream and finally reconstituted in 50 μL of MSTFA + 5% TMCS derivatizing agent using a microwave (2 min at 800W) for the derivatization for further chromatographic analysis.

2.2. Validation

The validation of the analytical method was carried out considering the guidelines provided by SOHT for drug testing in hair [55] and following the ANSI/ASB Standard 036 (1st edition) — Standard Practices for Method Validation in Forensic Toxicology [56]. The validation process involved evaluating several parameters, including selectivity, linearity, limits, precision (within-run, between-run, and combined within-run and between-run), accuracy, and recovery.

2.2.1. Selectivity

Selectivity describes how well a method can identify the target analyte without interference from the sample matrix or the preparation process. Following the established guidelines, this parameter was evaluated using blank hair samples from ten different donors, without the addition of the IS, to confirm the absence of interfering signals at the relevant retention times and transitions. For comparison, a pooled sample containing the IS together with AEME, EME, COC, COET, BEG, and NCOC at their respective lowest limit of quantification (LLOQ) was also analysed. No significant interferences were observed at the retention times or in the selected ion transitions of any analyte, therefore, the method was considered selective for all analytes.

2.2.2. Linearity and limits

To evaluate linearity, a pool of ten blank samples previously examined for selectivity was fortified to establish a working range, which included eight calibrators for all analytes, except for AEME, which only had six. The working range for AEME was 0.5–5 ng/mg, while for EME, COC, COET, BEG, and NCOC was 0.05–5 ng/mg. These working ranges

were tested over five days to establish a mathematical correlation between the analyte-to-IS peak area ratio and analyte concentration. Each analyte used a different IS for the construction of the calibration curve; AEME and COC both used COC-d3 as the IS, because there was no AEME-d3, which would be the perfect IS for AEME. EME and COET used EME-d3 and COET-d3, respectively. Finally, BEG and NCOC used the BEG-d3 as the IS, because there was no NCOC-d3 available, which would have been the best IS for NCOC. The criteria for a valid calibration model include a determination coefficient (R2) of at least 0.99 and an mean relative error not exceeding 20% for any calibrator. Table 4 summarizes the results for linearity and the respective LLOQ for each analyte.

The working range indicates the concentration levels that the laboratory commonly encounters in routine analysis. In the described method, the working ranges for all compounds are broad, providing a versatile spectrum capable of detecting both chronic and occasional COC users. This broad range resulted in the adoption of the transformation 1/x for the calibration curve to compensate for heteroskedasticity. Additionally, for COC, the cut-off value of 0.5 ng/mg, as specified by the SOHT guidelines, falls well within the COC range of 0.05-5 ng/mg, enabling effective identification of drug users [55]. Although the linearity for AEME did not produce the same results as the other analytes, the guidelines clearly state that no specific cutoff value has been established for AEME. Detecting AEME, regardless of its concentration, is sufficient to confirm COC consumption, particularly in its smoked form, which is more commonly associated with crack.

Table 4 – Linearity data (n=5)

Analyte	Weight	Linear range (ng/mg)	Linearity		R2*	LLOQ (ng/mg)
			Slope*	Intercept*		
AEME	1/x	0.5 - 5	1.378 ± 0.731	-0.119 ± 0.183	0.9941 ± 0.0032	0.5
EME		0.05 - 5	1.336 ± 0.616	0.078 ± 0.132	0.9962 ± 0.0032	0.05
COC		0.05 - 5	0.970 ± 0.341	0.022 ± 0.039	0.9943 ± 0.0026	0.05
COET		0.05 - 5	1.261 ± 0.759	0.007 ± 0.032	0.9905 ± 0.0005	0.05
BEG		0.05 - 5	0.916 ± 0.080	0.089 ± 0.058	0.9935 ± 0.0032	0.05
NCOC		0.05 - 5	1.678 ± 1.009	0.001 ± 0.030	0.9930 ± 0.0021	0.05

* Mean values ± standard deviation

The LLOQ was defined as the lowest concentration that can be quantified with acceptable precision and accuracy. It corresponded to the lowest non-zero calibrator within the working range. In this study, the LLOQ values were 0.05 ng/mg for EME, COC, COET,

BEG, and NCOC, while 0.5 ng/mg for AEME (Fig. 10). LOD was not systematically evaluated, so in this study, the LLOQ and LOD were considered the same for all analytes.

Figure 10 – Representative chromatogram at the LLOQ for all analysed compounds.

Despite the variety of approaches developed for hair analysis, none of them employed QuEChERS as a clean-up technique for the analysis of COC. The QuEChERS was used for the determination of COC only in classic samples such as whole blood, as in Dulaurent *et al.* [57] or urine as in Demile *et al.* [58]. This highlights the importance and novelty of the method developed in this study, since it integrates µ-QuEChERS for a clean-up technique in hair samples to determine COC and its metabolites by GC-MS/MS.

Also, with the goal of determining COC and BEG in hair samples, López *et al.* [59] used an ELISA screening and LC-MS/MS method, obtaining LOD values of 0.01 ng/mg and 0.001 ng/mg for COC and BEG, respectively. The LOQ values were 0.05 ng/mg and 0.005 ng/mg for COC and BEG, respectively. Although the author's method yielded greater sensitivity with smaller sample amounts (20-40 mg of hair) and without a clean-up step, it was only applied to two analytes and reported a very low specificity (10.8%), as well as a long-time of extraction (4-hour sonication).

Additionally, Moore *et al.* [60] developed a method using SPE extraction for the determination of COC, BEG, COET, and NCOC in human hair, using only 10 mg of hair sample and achieving LOD and LOQ values of 25 pg/mg and 50 pg/mg, respectively.

Although SPE is a common extraction and clean-up method, it has its disadvantages, such as the employment of single-use cartridges. Additionally, the herein proposed method adds AEME to the method which can be pertinent to differentiate crack cocaine users.

2.2.3. Precision and accuracy

A trustworthy analysis can measure concentrations repeatedly, obtaining results that are close to the true value with minimal dispersion between measurements. This reliability is characterised by the accuracy (mean relative error in percentage – RE (%)), which refers to the proximity between the mean of the measurements and the actual value, and precision (coefficient of variance – CV (%)), which indicates the variability across multiple measurements. When both accuracy and precision criteria are met, the method can be considered precise and accurate.

Between-run precision and accuracy were evaluated by spiking blank samples with the adopted calibrators concentrations per-analyte within the 5-day validation period. For within-run precision and accuracy, the blank samples were spiked with four concentration levels per analyte, except for AEME, as the concentration of 0.05 ng/mg could not be met. These concentration levels were then tested (n=5) on the same day that one calibration curve was also analyzed. The acceptance criteria for both parameters require that all values stay below 20%. In this study, the LLOQ precision and accuracy were also evaluated.

Combined within-run and between-run values were evaluated during the 5-day validation period, where both the between-run and the within-run values were gathered and combined.

Table 5 summarises all data obtained for precision and accuracy evaluation.

Table 5 – Between-run, within-run, and combined within-run and between-run precision and accuracy

		Between-run (n=5)			Within-run (n=5)			Combined within-run and between-run (n=15)		
Analyte	Spiked	Measured	CV(%)	RE(%)	Measured	CV(%)	RE(%)	Measured	CV (%)	RE (%)
AEME	0.5	0.54 ± 0.02	4.49	7.78	0.55 ± 0.001	0.23	10.84	0.55 ± 0.01	2.36	9.31
	1	0.99 ± 0.03	3.25	-1.40						
	2	1.82 ± 0.04	2.37	-8.86	1.96 ± 0.22	11.33	-1.96	1.89 ± 0.13	6.85	-5.41

	3	2.89 ± 0.09	3.08	-3.71						
	4	4.11 ± 0.17	4.07	2.80	3.76 ± 0.37	9.79	-5.91	3.94 ± 0.27	6.93	-1.56
	5	5.15 ± 0.13	2.46	3.01						
EME	0.05	0.05 ± 0.003	5.62	6.64	0.05±0.01	13.11	-7.78	0.05 ± 0.007	9.37	-0.57
	0.1	0.09 ± 0.01	5.33	-5.56						
	0.5	0.46 ± 0.03	6.59	-7.27	0.44±0.016	3.69	-12.22	0.45 ± 0.023	5.14	-9.75
	1	1.03 ± 0.08	7.69	2.90						
	2	2.06 ± 0.23	11.30	3.22	1.89 ± 0.05	2.60	-5.66	1.98 ± 0.14	6.95	-1.22
	3	2.96 ± 0.18	6.00	-1.26						
	4	3.93 ± 0.19	4.83	-1.69	3.76 ± 0.52	13.85	-5.99	3.85 ± 0.36	9.34	-3.84
	5	5.06 ± 0.20	4.04	1.10						
COC	0.05	0.05 ± 0.01	15.24	-0.51	0.05±0.01	12.98	-0.26	0.05 ± 0.01	14.11	-0.39
	0.1	0.10 ± 0.01	9.97	4.31						
	0.5	0.48 ± 0.03	6.58	-3.89	0.44 ± 0.019	4.41	-15.30	0.46 ± 0.025	5.50	-9.60
	1	0.98 ± 0.14	13.95	-1.77						
	2	2.01 ± 0.19	9.46	0.71	1.89 ± 0.11	5.76	-5.44	1.95 ± 0.15	7.61	-2.37

	3	3.03 ± 0.17	5.53	0.93						
	4	4.13 ± 0.25	6.00	3.19	4.23 ± 0.31	7.43	5.66	4.18 ± 0.28	6.72	4.43
	5	4.86 ± 0.34	6.91	-2.73						
COET	0.05	0.05 ± 0.005	10.39	-7.02	0.05 ± 0.01	10.99	-2.98	0.05 ± 0.008	10.69	-5.00
	0.1	0.10 ± 0.01	13.14	0.33						
	0.5	0.50 ± 0.01	2.72	-0.62	0.47 ± 0.06	13.75	-6.71	0.49 ± 0.04	8.24	-3.67
	1	1.03 ± 0.08	7.92	3.46						
	2	2.17 ± 0.14	6.27	8.55	2.01 ± 0.19	9.40	0.47	2.09 ± 0.17	7.84	4.51
	3	3.04 ± 0.38	12.44	1.25						
	4	4.06 ± 0.39	9.54	1.56	4.34 ± 0.53	12.33	8.45	4.20 ± 0.46	10.94	5.01
	5	4.75 ± 0.48	10.12	-4.93						
BEG	0.05	0.05 ± 0.01	9.53	7.50	0.05 ± 0.01	15.22	1.25	0.05 ± 0.01	12.38	4.38
	0.1	0.09 ± 0.01	14.14	-10.17						
	0.5	0.51 ± 0.01	2.32	1.20	0.47 ± 0.037	7.76	-5.64	0.49 ± 0.024	5.04	-2.22
	1	0.96 ± 0.09	8.90	-3.82						
	2	2.02 ± 0.17	8.56	0.78	1.93 ± 0.13	6.59	-3.64	1.98 ± 0.15	7.58	-1.43
	3	2.90 ± 0.28	9.82	-3.34						
	4	3.91 ± 0.35	9.00	-2.16	4.20 ± 0.34	8.17	5.05	4.06 ± 0.35	8.59	1.45
	5	5.19 ± 0.28	5.46	3.71						
NCOC	0.05	0.05 ± 0.01	14.15	3.28	0.05 ± 0.01	13.19	4.30	0.05 ± 0.01	13.67	3.79
	0.1	0.09 ± 0.01	7.77	-8.30						
	0.5	0.49 ± 0.05	9.49	-1.17	0.53 ± 0.061	11.60	5.35	0.51 ± 0.056	10.55	2.09
	1	1.04 ± 0.13	12.09	3.65						
	2	2.10 ± 0.30	14.45	5.13	1.93 ± 0.29	14.80	-3.34	2.02 ± 0.30	14.63	0.90
	3	2.85 ± 0.26	9.04	-5.38						
	4	3.95 ± 0.28	6.99	-1.15	4.06 ± 0.24	5.82	1.42	4.01 ± 0.26	6.41	0.14
	5	5.12 ± 0.51	9.94	1.98						

All concentrations in ng/mg; CV – Coefficient of variation; RE – Relative error [(measured concentration-spiked concentration/spiked concentration)] x 100; Mean values ± standard deviation.

