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Review Article

Detection of nicotine and cotinine in keratinized samples: A review

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Abstract
Nicotine, the primary addictive compound in tobacco and e-cigarette smoke, triggers a cycle of dependence and repeated use. Overconsumption of nicotine can be fatal when ingested at levels exceeding the median lethal dose (LD_{50}) of 6.5-13 mg/kg. Cotinine, the main metabolite of nicotine after consumption, is widely distributed throughout the body. Nicotine has a half-life of 6 to 8 hours, whereas cotinine has a half-life of 16 to 18 hours. The detection of nicotine and cotinine is widely employed in clinical toxicology, forensic toxicology, workplace testing, and related fields. Due to their rapid absorption rates, nicotine and cotinine are frequently analyzed in biological matrices such as blood, urine, and saliva. Nevertheless, their relatively short half-lives have shifted attention to keratinized matrices, including hair and nails, which offer superior utility for long-term monitoring. Drugs and xenobiotics are incorporated into keratinized tissues via systemic circulation during their growth phase, where they remain sequestered, providing a stable medium for retrospective analysis. This review examines contemporary methodologies for the detection and quantification of nicotine and cotinine in keratinized samples, emphasizing their potential in longitudinal toxicological assessments.

Keywords: e-cigarette, biomarkers, forensic chemistry, onychology, toxicology

Introduction

Nicotine ((S)-3-(1-methyl-2-pyrrolidyl)pyridine) is a naturally occurring addictive compound found in tobacco leaves, first used as a botanical insecticide in 1763 [1]. It is the primary alkaloid in tobacco, constituting approximately 95% of the total alkaloid content [2]. While nicotine drives the sustained and prolonged use of tobacco, it is not directly responsible for the harmful health effects associated with tobacco use [3]. Its addictive properties stem from the activation of nicotinic acetylcholine receptors (nAChRs), which induce rewarding and reinforcing effects, such as pleasure, arousal, and mood modulation [4]. These effects are mediated by dopamine release in the brain, a key component in the signaling of pleasure [5]. Addiction develops through neuroadaptation, requiring progressively higher doses of nicotine to achieve the same neurochemical effects. Over time, as tolerance builds, nicotine becomes essential to maintain normal brain function [4]. Cotinine, a minor alkaloid in tobacco, serves as the primary metabolite of nicotine in humans [6].

Smoking a cigarette results in the absorption of approximately 2 mg of nicotine, increasing the mean arterial plasma concentration to about 0.03 mg/L [7]. According to estimates in the literature, the lower fatal dose of nicotine ranges from 0.5 to 1 g, which corresponds to an oral lethal dose (LD50) of 6.5–13 mg/kg [8]. The evaluation of tobacco smoke exposure relies on biomarkers with several key attributes, including an appropriate half-life, high specificity, a clear dose-response relationship, suitability for detection in biospecimens, and quantifiable levels across a wide concentration range using current analytical techniques [9]. Additionally, an ideal biomarker should remain unaffected by the presence of other compounds during analysis and should not be influenced by environmental sources unrelated to tobacco smoke or e-cigarette vapors [10]. Identifying a suitable biomarker for tobacco is

critical, as accurate and precise exposure measurements are essential for confirming the use of nicotine-containing products and evaluating potential biological effects in long-term epidemiological studies [11].

Various biomarkers have been identified for assessing exposure to cigarettes or nicotine-containing products, including nicotine, cotinine, tobacco-specific nitrosamines (TSNAs), carbon monoxide, carboxyhemoglobin, carbonyl compounds, heavy metals, and thiocyanate ions [12]. These biomarkers have been successfully detected in biological matrices such as urine, blood, saliva, exhaled air, and respiratory fluids. Among them, nicotine, cotinine, and TSNAs are particularly specific to the use of nicotine-delivery products, likely due to their minimal influence from dietary and environmental exposures [9].

The detection of nicotine and cotinine is widely applied across various fields, including long-term monitoring of drug or medication consumption in clinical toxicology, determining causes of death or constructing deceased profiles in forensic toxicology, and supporting law enforcement investigations when other evidence is lacking [13]. Current assessments primarily focus on biospecimens such as urine, saliva, and blood due to the extensive distribution of nicotine and cotinine following inhalation. Their rapid circulation facilitates detection, with high concentrations present in body fluids [2]. Consequently, methods for analyzing nicotine and cotinine in these samples are well-established and reliable.

However, collecting biological fluids presents challenges, including the invasive nature of blood sampling, difficulties in handling and storage, and potential non-cooperation from individuals [14-16]. These limitations have prompted the exploration of alternative samples, such as keratinized matrices (nails and hair). The keratin composition of human nails allows for the incorporation and accumulation of xenobiotics through their porous surfaces [17]. Additionally, keratinized samples are easy to collect, resistant to degradation, and provide a practical solution for long-term monitoring [13]. Despite extensive research on nicotine and cotinine determination in biological fluids, studies focusing on keratinized samples remain relatively limited.

The literature was systematically searched across various platforms, including PubMed and Scopus, to identify studies containing relevant data. This review marks 26 years of progress in researching the detection of nicotine and cotinine in keratinized samples, spanning from 1997 to 2023. Previous reviews have highlighted key areas of focus, including the accuracy and feasibility of keratinized samples [18], recent advancements in detecting nicotine and cotinine across various sample types [19], and the use of keratinized specimens as biomarkers for exposure to tobacco smoke and drugs of abuse [20, 21].

This review provides a comprehensive summary of established methods for detecting nicotine and cotinine in keratinized specimens, offering an overview of this emerging field of research. Additionally, it examines sample treatment procedures, detailing their advantages and disadvantages to facilitate comparative analysis.

Pharmacokinetic of nicotine

Understanding the pharmacodynamics of nicotine is crucial for the detection of nicotine and cotinine, as it is influenced by several factors. Upon inhaling tobacco smoke, nicotine is distilled and carried as fine particles into the lungs. These particles are then rapidly absorbed into the pulmonary venous circulation and subsequently distributed into the arterial circulation as blood flows. At this stage, nicotine levels spike quickly in the blood, peaking at the completion of smoking [5, 2, 9]. Once nicotine enters the bloodstream, it is extensively distributed throughout the body, with a high affinity for organs such as the lungs, liver, kidneys, and spleen, and a lower affinity for adipose tissue. Additionally, nicotine readily crosses the blood-brain barrier, concentrating in the central nervous system, which limits the time required for tolerance to develop. This results in a potent pharmacological effect [2].