2.2.4. Recovery

To evaluate the recovery capability of the developed μ-QuEChERS method, a protocol was developed using three concentrations—low, medium, and high—for each analyte (0.5, 2, and 5 ng/mg for all analytes). By fortifying the sample with a mixture of analytes before and after the clean-up procedure, this parameter could be evaluated. The recovery rates ranged from 20–29 % for AEME, 24-27 % for EME, 33-42 % for COC, 43-72 % for COET, 25-48 % for BEG, and 27–37 % for NCOC (Table 6).

Comparing with the recoveries obtained for the same analytes but when applied another miniaturized technique to hair samples, the recoveries obtained in the herein proposed u-QuEChERS method, were better for AEME, EME, and BEG. In Rosado *et al.* [61] study the recoveries for AEME ranged from 4-6%, EME ranged from 1-3%, and BEG ranged from 21-28%. However, better recoveries were obtained by Rosado *et al.* [61] for COC (44-65%), COET (63-73%), and NCOC (36-44%), although the differences with the u-QuEChERS method were not considerable. This way, the obtained recoveries were considered satisfactory.

Table 6 – Recoveries (n=3)

Analyte	Low Concentration	Medium Concentration	High Concentration
AEME	19.77±3.73	28.71±3.61	28.42±4.43
EME	26.91±3.54	24.00±2.43	25.43±0.60
COC	32.94±4.26	40.04±2.60	41.94±2.04
COET	42.99±8.98	46.21±8.36	71.47±5.22
BEG	26.01±4.15	24.79±4.35	48.24±2.44
NCOC	36.57±1.00	37.41±4.46	27.35±3.70

*Mean values (%) ± standard deviation (%).

2.3. Method application to authentic samples

The developed method was applied to hair samples from individuals who attended an alternative music festival in Portugal in 2022. Table 7 lists the concentrations of all analytes, and the chromatogram of sample 1 is also presented (Fig. 11). These values offer a solid foundation for interpreting drug use.

In these samples, COC was detected in almost all of them (except for sample 3 and sample 7), although COC measured concentrations were below the cut-off values recommended

by SOHT (0.5 ng/mg), for all metabolites the measured concentrations were above the cut-off (0.05 ng/mg). COET and NCOC were detected only in two samples, in both sample 3 and sample 1, COET was detected, and NCOC was detected in sample 1, and both were above their cut-off values proposed by the SOHT. The same thing happened to EME and BEG, where they both appeared in only a few samples, but always above the cut-off values proposed by the SOHT. On the other hand, the SOHT does not have proposed cut-off values for the AEME; however, the values detected for sample 1, sample 2, and sample 8 were superior to the cut-off value of COC, which confirms the presence of AEME, and additionally, the consumption of COC in its smoked form.

Table 7 – Authentic hair samples measured concentrations

Analyte	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8
AEME	0.79	0.80	ND	ND	ND	ND	ND	0.76
EME	ND	ND	0.12	ND	0.10	ND	ND	ND
COC	0.19	0.20	ND	0.19	0.19	0.16	ND	0.23
COET	0.10	ND	0.11	ND	ND	ND	ND	ND
BEG	0.38	0.35	ND	ND	0.42	ND	ND	0.43
NCOC	0.57	ND	ND	ND	ND	ND	ND	ND

All concentrations in ng/mg; (ND = Not detected (<LLOQ))

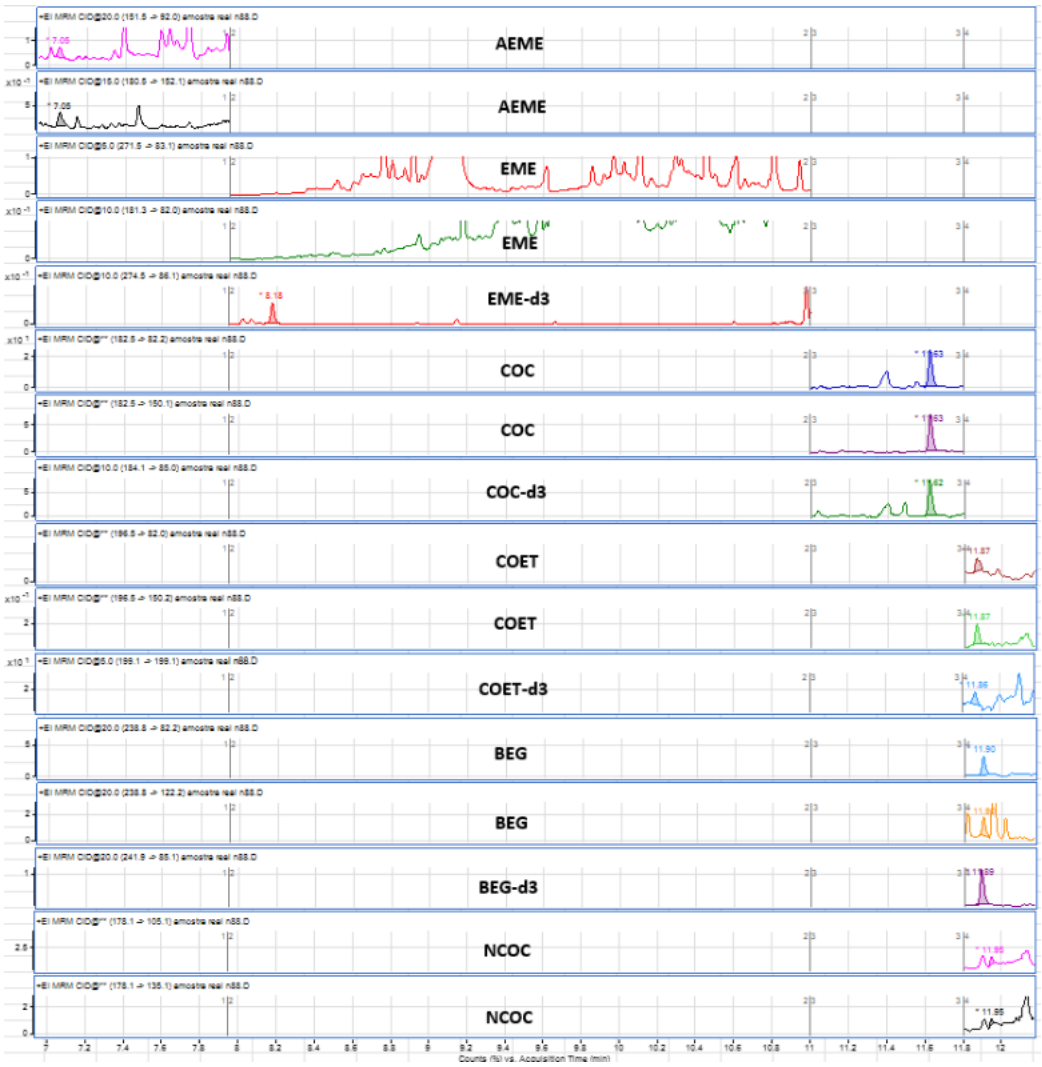


Figure 11 – Chromatogram obtained from sample 1

Chapter 4 | Conclusion

This study aimed to develop a new clean-up strategy for hair samples by adapting a method already used in other analytical contexts. Since hair is a particularly complex matrix, the µ-QuEChERS approach proved highly effective for isolating COC and its metabolites, delivering excellent sensitivity when applied. By optimising the pretreatment steps, it was possible to miniaturise the clean-up procedure. Bringing together green chemistry, safety, routine applicability, and cost-effectiveness within a single method is challenging. Over the years, many approaches applied to COC detection in hair have struggled to meet all of these requirements simultaneously. In contrast, the µ-QuEChERS method allows for the processing of multiple samples at once without reducing efficiency. It produces little waste and avoids the use of highly toxic reagents. Validation results confirmed the robustness of the method, with limits of detection and quantification, as well as linearity, comparable to established approaches. Its successful

application to authentic samples further demonstrated its effectiveness. The pre-treatment stage is simple and does not require specialised equipment, making the method practical and user-friendly for routine laboratory use. Overall, these findings are encouraging and suggest that the method could be extended to the analysis of other drugs of abuse in hair. The decisive factor lies in the careful optimisation of the μ-QuEChERS protocol, which allowed key challenges associated with the complexity of biological matrices such as hair to be overcome. Importantly, the method shows high potential for transferability and scalability in forensic laboratories, supporting its implementation in routine toxicological analysis.

Chapter 5 | Dissemination

The results obtained in the scope of this dissertation were disseminated through the following communications and publications:

- Potencial da metodologia QuEChERS para a análise de drogas de abuso em cabelo (poster) – Rodrigo Pelixo; Hugo Brito; José Luís Guedes; Mário Barroso; Tiago Rosado; Eugenia Gallardo. 1º Congresso ICAD (Instituto para os Comportamentos Aditivos e as Dependências); 16, 17 e 18 de junho de 2025; Castelo Branco, Portugal.
- QuEChERS miniaturization for hair analysis application in forensic toxicology routine (poster, aceite) – Rodrigo Pelixo; Hugo Brito; José Luís Guedes; Tiago Rosado; Mario Barroso; Eugenia Gallardo. 14º ENC (Encontro Nacional de Cromatografia); 4, 5 e 6 de dezembro de 2025; Funchal, Madeira.
- Sustainable and Green Approaches for Sample Preparation (in production). H Brito, S Calado, B Pires, L M Rosendo, M Barroso, T Rosado, E Gallardo. In Ecofriendly Approaches for Chemical Analysis: Concepts and Applications. Eds. Chaudhery Mustansar Hussain, Arpana Agrawal, Rüstem Keçili, and Chaudhery Ghazanfar Hussain, Wiley 2026.