Nicotine is metabolized into several metabolites, with the major one being cotinine, which accounts for approximately 70-80%. The metabolism of cotinine follows two main pathways: oxidation by the enzyme CYP2A6, which produces nicotine-Δ1′(5′)-iminium ion, and further catalysis by cytoplasmic aldehyde oxidase to generate the final product [22]. After metabolism, cotinine circulates and accumulates in several bodily fluids, such as saliva and blood, and is excreted in urine [23]. Nicotine has an average half-life of two hours and persists for 6 to 8 hours after smoking cessation. In comparison, cotinine has a longer half-life of 16 to 18 hours due to its lower plasma protein binding [9]. This longer half-life makes cotinine a favorable biomarker in keratinized samples, as its incorporation takes more time. Drugs in nails are incorporated through four main mechanisms: (1) external contamination, (2) drugs in sweat or sebum, and (3) and (4) through circulation in the blood during nail growth, both horizontally and vertically [24, 25]. However, the exact incorporation mechanisms of

endogenous substances, xenobiotics, and metabolites have yet to be fully understood.

Generally, only a portion of nicotine in an electronic cigarette liquid is aerosolized upon inhalation, hence the nicotine concentration in aerosol is critical to nicotine pharmacokinetic [26]. The pharmacokinetics of nicotine in E-cigarette (e-cig) have been a key area of research to elucidate the differences with tobacco cigarettes. Three key elements were studied by groups of researchers such as time to maximum blood concentration (T_{max}), particular maximum blood concentration (C_{max}), and overall blood nicotine exposure. Helen et al. [27] found that the C_{max} of plasma occurred between 2 to 5 minutes after puffing and had lower levels than tobacco smoking, suggesting that not all nicotine inhaled is absorbed through the lungs. Additionally, the nicotine delivery levels by e-cigarettes are reported to have similar or higher systemic retention compared to tobacco cigarettes [27]. In addition, a similar finding was documented where a single use of e-cigarettes has a lower nicotine intake and systemic exposure when compared to tobacco cigarettes [28]. Moreover, Goniewicz et al. [29] found that nicotine concentration in vapour produced by e-cig is relatively lower than in conventional cigarettes. Furthermore, e-cigs users were found to have lower C_{max} and plasma nicotine concentration than tobacco users, while nicotine was delivered at the fastest rate in usual cigarette users [30].

Additionally, Hajek et al. [31] studied the effect of e-liquid nicotine concentrations on nicotine delivery, where the effect was found to be negligible. It was also reported that the nicotine delivery of e-cigarettes is not as efficient as conventional cigarettes, with similar T_{max}, lower C_{max}, and plasma concentrations. These findings suggest that nicotine delivery from e-cigarettes is comparatively lower than that of tobacco cigarettes. However, the development of e-cigarettes is still advancing, and high-power settings employed in recent devices have shown to improve nicotine delivery [32]. These controversial results suggest that further investigation is required to fully elucidate the relationship between nicotine delivery from e-cigarettes under different puffing topography.

Due to the complexity of smoking, the levels of nicotine are often varied among smokers since smokers can manipulate the inhaled dose of nicotine. This can be affected by inhalation depth, puff volume, dilution extent, intensity and puffing rate [2]. In addition, nicotine metabolism can be affected by genetic variation, race, gender, oestrogen-containing hormones, and diseases [8].

To explain further, there are several main factors affecting nicotine metabolism into cotinine and absorption into nails which includes: (1) physiological effect, (2) medications, (3) smoking habits, and (4) racial and ethnic differences [33]. The first point, physiological influences include diet, age, gender, pregnancy, and diseases. Diet may affect the metabolism rate of nicotine to cotinine due to hepatic flow, where a 30% increase in liver blood flow may enhance the clearance of nicotine at approximately 40% after a meal [2]. Moreover, the consumption of menthol can induce CYP2A6 inhibition which inhibits the metabolism of nicotine to cotinine [34].

In addition to diet, nicotine metabolism is influenced by age. A study conducted by Kumboyono et al. [35] found that older male smokers have higher cotinine levels compared to younger smokers. This might be explained by the reduced clearance of nicotine in the elderly compared to adults [36]. However, the elderly have been reported to have lower nicotine metabolism due to reduced hepatic flow [2]. On the other hand, gender also plays a role in nicotine metabolism. A study conducted by Pérez-Martín et al. [37] reported that females have a faster metabolic rate of nicotine than males, which can be influenced by the action of sex hormones on CYP2A6.

In terms of pregnancy, pregnant smokers have significantly lower cotinine levels due to a 140% increase in cotinine clearance as hepatic flow increases [38]. This was demonstrated by Selby et al. [39], where pregnant smokers reported relatively lower serum nicotine levels compared to population-based values. Lastly, kidney failure has been reported to reduce the renal clearance of nicotine and cotinine, while also affecting the metabolic clearance of nicotine [40]. Additionally, the uptake of several drugs for treatment may result in CYP2A6 induction and inhibition, which further affects cotinine clearance. It has been documented that the use of oral contraceptives in women leads to higher clearance of nicotine and cotinine [41]. Methoxsalen, a medication used in photochemotherapy for psoriasis, has been reported to inhibit the metabolism of nicotine by inhibiting CYP2A6. This drug also reduces the first-pass metabolism of orally taken nicotine and decreases the clearance of nicotine administered subcutaneously [42, 43].

Interestingly, smoking habits have been found to reduce the clearance of cotinine. Tobacco smokers have slower nicotine clearance compared to non-smokers, while nicotine clearance was reported to increase by 14% after four days of smoking cessation in smokers [44, 45]. Lastly, nicotine metabolism is also affected by racial and ethnic differences. Studies have shown that whites have a faster rate of metabolism compared to both Black and Asian

individuals [46], which is further supported by findings that Blacks report lower rates of nicotine clearance than Whites [47].

Detection of nicotine and cotinine

The widespread use of tobacco smoke and e-cigarettes has highlighted the need for toxicant monitoring, both among users and non-users, particularly for policy-making and decision-making purposes [48]. The detection of nicotine and cotinine in smokers has been studied for years using various keratinized samples, such as hair and nails. These concentrations are often compared with those of non-smokers to establish a cut-off point or to understand the relationship between the two for more accurate identification. This section provides a comprehensive summary of the detection of these biomarkers in smokers, e-cigarette users, passive smokers, and non-smokers using keratinized samples.