Chapter 6 | References

[1] R.A. Goldstein, C. DesLauriers, A. Burda, K. Johnson-Arbor, Cocaine: history, social implications, and toxicity: a review, Semin Diagn Pathol 26 (2009) 10–17. https://doi.org/10.1053/j.semdp.2008.12.001.

[2] J. Jastrzębska, W.A. Daniel, Cocaine-Induced Time-Dependent Alterations in Cytochrome P450 and Liver Function, Int J Mol Sci 24 (2023). https://doi.org/10.3390/IJMS24021632.

[3] S.B. Karch, Cocaine: History, use, abuse, J R Soc Med 92 (1999) 393–397. https://doi.org/10.1177/014107689909200803/ASSET/01410768990920 0803.FP.PNG_V03.

[4] R. Roque Bravo, A.C. Faria, A.M. Brito-Da-costa, H. Carmo, P. Mladěnka, D. Dias da Silva, F. Remião, Cocaine: An Updated Overview on Chemistry, Detection, Biokinetics, and Pharmacotoxicological Aspects including Abuse Pattern, Toxins 2022, Vol. 14, Page 278 14 (2022) 278. https://doi.org/10.3390/TOXINS14040278.

[5] S.J. Chinta, J.K. Andersen, Dopaminergic neurons, Int J Biochem Cell Biol 37 (2005) 942–946. https://doi.org/10.1016/J.BIOCEL.2004.09.009.

[6] J.R. Richards, J.K. Le, Cocaine Toxicity, StatPearls (2023). https://www.ncbi.nlm.nih.gov/books/NBK430976/ (accessed February 24, 2025).

[7] N.L. Benowitz, Clinical Pharmacology and Toxicology of Cocaine, Pharmacol Toxicol 72 (1993) 3–12. https://doi.org/10.1111/J.1600-0773.1993.TB01331.X.

[8] J.B. Wang, J.R. Mantsch, l-tetrahydropalamatine: a potential new medication for the treatment of cocaine addiction, Future Med Chem 4 (2012) 177–186. https://doi.org/10.4155/FMC.11.166.

[9] J.K. Penberthy, N. Ait-Daoud, M. Vaughan, T. Fanning, Review of treatment for cocaine dependence, Curr Drug Abuse Rev 3 (2010) 49–62. https://doi.org/10.2174/1874473711003010049.

[10] C.S. Restrepo, J.A. Carrillo, S. Martínez, P. Ojeda, A.L. Rivera, A. Hatta, Pulmonary Complications from Cocaine and Cocaine-based Substances: Imaging Manifestations1, Https://Doi.Org/10.1148/Rg.274065144 27 (2007) 941–956. https://doi.org/10.1148/RG.274065144.

[11] M.J. Valente, F. Carvalho, M.L. Bastos, M. Carvalho, P.G. de Pinho, Development and validation of a gas chromatography/ion trap-mass spectrometry method for simultaneous quantification of cocaine and its metabolites benzoylecgonine and norcocaine: Application to the study of cocaine metabolism in human primary cultured renal cells, Journal of

Chromatography B 878 (2010) 3083–3088. https://doi.org/10.1016/J.JCHROMB.2010.09.010.

[12] P. Kintz, Hair analysis in forensic toxicology, Wiley Interdisciplinary Reviews: Forensic Science 1 (2019) e1196. https://doi.org/10.1002/WFS2.1196.

[13] L. Alonso, E. Fuchs, The hair cycle, J Cell Sci 119 (2006) 391–393. https://doi.org/10.1242/JCS.02793.

[14] Y. Niu, Y. Wang, H. Chen, X. Liu, J. Liu, Overview of the Circadian Clock in the Hair Follicle Cycle, Biomolecules 2023, Vol. 13, Page 1068 13 (2023) 1068. https://doi.org/10.3390/BIOM13071068.

[15] X. Lin, L. Zhu, J. He, Morphogenesis, Growth Cycle and Molecular Regulation of Hair Follicles, Front Cell Dev Biol 10 (2022) 899095. https://doi.org/10.3389/FCELL.2022.899095/PDF.

[16] L. Skender, V. Karačić, I. Brčić, A. Bagarić, Quantitative determination of amphetamines, cocaine, and opiates in human hair by gas chromatography/mass spectrometry, Forensic Sci Int 125 (2002) 120–126. https://doi.org/10.1016/S0379-0738(01)00630-2.

[17] M. Barroso, E. Gallardo, D.N. Vieira, M. López-Rivadulla, J.A. Queiroz, Hair: A Complementary Source of Bioanalytical Information in Forensic Toxicology, Bioanalysis 3 (2011) 67–79. https://doi.org/10.4155/BIO.10.171.

[18] M. Barroso, E. Gallardo, Hair Analysis for Forensic Applications: is The Future Bright?, Bioanalysis 6 (2014) 1–3. https://doi.org/10.4155/BIO.13.291.

[19] C. Jurado, Forensic Applications of Hair Analysis, Hair Analysis in Clinical and Forensic Toxicology (2015) 241–273. https://doi.org/10.1016/B978-0-12-801700-5.00009-1.

[20] C.M. Selavka, F. Rieders, The determination of cocaine in hair: a review, Forensic Sci Int 70 (1995) 155–164. https://doi.org/10.1016/0379-0738(94)01622-C.

[21] M. Barroso, E. Gallardo, J.A. Queiroz, Bioanalytical Methods for the Determination of Cocaine and Metabolites in Human Biological Samples, Bioanalysis 1 (2009) 977–1000. https://doi.org/10.4155/BIO.09.72.

[22] M. Janicka, A. Kot-Wasik, J. Namieśnik, Analytical procedures for determination of cocaine and its metabolites in biological samples, TrAC Trends in Analytical Chemistry 29 (2010) 209–224. https://doi.org/10.1016/J.TRAC.2009.12.005.

[23] K. Lachenmeier, F. Musshoff, B. Madea, Determination of opiates and cocaine in hair using automated enzyme immunoassay screening methodologies followed by gas chromatographic–mass spectrometric (GC–MS) confirmation, Forensic Sci Int 159 (2006) 189–199. https://doi.org/10.1016/J.FORSCIINT.2005.08.009.

[24] N. Gambier, J. Warling, N. Van Elsue, M. Yegles, AEME production in cocaine positive hair after thermal hair treatment, Forensic Sci Int 302 (2019) 109894. https://doi.org/10.1016/J.FORSCIINT.2019.109894.

[25] M. Anastassiades, S.J. Lehotay, D. Štajnbaher, F.J. Schenck, Fast and Easy Multiresidue Method Employing Acetonitrile Extraction/Partitioning and "Dispersive Solid-Phase Extraction" for the Determination of Pesticide Residues in Produce, J AOAC Int 86 (2003) 412–431. https://doi.org/10.1093/JAOAC/86.2.412.

[26] R. Perestrelo, P. Silva, P. Porto-Figueira, J.A.M. Pereira, C. Silva, S. Medina, J.S. Câmara, QuEChERS - Fundamentals, relevant improvements, applications and future trends, Anal Chim Acta 1070 (2019) 1–28. https://doi.org/10.1016/J.ACA.2019.02.036.

[27] A. Gałuszka, Z. Migaszewski, J. Namieśnik, The 12 principles of green analytical chemistry and the SIGNIFICANCE mnemonic of green analytical practices, TrAC Trends in Analytical Chemistry 50 (2013) 78–84. https://doi.org/10.1016/J.TRAC.2013.04.010.

[28] M. González-Curbelo, B. Socas-Rodríguez, A. V. Herrera-Herrera, J. González-Sálamo, J. Hernández-Borges, M. Rodríguez-Delgado, Evolution and applications of the QuEChERS method, TrAC Trends in Analytical Chemistry 71 (2015) 169–185. https://doi.org/10.1016/J.TRAC.2015.04.012.

[29] S. Seo, H.-D. Cho, J.-Y. Lee, Y.-Y. Kim, Y. Lee, E. Jo, G. Kim, S.-H. Jeon, S. Park, S.B. Han, Analysis of cotinine and 3-hydroxycotinine in human saliva using QuEChERS purification coupled to liquid chromatography-tandem mass spectrometry (LC-MS/MS), 한국분석과학회 학술대회 (2019) 239–239. https://www.dbpia.co.kr/journal/articleDetail?nodeId=NODE09264105 (accessed August 25, 2025).

[30] L. Correia-Sá, S. Norberto, C. Delerue-Matos, C. Calhau, V.F. Domingues, Micro-QuEChERS extraction coupled to GC–MS for a fast determination of Bisphenol A in human urine, Journal of Chromatography B 1072 (2018) 9–16. https://doi.org/10.1016/J.JCHROMB.2017.10.060.

[31] K. Usui, Y. Hayashizaki, M. Hashiyada, M. Funayama, Rapid drug extraction from human whole blood using a modified QuEChERS

extraction method, Leg Med 14 (2012) 286–296. https://doi.org/10.1016/J.LEGALMED.2012.04.008.

[32] Á. Santana-Mayor, B. Socas-Rodríguez, A. V. Herrera-Herrera, M.Á. Rodríguez-Delgado, Current trends in QuEChERS method. A versatile procedure for food, environmental and biological analysis, TrAC Trends in Analytical Chemistry 116 (2019) 214–235. https://doi.org/10.1016/J.TRAC.2019.04.018.

[33] S.N. Rivai, S. Kristianto, R.E. Putri, A. Fatiqin, M.H.Z. Abidin, Modification of the QuEChERS Method for Drug Analysis in Biological Sample: A Review, Journal of Multidisciplinary Applied Natural Science 5 (2025) 523–535. https://doi.org/10.47352/JMANS.2774-3047.261.

[34] M.P. Dybowski, A.L. Dawidowicz, Application of the QuEChERS procedure for analysis of Δ9-tetrahydrocannabinol and its metabolites in authentic whole blood samples by GC–MS/MS, Forensic Toxicol 36 (2018) 415–423. https://doi.org/10.1007/S11419-018-0419-8/TABLES/3.

[35] J. de M. Campêlo, T.B. Rodrigues, J.L. Costa, J.M. Santos, Optimization of QuEChERS extraction for detection and quantification of 20 antidepressants in postmortem blood samples by LC-MS/MS, Forensic Sci Int 319 (2021) 110660. https://doi.org/10.1016/J.FORSCIINT.2020.110660.