Nicotine and cotinine in hair

Various studies have focused on detecting nicotine and cotinine in hair. Kim et al. [49] developed a simplified method for analyzing nicotine metabolites, including trans-3'-hydroxycotinine (3-HCOT) and cotinine, in nail and hair samples using a streamlined solid-phase microextraction (QuEChERS) technique, with detection performed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The sample preparation process was optimized by evaluating the efficiency of decontaminating solvents, extraction solutions, and different packing materials for QuEChERS. The mean cotinine concentrations obtained for hair and nails were 7.6 pg/mg (10.2–1157.2 pg/mg) and 13.7 pg/mg (10.0–21.4 pg/mg), respectively. Based on these results, cotinine was determined to be a more suitable indicator for indirect nicotine exposure in keratinized samples, as it was more frequently detected. The authors also compared their method with previous ones, demonstrating that the current approach had higher recovery rates and lower relative standard deviation, making it more suitable for clinical use.

Additionally, Cashman and Nutt [50] developed a procedure for detecting nicotine and cotinine in hair using gas chromatography-tandem mass spectrometry (GC-MS/MS). Their findings revealed that concentrations of both compounds were approximately 37% higher in conventional tobacco smokers compared to e-cigarette users. Furthermore, the hair of e-cigarette users showed higher levels of cotinine than nicotine, which is believed to be due to the fact that nicotine is more readily absorbed, leading to increased cotinine levels as a metabolite.

A study assessing active and passive tobacco smoke exposure by measuring cotinine and nicotine levels in hair samples using LC-MS/MS was conducted, and the results were compared with self-reported data [51]. The developed method successfully distinguished between passive and active smokers, with both compounds detected at significantly higher levels in active smokers than in passive smokers. The mean nicotine concentration in passive smokers was 1.88 ± 1.85 ng/mg, compared to 43.12 ± 34.81 ng/mg in active smokers. Cotinine concentrations were 0.022 ± 0.028 ng/mg in passive smokers and 0.655 ± 0.616 ng/mg in active smokers. Additionally, nicotine and cotinine were detected in participants who reported no exposure to tobacco smoke, suggesting that self-reports can only serve as supportive documentation for long-term tobacco exposure.

A correlation study between hair nicotine levels and reported tobacco smoke exposure was conducted by Pattemore et al. [52], analyzing hair samples from children at different time points—birth, and at ages 3, 6, and 15 months—using HPLC. Parents were asked to complete a 15-month questionnaire detailing demographic information, tobacco smoke exposure after birth, smoking status during pregnancy, and other related factors. The study revealed a clear difference in the mean nicotine levels in the hair of children exposed to tobacco smoke compared to those not exposed, with levels reported at 1.32 ng/mg and 0.28 ng/mg, respectively. Furthermore, hair nicotine levels at 15 months were positively correlated with the estimated daily number of cigarettes smoked during pregnancy, as well as the combined total of cigarettes smoked per day by both parents. This suggests that the smoking history of a mother can be predicted by detecting nicotine levels in her child's hair. Additionally, the study found that an increase in hair nicotine levels at 15 months was associated with the presence of additional smokers in the household.

A pilot study conducted by Tzatzarakis et al. [53] aimed to assess the usefulness and validity of hair samples for detecting cotinine and nicotine as biomarkers of exposure to second-hand smoke (SHS) in both infants and adults. The study analyzed 66 hair samples using high-performance liquid chromatography (HPLC), including samples from infants, non-smoking adults, and active smokers. For the infants, it was found that those with both parents who smoke had higher hair nicotine levels compared to those with either one parent who smokes or no exposure to SHS, with mean concentrations of 11.98, 5.52, and 0.72 ng/mg, respectively. In adults, smokers showed significantly higher concentrations of hair nicotine and cotinine compared to non-smokers, with mean values of nicotine at 27.97 ng/mg versus 1.49 ng/mg, and cotinine at 1.16 ng/mg versus 0.13 ng/mg. Among smokers, a dose-response

relationship was observed between the number of daily cigarettes smoked and hair nicotine and cotinine levels. The study established a significant difference between smokers and non-smokers, demonstrating that hair nicotine and cotinine are reliable indicators for distinguishing between these groups.

An earlier study conducted by Groner et al. [54] aimed to determine whether a correlation existed between children's exposure to second-hand smoke (SHS) and nicotine levels in hair, using HPLC. A total of 115 hair samples were analyzed, including 61 from youth and 54 from toddlers. The results revealed that hair nicotine levels were higher in toddlers compared to older children, with mean concentrations of 1.90 ng/mg versus 0.48 ng/mg, respectively. The underlying mechanisms for this difference remain unknown.

A headspace-solid phase microextraction (HS-SPME) method followed by GC-MS analysis was optimized for determining nicotine levels in hair samples from non-smokers [55]. After validating the method, 100 hair specimens from children aged 5 to 12 were collected to assess environmental tobacco smoke (ETS) exposure. Nicotine was successfully detected in all samples, with significantly higher concentrations in the exposed group compared to the non-exposed group, yielding mean nicotine concentrations of 2.57 ng/mg and 0.76 ng/mg, respectively. Additionally, the study concluded that hair cotinine is less suitable as an indicator for low ETS exposure due to many samples falling below the detection limit of 0.02 ng/mg for the proposed method.

A similar study earlier optimized GC-MS extraction for detecting nicotine in hair samples from both children and adults with SHS exposure [56]. Nicotine was successfully identified in all samples, with low mean concentrations of 0.42 ng/mg in adults and 0.88 ng/mg in children. This study suggests that hair samples are a feasible biomarker for evaluating SHS exposure.