[36] Z. Zhang, H. Cheng, Recent Development in Sample Preparation and Analytical Techniques for Determination of Quinolone Residues in Food Products, Crit Rev Anal Chem 47 (2017) 223–250. https://doi.org/10.1080/10408347.2016.1266924.

[37] R. Pelixo, M. Barroso, T. Rosado, E. Gallardo, Determination of ketamine and norketamine in hair samples by μ-QuEChERS and gas chromatography coupled to tandem mass spectrometry, Microchemical Journal 213 (2025) 113770. https://doi.org/10.1016/J.MICROC.2025.113770.

[38] A.R.S. Mateus, S.C. Barros, A. Pena, A. Sanches-Silva, Miniaturized QuEChERS extraction (μQuEChERS) combined with HPLC-MS/MS as new analytical method for determination of 105 residues of pesticides in fruit by-products extracts, Food Chem 481 (2025) 143898. https://doi.org/10.1016/J.FOODCHEM.2025.143898.

[39] Y. Yamasaki, I. Aloisi, H. Mol, Miniaturization of the QuEChERS method in fruits and vegetables without cryogenic milling: approach to greener analysis of pesticide residues, Food Additives and Contaminants - Part A 42 (2025) 902–913. https://doi.org/10.1080/19440049.2025.2502014;WEBSITE:WEBSITE:TFOPB;PAGEGROUP:STRING:PUBLICATION.

[40] E.F. Júnior, V.S. Bitencourt, Á.B.M. de Souza, E.D. Caldas, Target analysis of psychoactive drugs in oral fluid by QuEChERS extraction and LC-MS/MS, J Pharm Biomed Anal 244 (2024). https://doi.org/10.1016/J.JPBA.2024.116139.

[41] A. Di Trana, G. Sprega, G. Kobidze, O. Taoussi, A.F. Lo Faro, G. Bambagiotti, E. Montanari, M.S. Fede, J. Carlier, A. Tini, F.P. Busardò, A. Di Giorgi, S. Pichini, QuEChERS Extraction and Simultaneous Quantification in GC-MS/MS of Hexahydrocannabinol Epimers and Their Metabolites in Whole Blood, Urine, and Oral Fluid, Molecules 29 (2024). https://doi.org/10.3390/MOLECULES29143440.

[42] M. Pajewska-Szmyt, E. Sinkiewicz-Darol, U. Bernatowicz-Łojko, T. Kowalkowski, R. Gadzała-Kopciuch, B. Buszewski, QuEChERS extraction coupled to GC-MS for a fast determination of polychlorinated biphenyls in breast milk from Polish women, Environ Sci Pollut Res Int 26 (2019) 30988–30999. https://doi.org/10.1007/S11356-019-06201-Y.

[43] M.C. Bruzzoniti, L. Checchini, R.M. De Carlo, S. Orlandini, L. Rivoira, M. Del Bubba, QuEChERS sample preparation for the determination of pesticides and other organic residues in environmental matrices: A critical review, Anal Bioanal Chem 406 (2014) 4089–4116. https://doi.org/10.1007/S00216-014-7798-4/FIGURES/2.

[44] P.T. Anastas, J.C. Warner, Green Chemistry: Theory and Practice, (2000). https://doi.org/10.1093/OSO/9780198506980.001.0001.

[45] O. Coskun, Separation techniques: Chromatography, North Clin Istanbul 3 (2016) 156–60. https://doi.org/10.14744/nci.2016.32757.

[46] K.D. Bartle, P. Myers, History of gas chromatography, TrAC Trends in Analytical Chemistry 21 (2002) 547–557. https://doi.org/10.1016/S0165-9936(02)00806-3.

[47] F.W. McLafferty, Tandem Mass Spectrometry, Science (1979) 214 (1981) 280–287. https://doi.org/10.1126/SCIENCE.7280693.

[48] A. Tryenk, SKOOG - Química analítica VOLUME ÚNICO (8ª ed), Fundamentos de Química Analítica (2006). https://www.academia.edu/120793597/SKOOG_Qu%C3%ADmica_anal%C3%ADtica_VOLUME_%C3%9ANICO_8a_ed_ (accessed September 30, 2025).

[49] H.Monroe. McNair, J.M.. Miller, N.H.. Snow, Basic gas chromatography, (2019).

[50] E. Stashenko, J. Ren, Gas Chromatography-Mass Spectrometry, Advances in Gas Chromatography (2014). https://doi.org/10.5772/57492.

[51] O.I. Savolainen, A.S. Sandberg, A.B. Ross, A Simultaneous Metabolic Profiling and Quantitative Multimetabolite Metabolomic Method for Human Plasma Using Gas-Chromatography Tandem Mass Spectrometry, J Proteome Res 15 (2016) 259–265. https://doi.org/10.1021/ACS.JPROTEOME.5B00790/SUPPL_FILE/PR5B00790_SI_001.PDF.

[52] S.J. Lehotay, M. O'Neil, J. Tully, A.V. García, M. Contreras, H. Mol, V. Heinke, T. Anspach, G. Lach, R. Fussell, K. Mastovska, M.E. Poulsen, A. Brown, W. Hammack, J.M. Cook, L. Alder, K. Lindtner, M.G. Vila, M. Hopper, A. De Kok, M. Hiemstra, F. Schenck, A. Williams, A. Parker, Determination of pesticide residues in foods by acetonitrile extraction and partitioning with magnesium sulfate: collaborative study, J AOAC Int 90 (2007) 485–520. https://doi.org/10.1093/jaoac/90.2.485.

[53] B. Yuan, D. Zhao, W. Lyu, Z. Yin, D. Kshatriya, J.E. Simon, N.T. Bello, Q. Wu, Development and validation of a micro-QuEChERS method with high-throughput enhanced matrix removal followed with UHPLC-QqQ-MS/MS for analysis of raspberry ketone-related phenolic compounds in adipose tissues, Talanta 235 (2021) 122716. https://doi.org/10.1016/J.TALANTA.2021.122716.

[54] E. Lehmann, C. Oltramare, L.F. de Alencastro, Development of a modified QuEChERS method for multi-class pesticide analysis in human hair by GC-MS and UPLC-MS/MS, Anal Chim Acta 999 (2018) 87–98. https://doi.org/10.1016/J.ACA.2017.11.009.

[55] D. Favretto, G. Cooper, M. Andraus, F. Sporkert, R. Agius, B. Appenzeller, M. Baumgartner, T. Binz, V. Cirimele, R. Kronstrand, M. del Mar Ramirez, S. Strano-Rossi, M. Uhl, M. Vincenti, M. Yegles, The Society of Hair Testing consensus on general recommendations for hair testing and drugs of abuse testing in hair, Drug Test Anal 15 (2023) 1042–1046. https://doi.org/10.1002/DTA.3526;CTYPE:STRING:JOURNAL.

[56] Standard Practices for Method Validation in Forensic Toxicology, Colorado Springs (2019). www.asbstandardsboard.org.

[57] S. Dulaurent, S. El Balkhi, L. Poncelet, J.M. Gaulier, P. Marquet, F. Saint-Marcoux, QuEChERS sample preparation prior to LC-MS/MS determination of opiates, amphetamines, and cocaine metabolites in whole blood, Anal Bioanal Chem 408 (2016) 1467–1474. https://doi.org/10.1007/S00216-015-9248-3/FIGURES/3.

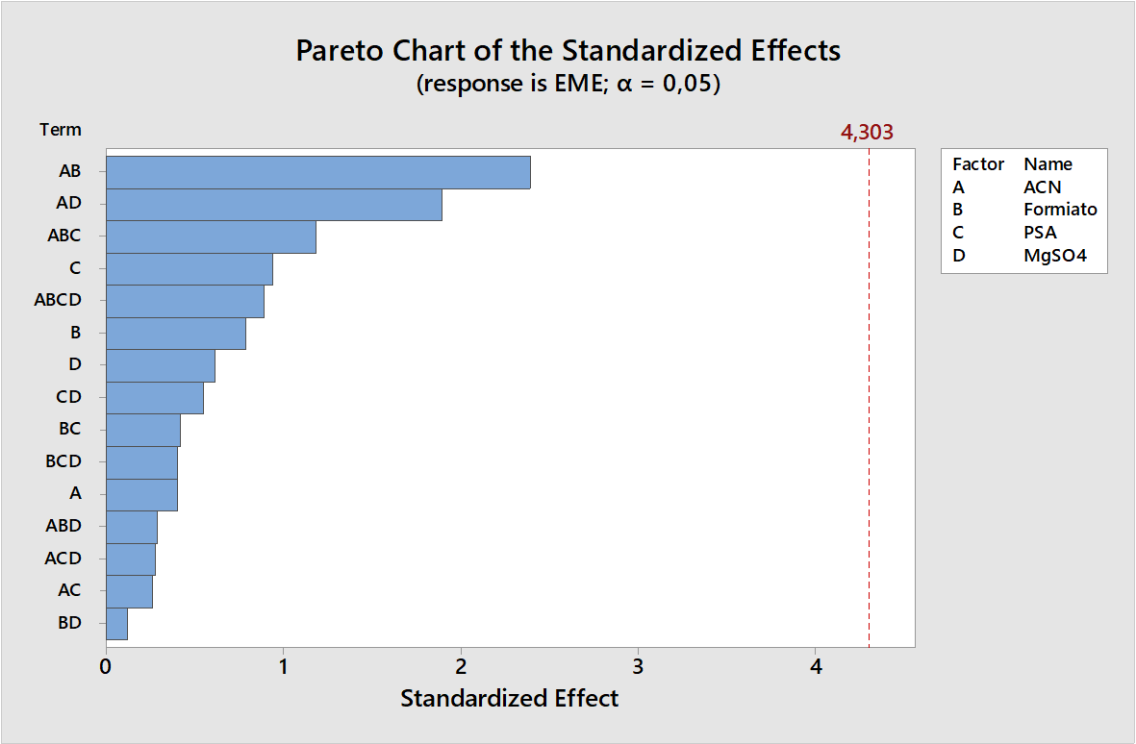
[58] L. Demlie Mihretu, E. Niyonizera, C. Karangwa, J. Kabera, S. Habumugisha, Technology and Engineering Management ISSN 2620-1755, J Agron Technol Eng Manag 2025 (n.d.) 1551. https://doi.org/10.55817/IDJI4816.