A previous study conducted in Malaysia used GC-MS to determine nicotine levels in hair among university students, who were categorized into four groups: active smokers, non-smokers, passive smokers, and ex-smokers [57]. The results showed that nicotine was detected in all hair samples, with the highest levels found in active smokers, followed by ex-smokers, passive smokers, and non-smokers, with mean concentrations of 26.25, 4.59, 2.94, and 1.02 ng/mg, respectively. Significant differences in nicotine levels were observed between the groups. Additionally, environmental tobacco smoke was assessed by developing a simplified detection method using a molecularly imprinted polymer (MIP) as the selective sorbent for solid-phase extraction (SPE), followed by HPLC analysis of nicotine levels in hair samples from non-smokers and smokers [58]. The results revealed a wide range of nicotine concentrations in hair, with levels of 5.1–69.5 ng/mg in smokers and 0.5–9.3 ng/mg in non-smokers. A positive association was observed between ETS exposure levels and nicotine concentrations in hair. However, the study faced a limitation in differentiating between non-smokers and passive smokers due to the overlapping range of nicotine concentrations.

A simultaneous procedure for determining nicotine and cotinine in hair was developed using HPLC with various extraction methods, differing from those used in recent studies [59]. The findings revealed that the mean concentrations of nicotine and cotinine were significantly higher in smokers, with levels of 39.0 ng/mg and 2.5 ng/mg, respectively. In contrast, cotinine was not detected in any non-smokers (1.9 ng/mg in smokers). Additionally, no correlation was found between nicotine and cotinine concentrations, which may be attributed to individual variability in nicotine metabolism and the differing metabolic rates of cotinine. Therefore, it is suggested to measure both nicotine and cotinine to more accurately assess tobacco smoke exposure.

Nicotine and cotinine in nails

Detection of nicotine and cotinine remains limited due to a lack of understanding of the mechanisms behind drug incorporation. A study conducted by Mari et al. [60] investigated the effectiveness of using newborn nails to monitor in utero drug exposure, including methadone, cocaine, benzoylecgonine, nicotine, morphine, and cotinine. Fingernails and toenails were collected from newborns within their first three months of life, and mothers completed a questionnaire regarding their smoking status or exposure to second-hand smoke (SHS). The results revealed that approximately 52% of the samples tested positive for nicotine and cotinine, indicating exposure to tobacco smoke. Among the samples that tested positive for both compounds, only 12.1% of mothers admitted smoking during pregnancy. However, positive detections of either nicotine or cotinine were also found in samples from non-smoking babies, suggesting passive nicotine inhalation during pregnancy.

Meanwhile, Al-Delaimy et al. [61] explored the feasibility of using toenail nicotine levels as a novel indicator of tobacco exposure by comparing these levels with self-reported tobacco exposure in a large

cohort study. A total of 2,485 samples were analyzed using LC-MS after extraction. The study found that active smokers had the highest nicotine concentrations in their toenails, followed by non-smokers with second-hand smoke (SHS) exposure, and non-smokers without SHS exposure, with mean concentrations of 1.77, 0.14, and 0.10 ng/mg, respectively. A significant difference was observed between these groups, though there was considerable overlap in nicotine levels based on reported smoking status. Overall, the results suggest that toenail nicotine levels can effectively measure tobacco smoke exposure and provide additional information not captured by self-reported data.

A study focused on the detection of nicotine and cotinine in toenails using GC-MS, and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol(NNAL) using LC-MS/MS. The newly developed method successfully detected nicotine and cotinine at low detection limits of 0.01 and 0.012 ng/mg, respectively. Additionally, a correlation was found between the concentrations of cotinine and NNAL in smokers' toenails, demonstrating that both are robust biomarkers for assessing long-term tobacco exposure [62].

The use of toenails as a biomarker for tobacco smoke exposure has been explored in earlier studies. One such study involved 106 participants who were categorized into four groups: (1) never smokers or past smokers with no exposure to environmental tobacco smoke (ETS); (2) passive smokers; (3) active smokers without ETS exposure; and (4) active smokers with ETS exposure. The findings revealed a significant difference in toenail nicotine levels between passive smokers and non-exposed individuals, with mean concentrations of 0.28 ng/mg and 0.08 ng/mg, respectively, among non-active smokers. In the active smoker group, a clear correlation was observed between the number of cigarettes smoked daily and nicotine levels, with higher nicotine concentrations associated with increased cigarette consumption. Additionally, similar nicotine levels were found among active and passive smokers. The results also demonstrated a correlation between toenail nicotine levels and cigarette smoke exposure, indicating that toenails are a valid biomarker for assessing both active and passive tobacco smoke exposure [20].

Sample preparation of keratinised sample for nicotine and cotinine detection

Different methods of sample preparation for keratinized samples in extracting nicotine and cotinine have been developed and validated. These methods have been proven robust by the authors for instrumental analysis, mainly chromatographic techniques. This section outlines the sample pretreatment steps prior to analysis from various studies. **Table 1** shows the different methods of extraction and determination of these two biomarkers in the mentioned studies. To summarize, samples were decontaminated, digested, and extracted under basic conditions using NaOH to enhance the extraction of cotinine. This approach relies on the principle that the pKa value of cotinine is 4.8, making it a strong basic drug [63]. Therefore, in a high pH environment, cotinine exists in its basic (unionized) form, which can be readily extracted by organic solvents, and vice versa [64]. After extracting the analyte of interest, sample cleaning and preconcentration are performed using solid-phase extraction, nitrogen blowing to reconstitute the sample in a suitable solvent, and direct instrument solid-phase microextraction to enhance recovery, sensitivity, and specificity.

Table 1. Summary of past studies on determination of nicotine and cotinine in keratinized samples

Author (s)	Sample treatment	Instrumentation	Advantages and Disadvantages
Kim et al. [49]	Samples were treated with 0.1% sodium dodecyl sulfate (SDS) aqueous solution, then digested with 1 M NaOH at room temperature (20–25°C). Samples were then passed through QuEChERS (a solid phase microextraction technique) consisting 800mg of magnesium sulfate, 200mg of sodium chloride, and 150mg of PSA.	LC–MS/MS	Advantage: • Sample cleaning and concentrated with QuEChERS provide better resolution and recovery. Disadvantage: • High cost of QuEChERS • Lengthy sample preparation