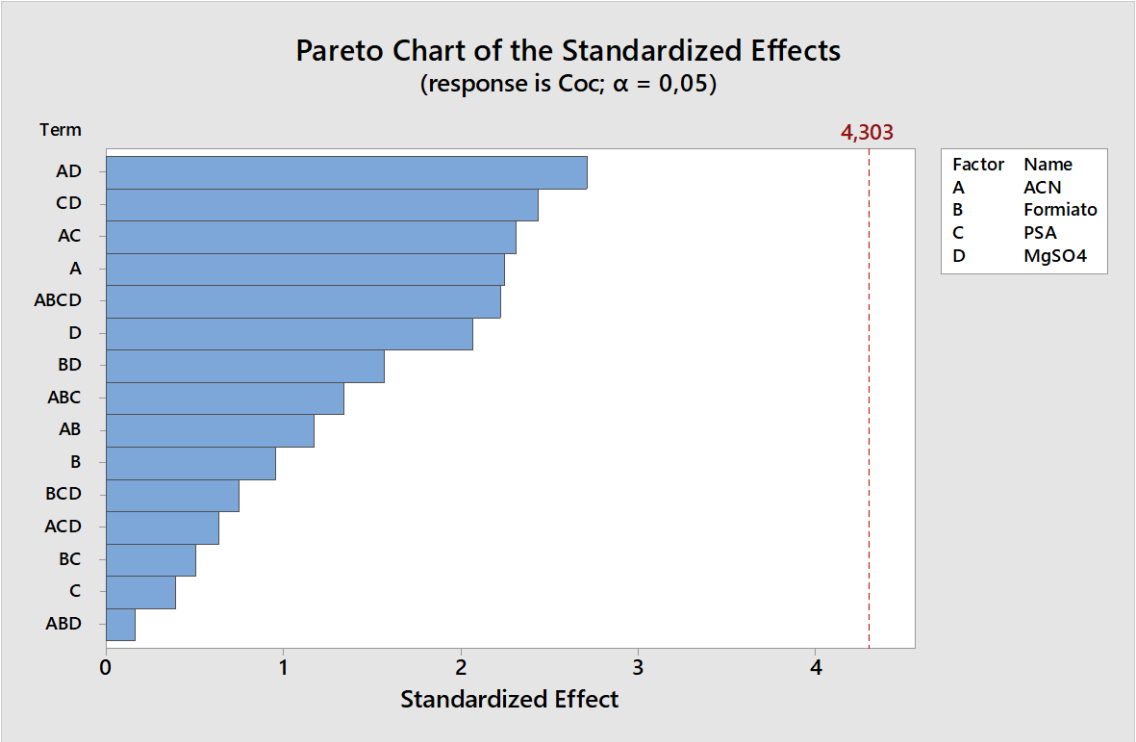
[59] P. López, S. Martello, A.M. Bermejo, E. De Vincenzi, M.J. Tabernero, M. Chiarotti, Validation of ELISA screening and LC-MS/MS confirmation methods for cocaine in hair after simple extraction, Anal Bioanal Chem 397 (2010) 1539–1548. https://doi.org/10.1007/S00216-010-3684-X/TABLES/5.

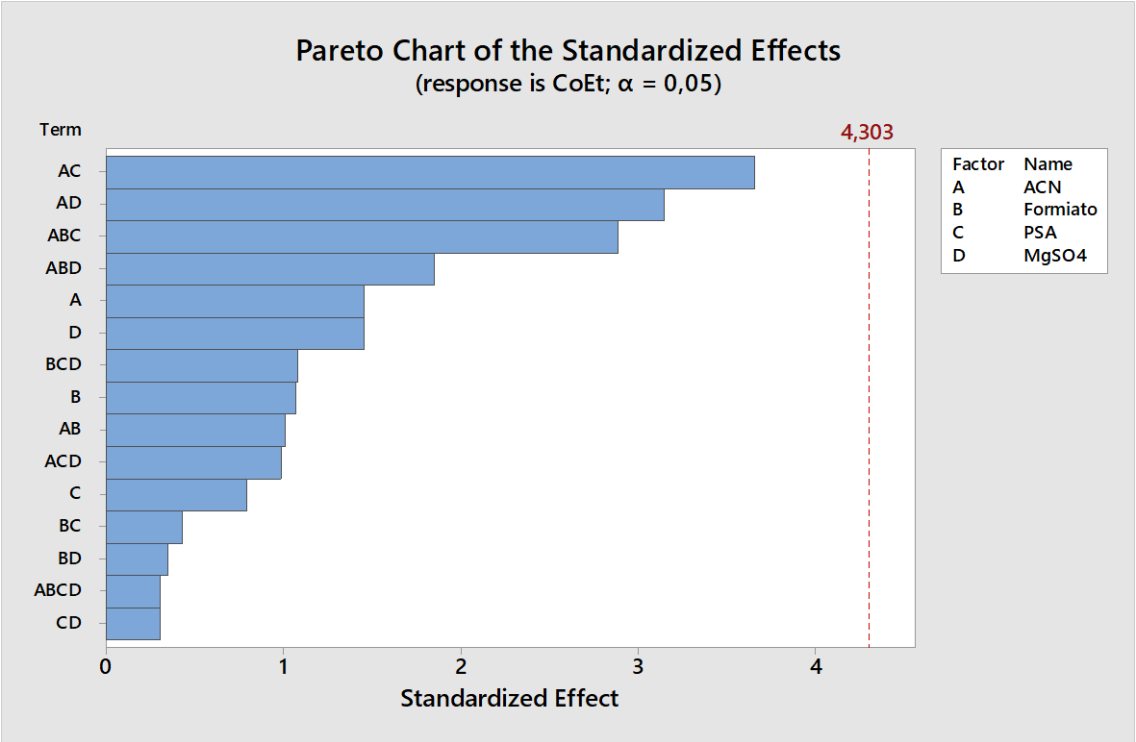
[60] C. Moore, C. Coulter, K. Crompton, Determination of cocaine, benzoylecgonine, cocaethylene and norcocaine in human hair using solid-phase extraction and liquid chromatography with tandem mass spectrometric detection, Journal of Chromatography B 859 (2007) 208–212. https://doi.org/10.1016/J.JCHROMB.2007.09.037.

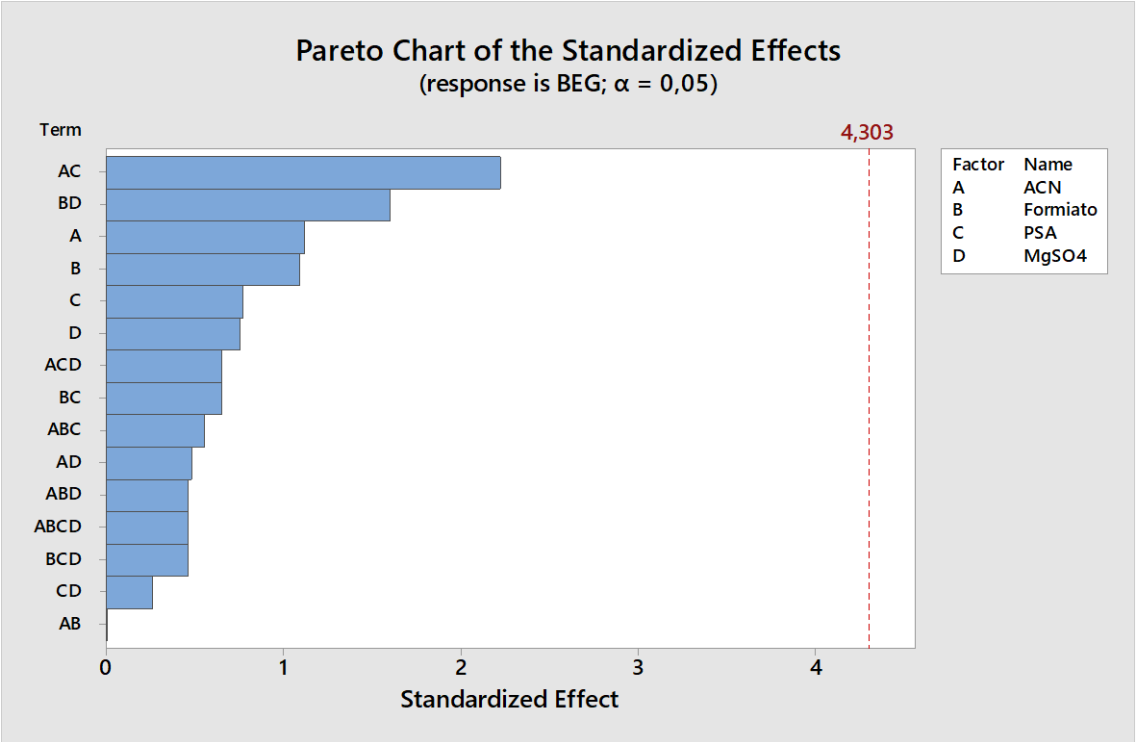
[61] T. Rosado, E. Gallardo, D.N. Vieira, M. Barroso, New miniaturized clean-up procedure for hair samples by means of microextraction by packed sorbent: determination of cocaine and metabolites, Anal Bioanal Chem 412 (2020) 7963–7976. https://doi.org/10.1007/S00216-020-02929-6/FIGURES/2.

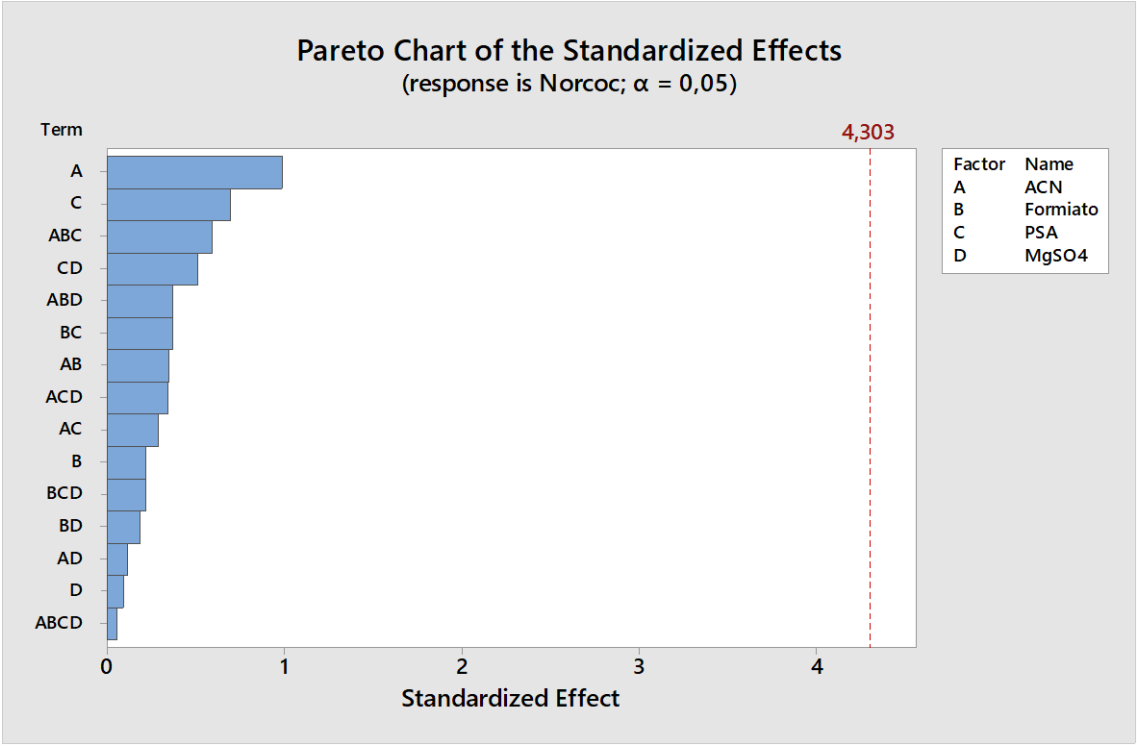
Annexes


Pareto Chart of the Standardized Effects
(response is EME; α = 0,05)
Term
4,303
AB
AD
ABC
C
ABCD
B
D
CD
BC
BCD
A
ABD
ACD
AC
BD
0
1
2
3
4
Standardized Effect
Factor Name
A ACN
B Formiato
C PSA
D MgSO4