Cashman and Nutt [50]	20-25 mg of hair samples were washed thrice with 3 mL of DCM by vortex. Samples were digested with 3 ml of 0.5N NaOH for 4 hours at room temperature. The solution was transferred to an Extrelut-3 glass column, which was preconditioned with 10 ml of DCM and left dry overnight. After passing through the extractant, 8 ml of dichloromethane-isopropyl alcohol (9:l) was used to elute the analytes. The DCM washes and the eluents from solid-phase extractions columns, added with 500 µL of methanolic HCl (25 mM), were evaporated to dryness, dissolved in 100 µL of HPLC buffer and analysed.	GC-MS/MS	Advantage: • Sample cleaning and concentrated with Extrelut-3 glass column provide better resolution and recovery. Disadvantage: • High cost of Extrelut-3 glass column • Lengthy sample preparation
Inukai et al. [51]	Hair samples undergone three rounds of sonication in 1 mL DCM for 3 minutes each and air dried. Around 1–2 mg of sample was added with 1mL of distilled water and internal standard, followed by extraction at 80◦C for 30 minutes. The sample was then cool, centrifuged, and transferred for analysis.	In-tube SPME LC–MS/MS	Advantage: • Simple sample preparation Disadvantage: • High cost of in-tube SPME LC-MS/MS
Pattemore et al. [52]	2–4 mg of samples was washed without agitation for 90 min in 2 ml of DCM at room temperature (21°C). DCM was then aspirated off and dried under 501°C. Samples were then subjected for overnight digestion overnight in 2 ml of 1N NaOH at 50°C. Nicotine was extracted in 4mL of diethyl ether by vortexing 40-60 s. The ether was then transferred out and added with 100 µL of 0.1% HCl in methanol, dried and redissolved with mobile phase.	HPLC with electrochemical detection	Advantage: • Straightforward sample pretreatment.
Tzatzarakis et al. [53]	Hair samples were cleaned twice with water, hexane and DCM and dried at 50°C. Digestion occurs for 90 min at 60°C with 2 ml 1M NaOH. Samples were extracted mechanically twice with 3 ml of DCM for 15 minutes. Then, 50 ml of 1M of HCl was added to adjust pH to 2, then dry with nitrogen at 30°C, dissolved in 100 µL methanol.	LC-MS	Advantage: • Good sensitivity of LCMS Disadvantage: • Lengthy sample preparation and generate waste

Groner et al. [54]	2–4 mg of samples was washed without agitation for 90 min in 2 ml of DCM at room temperature (21°C). DCM was then aspirated off and dried under 50°C. Samples were then digested overnight in 2 ml of 1N NaOH at 50°C. Nicotine was extracted in 4mL of diethyl ether by vortexing 40-60 s. The ether was then transferred out and added with 100 µL of 0.1% HCl in methanol, dried and redissolved with mobile phase.	HPLC with electrochemical detection	Advantage: • Straightforward sample pretreatment.
Lukrica et al. [55]	Around 2 cm long of hair sample was decontaminated with 2 mL of DCM for 2 min at ambient temperature and air dried. Then, 25 mg of the sample was taken, added with 25 mL of the IS solution of diphenylamine, 0.5 g of NaCl and 1 mL of 1M NaOH. The samples were heated at 80°C for 60 minutes then extracted with a PA fibre at 80°C for 15 minutes. The SPME fibre was inserted into the injection for analysis.	GC-MS	Advantage: • Usage of SPME fibre preconcentrate the analyte Disadvantage: • High cost of SPME fibre
Kim et al. [56]	30 mg of samples were washed using 3 mL of DCM by sonication and dried.1.5 mL of 1 M NaOH and 70 ng of internal standard (Nicotine-d3) were added and incubated at 50°C for 24 hours. After incubation, 3.5 mL of diethyl ether was added and shaken for 60 minutes, and finally centrifuged. The organic phase in was then transferred and added with 17.5 µL octanol. The samples were then extracted again with diethyl ether. The organic layer from combined extractions was thermally evaporated. Finally, 52.5 µL of methanol was added to the remaining solution of octanol and nicotine, bringing the volume to 70 µL.	GC-MS	Advantage: • Great sensitivity of GCMS Disadvantage: • Lengthy sample preparation
Man et al. [57]	Samples were washed twice using DCM with 15 minutes of sonication and dried overnight at 30°C. After washing, samples were mixed with 100 µL of 1M NaOH, 50 uL of internal standard and left overnight for digestion at room temperature. Then, 1 mL of distilled water was added, briefly mixed and centrifuged. The clear supernatant was recovered and extracted using 0.4 mL solvent mixture (methanol:chloroform (1:3)). The extract was centrifuged and dried over anhydrous sodium sulphate and inject for analysis.	GC-MS	Advantage: • High sensitivity of GCMS Disadvantage: • Usage of toxic solvent such as chloroform

Mari et al. [57]	A 10 mg nail sample was washed with 1 ml of methanol. Nalorphine was added as an internal standard, then incubated overnight with 0.1N HCl, solid phase extracted (Bond Elut Certify LRC cartridges) using the manufacturer's method proposed, derivatized with 50 ml of BSTFA with 1% TMCS and analysed.	GC-MS	Advantage: • Sample is preconcentrated with solid phase extraction Disadvantage: • Required a lot of specific chemicals to perform
Al-Delaimy and Willett [61]	2–4 mg of samples are washed without agitation for 90 min in 2 ml of DCM at room temperature (21°C). DCM was then aspirated off and dried under 50°C. Samples were then digested overnight in 2 ml of 1N NaOH at 50°C. Nicotine was extracted in 4mL of diethyl ether by vortexing 40-60 s. The ether was then transferred out and added with 100 µL of 0.1% HCl in methanol, dried and redissolved with mobile phase.	HPLC with electrochemical detection	Advantage: Straightforward sample pretreatment.
Yang et al. [58]	Hair samples of 20mg were washed thrice with 3.0 ml of DCM by vortex and dried. Then, the samples were subjected for digestion with 500 µL of 1M NaOH for 14 hours at 50°C and then centrifuged. The clear supernatant was diluted with a buffer of ammonium acetate + ammonia at pH 10.0. Next, a 1.0 ml aliquot was passed through the nicotine MISPE.	HPLC-PDA	Advantage: • Good recovery with HPLC-PDA Disadvantage: • Lengthy sample preparation
Stepanov et al. [62]	Around 20-30 mg of toenails were washed with 2 mL DCM under room temperature at 90-120 minutes without agitation and dried under 50°C. Then, 0.5mL of 1M NaOH was added for digestion at 50°C overnight. After that, the sample was added with an internal standard, 1 mL of DCM, and 0.5 mL of 25% potassium carbonate for liquid-liquid extraction. The organic phase was transferred and mixed with 200 µL of MeOH and dried to a volume of 100 µL for analysis.	GC-MS	Advantage: • Better sensitivity of GCMS Disadvantage: • Lengthy sample preparation