Pareto Chart of the Standardized Effects
(response is Coc; α = 0,05)
Term
4,303
AD
CD
AC
A
ABCD
D
BD
ABC
AB
B
BCD
ACD
BC
C
ABD
0
1
2
3
4
Standardized Effect
Factor Name
A ACN
B Formiato
C PSA
D MgSO4


Pareto Chart of the Standardized Effects
(response is CoEt; α = 0,05)
Term
4,303
AC
AD
ABC
ABD
A
D
BCD
B
AB
ACD
C
BC
BD
ABCD
CD
0
1
2
3
4
Standardized Effect
Factor Name
A ACN
B Formiato
C PSA
D MgSO4


Pareto Chart of the Standardized Effects
(response is BEG; α = 0,05)
Term
4,303
AC
BD
A
B
C
D
ACD
BC
ABC
AD
ABD
ABCD
BCD
CD
AB
0
1
2
3
4
Standardized Effect
Factor Name
A ACN
B Formiato
C PSA
D MgSO4


Pareto Chart of the Standardized Effects
(response is Norcoc; α = 0,05)
Term
4,303
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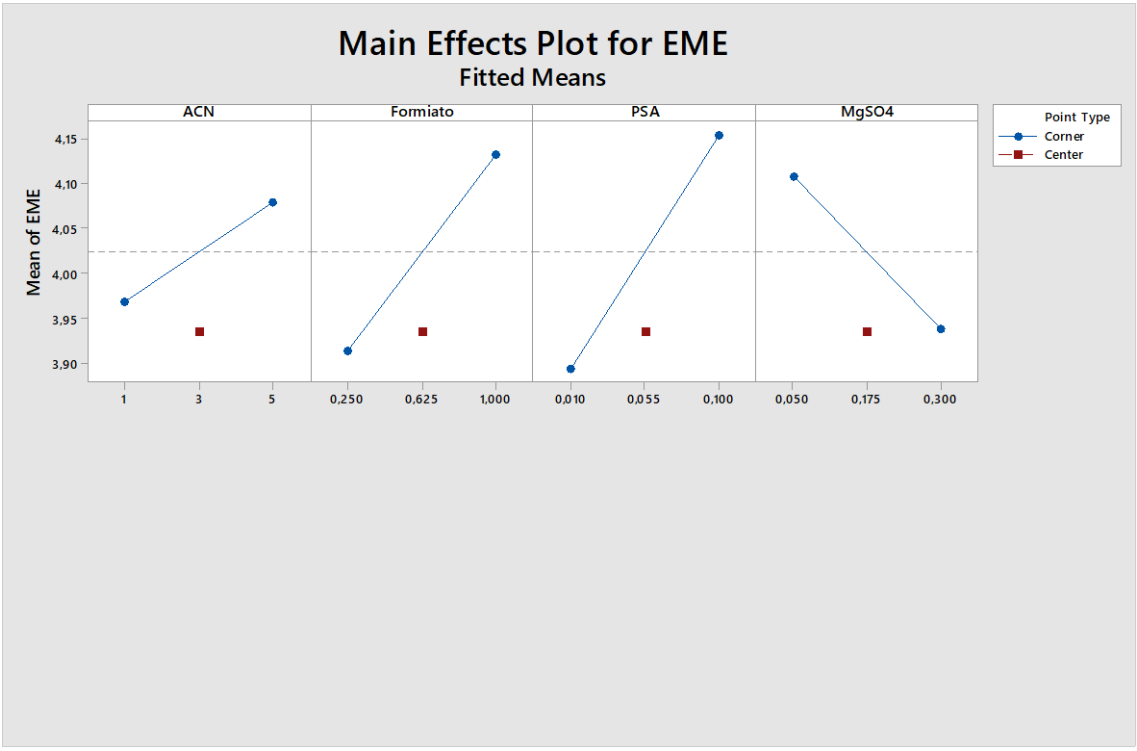
C
ABC
CD
ABD
BC
AB
ACD
AC
B
BCD
BD
AD
D
ABCD
0
1
2
3
4
Standardized Effect
Factor Name
A ACN
B Formiato
C PSA
D MgSO4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Fitted Means
ACN
Formiato
PSA
MgSO4
Mean of EME
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4,10
4,05
4,00
3,95
3,90
1
3
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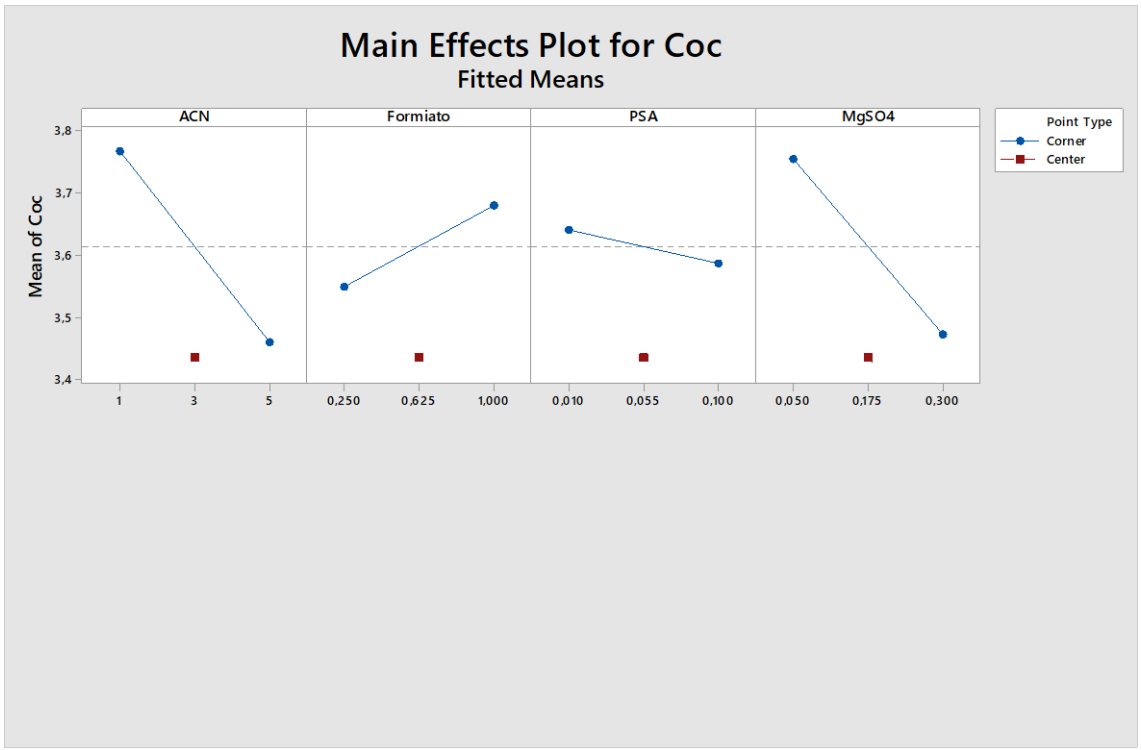
0,625
1,000
0,010
0,055
0,100
0,050
0,175
0,300
Point Type
Corner
Center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ACN
Formiato
PSA
MgSO4
Mean of Coc
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3,7
3,6
3,5
3,4
1
3
5