Chetiyanukornkul et al. [59]	Hair samples of 10 mg were washed three times without agitation for 10 minutes in 3 mL of DCM, dried, sonicated for 2 h with 400 µL of 2.5 M NaOH, mixed with internal standard and extracted twice with 400 µL of DCM. The combined organic phase was mixed with 500 µL of 25 mM HCl in methanol, evaporated to dryness completely, redissolved in the mobile phase for analysis.	LC-ESI-MS	Advantage: • Great sensitivity of LC-ESI-MS Disadvantage: • Lengthy sample preparation
Al-Delaimy et al. [20]	2–4 mg of samples was washed without agitation for 90 min in 2 ml of DCM at room temperature (21°C). DCM was then aspirated off and dried under 50°C. Samples were then digested overnight in 2 ml of 1N NaOH at 50°C. Nicotine was extracted in 4mL of diethyl ether by vortexing 40-60 s. The ether was then transferred out and added with 100 µL of 0.1% HCl in methanol, dried and redissolved with mobile phase.	HPLC with electrochemical detection	Advantage: Straightforward sample pretreatment

Conclusion

Various sample preparation methods and detection techniques have been explored to evaluate nicotine and cotinine concentrations in keratinized samples. These studies demonstrate the feasibility of using hair and nails as biospecimens for long-term drug monitoring. However, establishing a cutoff point and considering factors such as vaping status and second-hand smoke exposure are essential when interpreting the results. Several limitations have been identified in the literature. First, there is often a lack of comprehensive information on smoking status and behavior, which complicates the interpretation of metabolic accumulation levels. Second, significant variation in nicotine and cotinine concentrations is observed both between and within studies. This variability can be attributed to factors such as exposure history, environmental ventilation, duration of exposure, and individual inhalation capacity.

Additionally, identifying these biomarkers in keratinized samples remains challenging, as research into drug incorporation into nails is still ongoing, and various factors could contribute to the mechanism. Currently, no single analysis method provides a definitive measurement of nicotine and cotinine concentrations without comprehensive information. Hence, it is crucial to study the correlation between analyte concentrations in the matrix and the exposure period. Finally, despite extensive research, the correlations between different analysis methods have not been fully elucidated. Overall, while keratinized samples offer promising avenues for long-term monitoring of tobacco exposure, addressing these limitations and establishing standardized methods is crucial for accurate assessment.

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References

1. Gupta, P. K. (2016). Toxic effects of pesticides (agrochemicals). *Fundamentals of Toxicology*, 185-202.
2. Benowitz, N. L., Hukkanen, J., and Jacob, P. III. (2009). Nicotine chemistry, metabolism, kinetics, and biomarkers. In *Nicotine Psychopharmacology* (pp. 29-60).
3. Foll, B. L., Piper, M. E., Fowler, C. D., Tonstad, S., Bierut, L., Lu, L., and Hall, W. D. (2022). Tobacco and nicotine use. *Nature Reviews Disease Primers, 8*(1): 19.
4. Benowitz, N. L. (2009). Pharmacology of nicotine: Addiction, smoking-induced disease, and therapeutics. *Annual Review of Pharmacology and Toxicology,* 49: 57-71.
5. Prochaska, J. J., and Benowitz, N. L. (2016). The past, present, and future of nicotine addiction therapy. *Annual Review of Medicine, 67*: 467-486.
6. Tan, X., Vrana, K., and Ding, Z. M. (2021). Cotinine: Pharmacologically active metabolite of nicotine and neural mechanisms for its actions. *Frontiers in Behavioral Neuroscience,* 15:

758252.
7. Gourlay, S. G., and Benowitz, N. L. (1997). Arteriovenous differences in plasma concentration of nicotine and catecholamines and related cardiovascular effects after smoking, nicotine nasal spray, and intravenous nicotine. *Clinical Pharmacology and Therapeutics,* 62(4): 453-463.
8. Hukkanen, J., Jacob, P., and Benowitz, N. L. (2005). Metabolism and disposition kinetics of nicotine. *Pharmacological Reviews, 57*(1): 79-115.
9. Schick, S. F., Blount, B. C., Jacob, P., Saliba, N. A., Bernert, J. T., El Hellani, A., Jatlow, P., Pappas, R. S., Wang, L., Foulds, J., Ghosh, A., Hecht, S. S., Gomez, J. C., Martin, J. R., Mesaros, C., Srivastava, S., St. Helen, G., Tarran, R., Lorkiewicz, P. K., Blair, I. A., Kimmel, H. L., Doerschuk, C. M., Benowitz, N. L., and Bhatnagar, A. (2017). Biomarkers of exposure to new and emerging tobacco delivery products. *American Journal of Physiology. Lung Cellular and Molecular Physiology,* 313(3): L425-L452.
10. Dhar, P. (2004). Measuring tobacco smoke exposure: Quantifying nicotine/cotinine concentration in biological samples by colorimetry, chromatography, and immunoassay methods. *Journal of Pharmaceutical and Biomedical Analysis,* 35(1): 155-168.
11. Goniewicz, M. L. (2023). Biomarkers of electronic nicotine delivery systems (ENDS) use. *Addiction Neuroscience,* 6: 100077.
12. Goniewicz, M. L., Knysak, J., Gawron, M., Kosmider, L., Sobczak, A., Kurek, J., and Benowitz, N. (2014). Levels of selected carcinogens and toxicants in vapour from electronic cigarettes. *Tobacco Control,* 23(2): 133-139.
13. Solimini, R., Minutillo, A., Kyriakou, C., Pichini, S., Pacifici, R., and Busardo, F. P. (2017). Nails in forensic toxicology: An update. *Current Pharmaceutical Design, 23*(36): 5468-5479.
14. Avila-Tang, E., Al-Delaimy, W. K., Ashley, D. L., Benowitz, N., Bernert, J. T., Kim, S., and Hecht, S. S. (2013). Assessing secondhand smoke using biological markers. *Tobacco Control,* 22(3): 164-171.
15. Al-Delaimy, W. K., Stampfer, M. J., Manson, J. E., and Willett, W. C. (2008). Toenail nicotine levels as predictors of coronary heart disease among women. *American Journal of Epidemiology,* 167(11): 1342-1348.
16. Kim, S., Apelberg, B. J., Avila-Tang, E., Hepp, L., Yun, D., Samet, J. M., and Breysse, P. N. (2014). Utility and cutoff value of hair nicotine as a biomarker of long-term tobacco smoke exposure, compared to salivary cotinine. *International Journal of Environmental Research and Public Health,* 11(8): 8368-8382.
17. Muddasani, S., Lin, G., Hooper, J., and Sloan, S. B. (2021). Nutrition and nail disease. *Clinical Dermatology,* 39: 819-828.
18. Bandoli, G., Anunziata, F., Bogdan, R., Zilverstand, A., Chaiyachati, B. H., Gurka, K. K., ... and Bakhireva, L. N. (2024). Assessment of substance exposures in nail clipping samples: A systematic review. *Drug and Alcohol Dependence,* 254: 111038.
19. Marques, H., Cruz-Vicente, P., Rosado, T., Barroso, M., Passarinha, L. A., and Gallardo, E. (2021). Recent developments in the determination of biomarkers of tobacco smoke exposure in biological specimens: A review. *International Journal of Environmental Research and Public Health,* 18(4): 1768.
20. Al-Delaimy, W. K., Mahoney, G. N., Speizer, F. E., and Willett, W. C. (2002). Toenail nicotine levels as a biomarker of tobacco smoke exposure. *Cancer Epidemiology Biomarkers & Prevention,* 11(11): 1400-1404.
21. Cappelle, D., Yegles, M., Neels, H., van Nuijs, A. L. N., De Doncker, M., Maudens, K., Covaci, A., and Crunelle, C. L. (2014). Nail analysis for the detection of drugs of abuse and pharmaceuticals: A review. *Forensic Toxicology,* 33(1): 12-36.
22. Tricker, A. R. (2006). Biomarkers derived from nicotine and its metabolites: A review. *Contributions to Tobacco & Nicotine Research,* 22(3): 147-175.
23. Raja, M., Garg, A., Yadav, P., Jha, K., and Handa, S. (2016). Diagnostic methods for detection of cotinine level in tobacco users: A review. *Journal of Clinical and Diagnostic Research:* 10(3): ZE04.
24. Hill, V. A., Stowe, G. N., Paulsen, R. B., and Schaffer, M. (2018). Nail analysis for drugs: A role in workplace testing? *Journal of Analytical Toxicology,* 42(6): 425-436.
25. Madry, M. M., Steuer, A. E., Binz, T. M., Baumgartner, M. R., and Kraemer, T. (2014). Systematic investigation of the incorporation mechanisms of zolpidem in fingernails. *Drug Testing and Analysis,* 6(6): 533-541.
26. Schroeder, M. J., and Hoffman, A. C. (2014). Electronic cigarettes and nicotine clinical pharmacology. *Tobacco Control,* 23: 30-35.
27. St Helen, G., Havel, C., Dempsey, D. A., Jacob, P. and Benowitz, N. L. (2016). Nicotine delivery, retention and pharmacokinetics from various electronic cigarettes. *Addiction,* 111(3): 535-544.
28. St. Helen, G., Nardone, N., Addo, N., Dempsey, D., Havel, C., Jacob III, P., and Benowitz, N. L.

(2020). Differences in nicotine intake and effects from electronic and combustible cigarettes among dual users. *Addiction*, 115(4): 757-767.
29. Goniewicz, M. L., Knysak, J., Gawron, M., Kos mider, L., Sobczak, A., Kurek, J., and Benowitz, N. (2014). Levels of selected carcinogens and to xicants in vapour from electronic cigarettes. *Tobacco Control*, 23(2): 133-139.
30. McDermott, S., Reichmann, K., Mason, E., Fearon, I. M., O'Connell, G., and Nahde, T. (2023). An assessment of nicotine pharmacokinetics and subjective effects of the pulze heated tobacco system compared with cigarettes. *Scientific Reports*, 13(1): 9037.
31. Hajek, P., Przulj, D., Phillips, A., Anderson, R., and McRobbie, H. (2017). Nicotine delivery to users from cigarettes and from different types of e-cigarettes. *Psychopharmacology*, 234: 773-779.
32. Farsalinos, K. E., Gillman, G., Poulas, K., and Voudris, V. (2015). Tobacco-specific nitrosamines in electronic cigarettes: Comparison between liquid and aerosol levels. *International Journal of Environmental Research and Public Health*, 12(8): 9046-9053.
33. Akrodou, Y. M. (2015). CYP2A6 polymorphisms may strengthen individualized treatment for nicotine dependence. *Scientifica*, 2015(1): 491514.
34. Benowitz, N. L., Herrera, B., and Jacob, P. (2004). Mentholated cigarette smoking inhibits nicotine metabolism. *Journal of Pharmacology and Experimental Therapeutics*, 310(3): 1208-1215.
35. Kumboyono, K., Chomsy, I. N., Hidayat, W. S., Hakim, A. K., Shalshabilla, N. N., Sujuti, H., and Wihastuti, T. A. (2023). Factors affecting the serum cotinine level of male smokers in Malang, Indonesia. *International Journal of Medical Toxicology and Forensic Medicine*, 13(3): 40130-40130.
36. Molander, L., Hansson, A., and Lunell, E. (2001). Pharmacokinetics of nicotine in healthy elderly people. *Clinical Pharmacology & Therapeutics*, 69(1): 57-65.
37. Pérez-Martín, H., Lidón-Moyano, C., González-Marrón, A., Fu, M., Pérez-Ortuño, R., Ballbè, M., and Martínez-Sánchez, J. M. (2023). Variation in nicotine metabolization according to biological factors and type of nicotine consumer. *Healthcare*, 11(2): 179.
38. Dempsey, D., Jacob, P., & Benowitz, N. L. (2002). Accelerated metabolism of nicotine and cotinine in pregnant smokers. *Journal of Pharmacology and Experimental Therapeutics*, 301(2): 594-598.
39. Selby, P., Hackman, R., Kapur, B., Klein, J., and Koren, G. (2001). Heavily smoking women who cannot quit in pregnancy: Evidence of pharmacokinetic predisposition. *Therapeutic Drug Monitoring*, 23(3): 189-191.
40. Molander, L., Hansson, A., Lunell, E., Alainentalo, L., Hoffmann, M., and Larsson, R. (2000). Pharmacokinetics of nicotine in kidney failure. *Clinical Pharmacology & Therapeutics*, 68(3): 250-260.
41. Benowitz, N. L., Lessov-Schlaggar, C. N., Swan, G. E., and Jacob III, P. (2006). Female sex and oral contraceptive use accelerate nicotine metabolism. *Clinical Pharmacology & Therapeutics*, *79*(5): 480-488.
42. Sellers, E. M., Kaplan, H. L., and Tyndale, R. F. (2000). Inhibition of cytochrome P450 2A6 increases nicotine's oral bioavailability and decreases smoking. *Clinical Pharmacology & Therapeutics*, 68(1): 35-43.
43. Sellers, E. M., Ramamoorthy, Y., Zeman, M. V., Djordjevic, M. V., and Tyndale, R. F. (2003). The effect of methoxsalen on nicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) metabolism in vivo. *Nicotine & Tobacco Research*, *5*(6): 891-899.
44. Benowitz, N. L., and Jacob III, P. (1993). Nicotine and cotinine elimination pharmacokinetics in smokers and nonsmokers. *Clinical Pharmacology & Therapeutics*, 53(3): 316-323.
45. Benowitz, N. L., and Jacob III, P. (2000). Effects of cigarette smoking and carbon monoxide on nicotine and cotinine metabolism. *Clinical Pharmacology & Therapeutics, 67*(6): 653-659.
46. Rubinstein, M. L., Shiffman, S., Rait, M. A., and Benowitz, N. L. (2013). Race, gender, and nicotine metabolism in adolescent smokers. *Nicotine & Tobacco Research*, 15(7): 1311-1315.
47. Schwartz, E. K., Palmisano, A. N., Gueorguieva, R., DeVito, E. E., and Sofuoglu, M. (2023). Examining racial differences in smoking outcomes among smokers enrolled in an intravenous nicotine infusion study. *Addictive Behaviors*, 140: 107615.
48. Baird, C. (2020). Electronic nicotine delivery systems aka vapes and public policy. *Journal of Addictions Nursing*, 31(4): 310-311.
49. Kim, J., Cho, H. D., Suh, J. H., Lee, J. Y., Lee, E., Jin, C. H., and Han, S. B. (2020). Analysis of nicotine metabolites in hair and nails using QuEChERS method followed by liquid chromatography–tandem mass spectrometry. *Molecules, 25*(8): 1763.
50. Cashman, L., and Nutt, J. (2019). A comparison of levels of nicotine and cotinine in hair of tobac co smokers and users of e-cigarettes using GC-MS. *Toxicologie Analytique et Clinique*, 31(2): S