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1,000
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0,055
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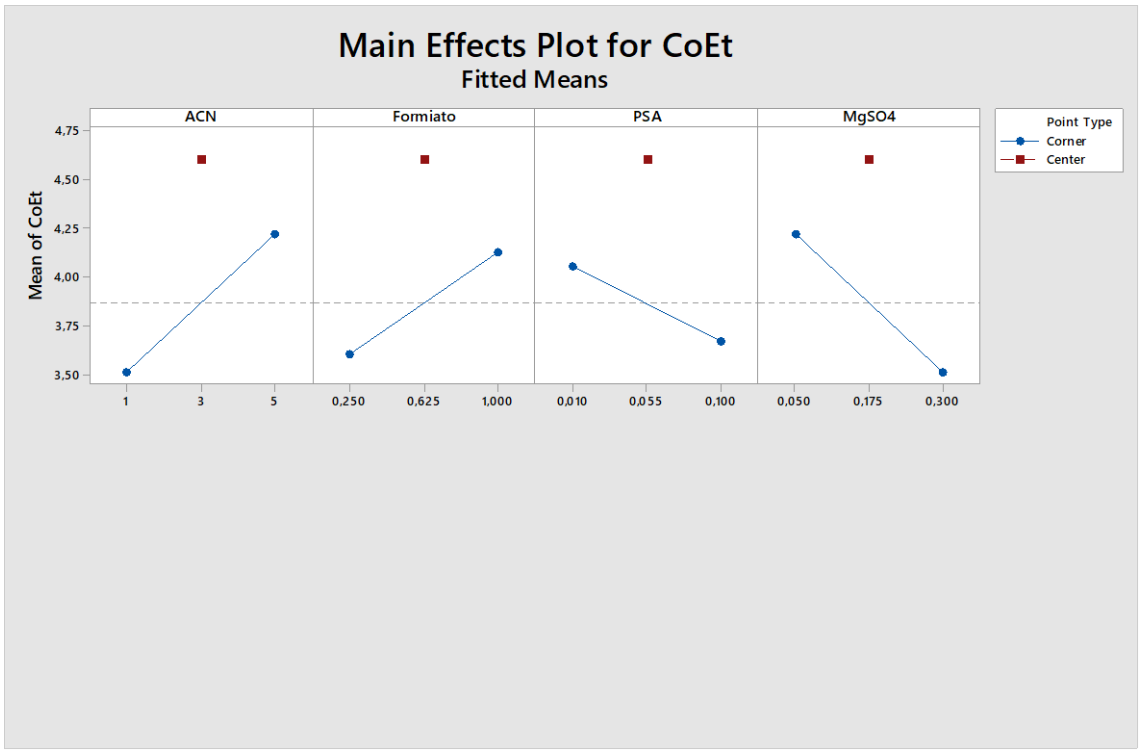
0,050
0,175
0,300
Point Type
Corner
Center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Fitted Means
ACN
Formiato
PSA
MgSO4
Mean of CoEt
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4,25
4,00
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Point Type
Corner
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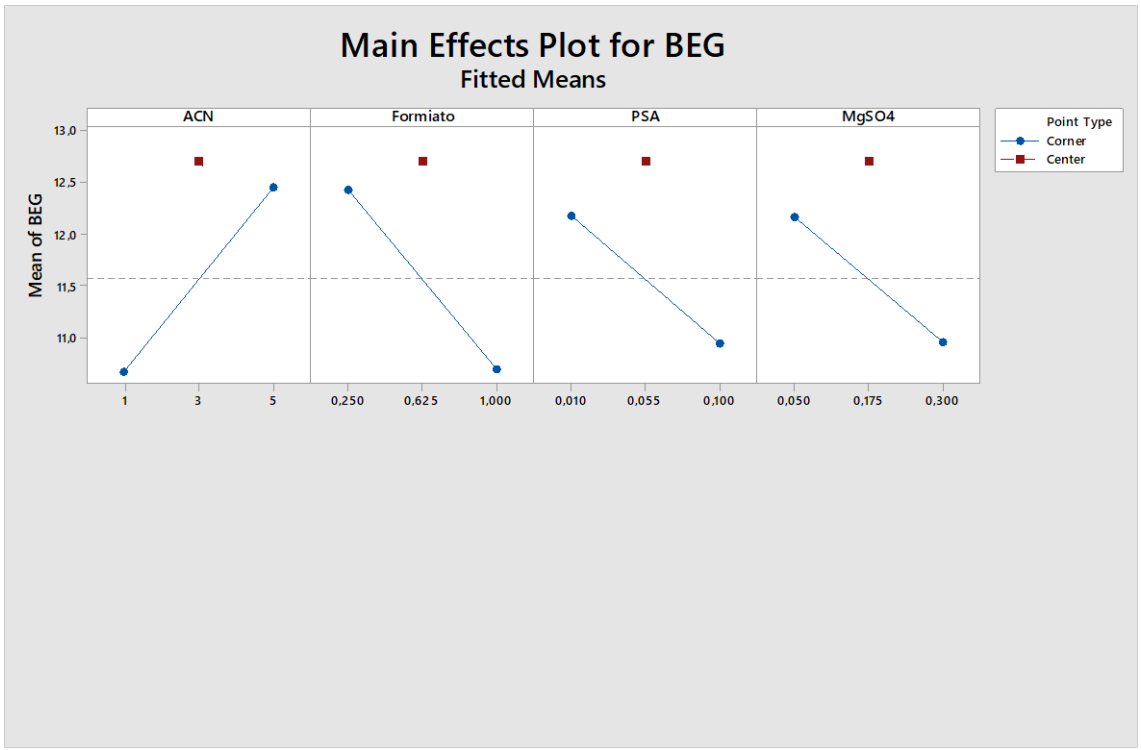
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Fitted Means
ACN
Formiato
PSA
MgSO4
Mean of BEG
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12,5
12,0
11,5
11,0
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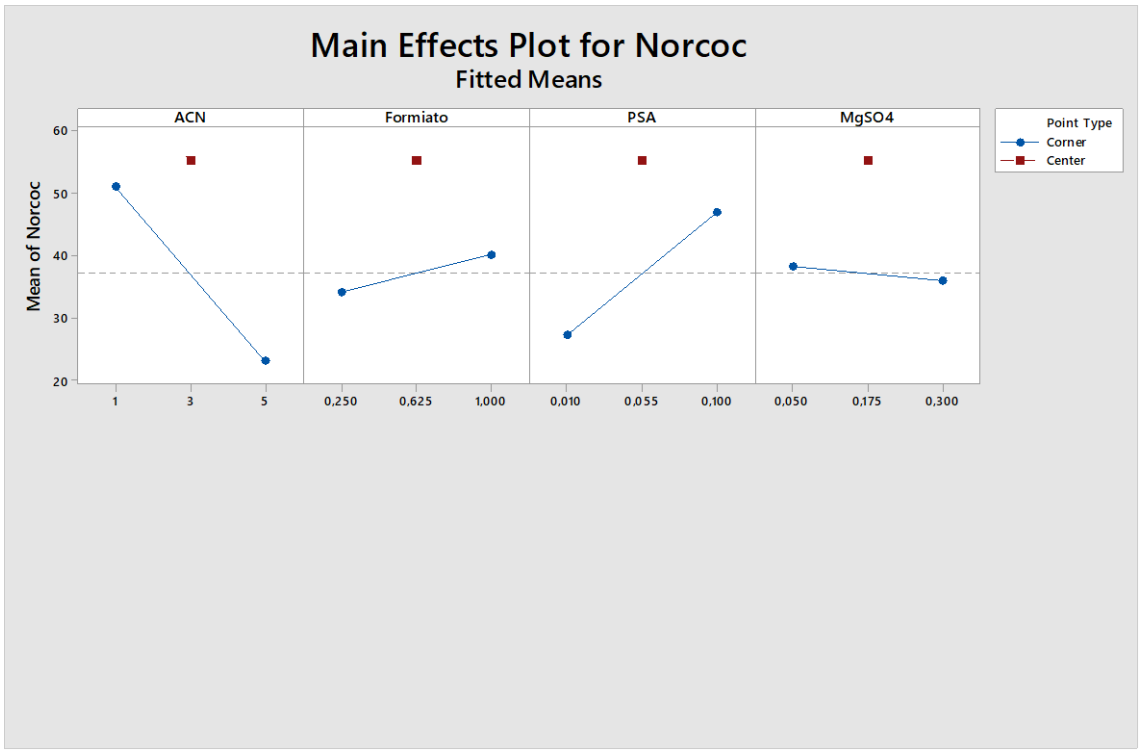
Point Type
Corner
Center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Fitted Means
ACN
Formiato
PSA
MgSO4
Mean of Norcoc
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40
30
20
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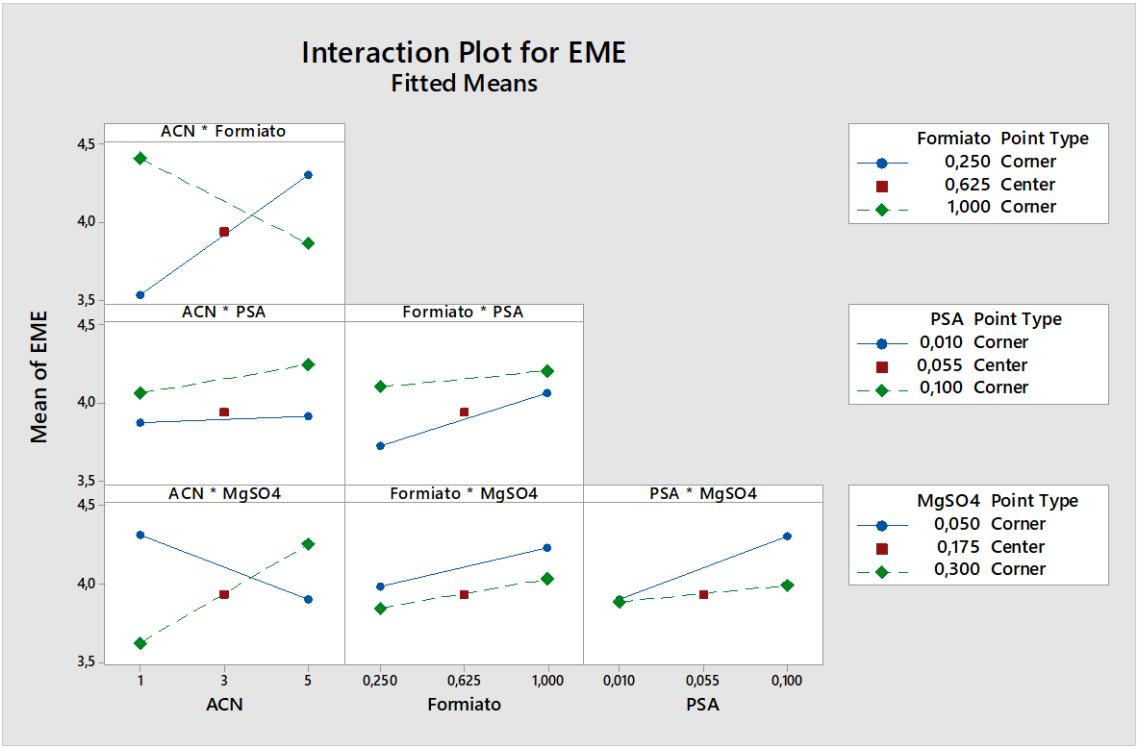
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Fitted Means
ACN * Formiato
ACN * PSA
Formiato * PSA
ACN * MgSO4
Formiato * MgSO4
PSA * MgSO4
Mean of EME
4,5
4,0
3,5
1
3
5
ACN
0,250
0,625
1,000
Formiato
0,010
0,055
0,100
PSA
Formiato Point Type
0,250 Corner
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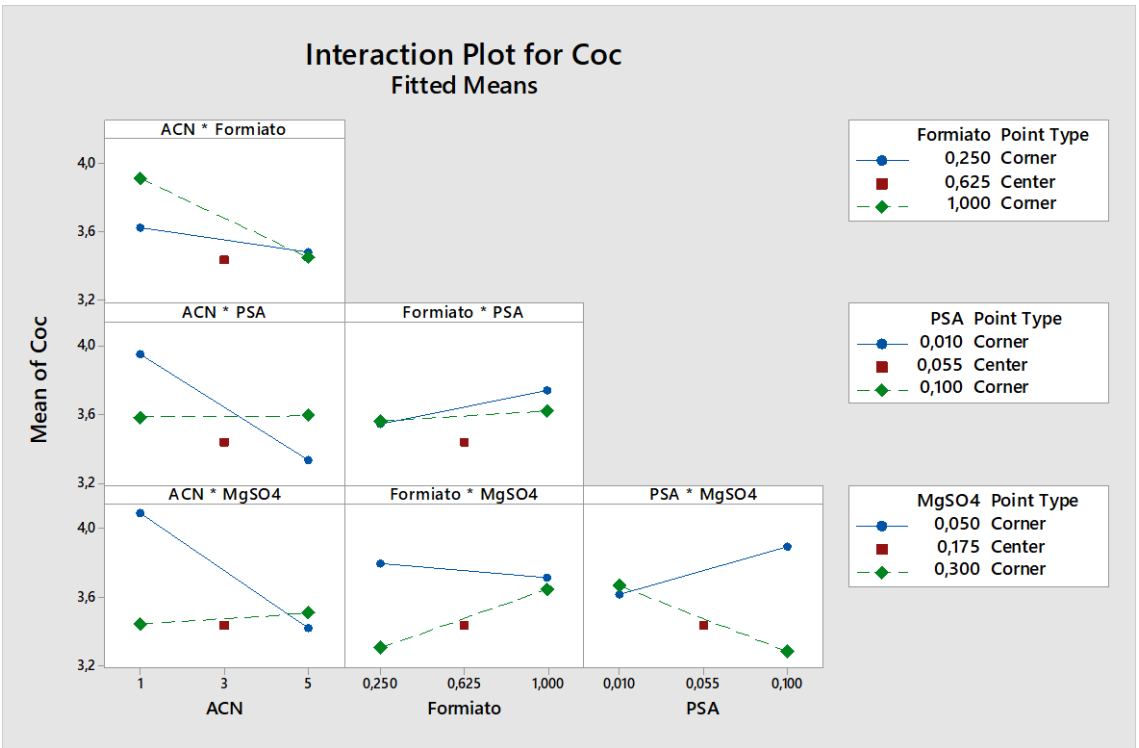
0,300 Corner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Fitted Means
ACN * Formiato
ACN * PSA
Formiato * PSA
ACN * MgSO4
Formiato * MgSO4
PSA * MgSO4
Mean of Coc
4,0
3,6
3,2
1
3
5
ACN
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0,625
1,000
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0,010
0,055
0,100
PSA
Formiato Point Type
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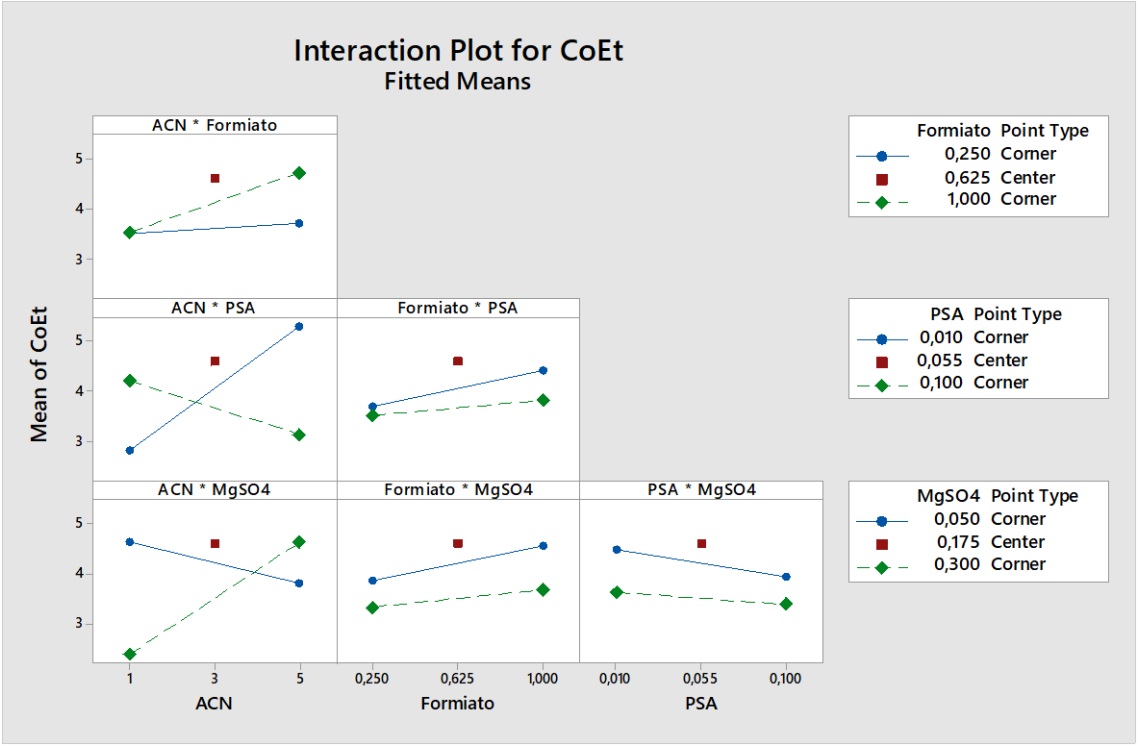
Interaction Plot for CoEt
Fitted Means
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ACN * PSA
Formiato * PSA
ACN * MgSO4
Formiato * MgSO4
PSA * MgSO4
Mean of CoEt
ACN
Formiato
PSA
Formiato Point Type
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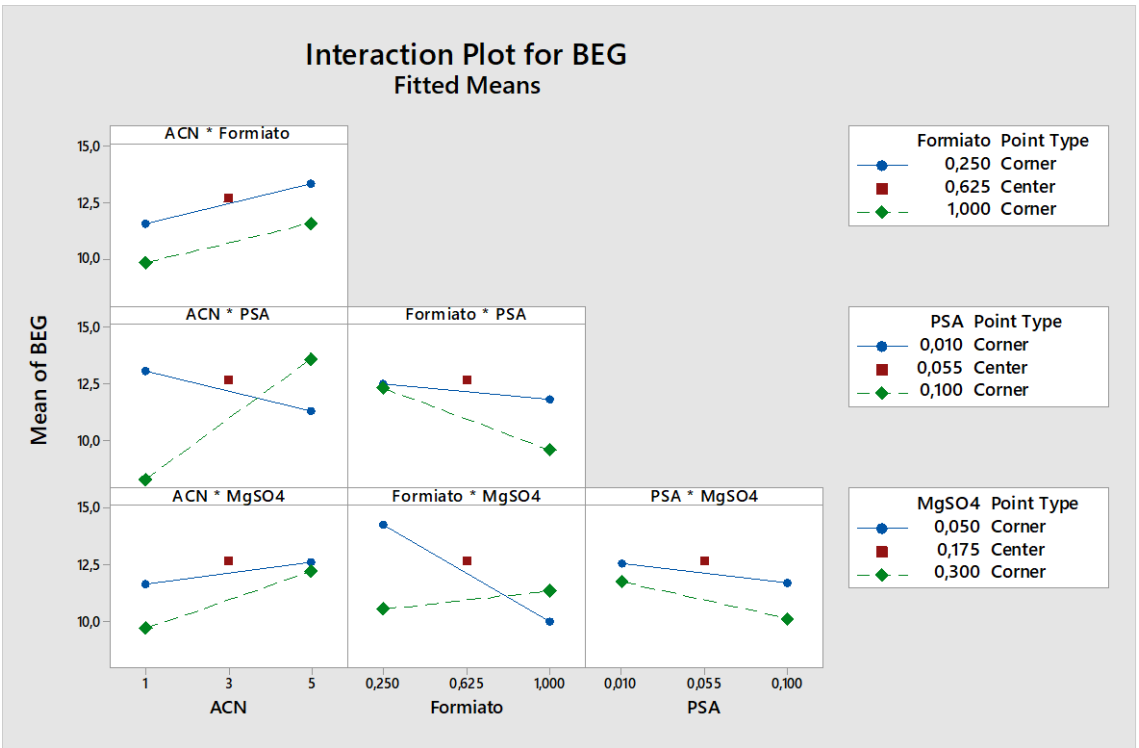
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ACN * MgSO4
Formiato * MgSO4
PSA * MgSO4
Mean of BEG
ACN
Formiato
PSA
Formiato Point Type
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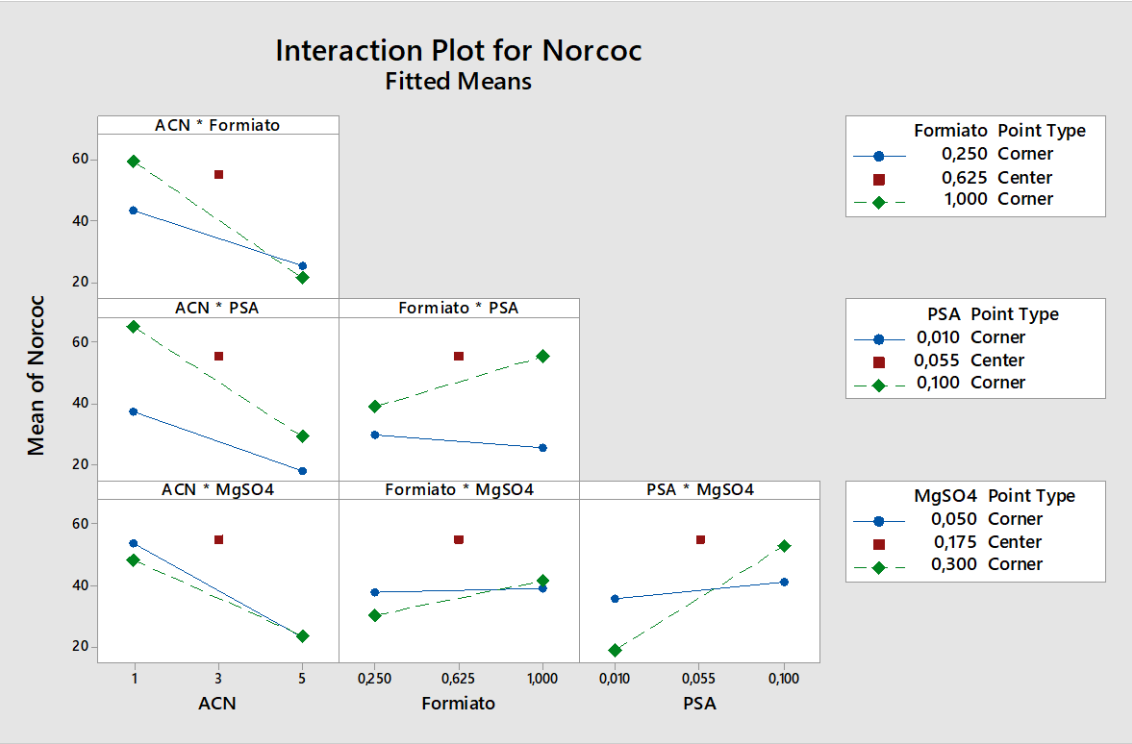
Interaction Plot for Norcoc
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ACN * MgSO4
Formiato * MgSO4
PSA * MgSO4
Mean of Norcoc
60
40
20
1
3
5
ACN
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0,625
1,000
Formiato
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PSA
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PSA Point Type
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