83.
51. Inukai, T., Kaji, S., and Kataoka, H. (2018). Anal -ysis of nicotine and cotinine in hair by on-line in-tube solid-phase microextraction coupled with liquid chromatography-tandem mass spectrometry as biomarkers of exposure to tobacco smoke. *Journal of Pharmaceutical and Biomedical Analysis,* 156: 272-277.
52. Pattemore, P. K., Silvers, K. M., Frampton, C. M., Wickens, K., Ingham, T., Fishwick, D., Crane, J., Town, G. I., Epton, M. J., and New Zealand Asthma and Allergy Cohort Study Group. (2018). Hair nicotine at 15 months old, tobacco exposure, and wheeze or asthma from 15 months to 6 years old. *Pediatric Pulmonology, 53*(4): 443-451.
53. Tzatzarakis, M. N., Vardavas, C. I., Terzi, I., Kavalakis, M., Kokkinakis, M., Liesivuori, J., and Tsatsakis, A. M. (2012). Hair nicotine/cotinine concentrations as a method of monitoring exposure to tobacco smoke among infants and adults. *Human & Experimental Toxicology,* 31(3): 258-265.
54. Groner, J. A., Huang, H., Nicholson, L., Kuck, J., Boettner, B., and Bauer, J. A. (2012). Secondhand smoke exposure and hair nicotine in children: Age-dependent differences. *Nicotine & Tobacco Research,* 14(9): 1105-1109.
55. Lukrica, Z., Brčić Karačonji, I., Brajenović, N., and Skender, L. (2011). Optimization of a solid-phase microextraction method for the analysis of nicotine in hair. *Journal of Separation Science,* 34(19): 2726-2731.
56. Kim, S. R., Wipfli, H., Avila-Tang, E., Samet, J. M., and Breysse, P. N. (2009). Method validation for measurement of hair nicotine level in nonsmokers. *Biomedical Chromatography: BMC,* 23(3): 273-279.
57. Man, C. N., Ismail, S., Harn, G. L., Lajis, R., and Awang, R. (2009). Determination of hair nicotine by gas chromatography–mass spectrometry. *Journal of Chromatography B,* 877(3): 339-342.
58. Yang, J., Hu, Y., Cai, J. B., Zhu, X. L., Su, Q. D., Hu, Y. Q., and Liang, F. X. (2007). Selective hair analysis of nicotine by molecular imprinted solid-phase extraction: An application for evaluating tobacco smoke exposure. *Food and Chemical Toxicology,* 45(6): 896-903.
59. Chetiyanukornkul, T., Toriba, A., Kizu, R., Kimura, K., and Hayakawa, K. (2004). Hair analysis of nicotine and cotinine for evaluating tobacco smoke exposure by liquid chromatography-mass spectrometry. *Biomedical Chromatography: BMC,* 18(9): 655-661.
60. Mari, F., Politi, L., and Bertol, E. (2008). Nails of newborns in monitoring drug exposure during pregnancy. *Forensic Science International,* 179(2–3): 176-180.
61. Al-Delaimy, W. K., and Willett, W. C. (2008). Measurement of tobacco smoke exposure: Comparison of toenail nicotine biomarkers and self-reports. *Cancer Epidemiology, Biomarkers & Prevention,* 17(5): 1255-1261.
62. Stepanov, I., Feuer, R., Jensen, J., Hatsukami, D., and Hecht, S. S. (2006). Mass spectrometric quantitation of nicotine, cotinine, and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol in human toenails. *Cancer Epidemiology, Biomarkers & Prevention,* 15(12): 2378-2383.
63. Axente, R. E., Stan, M., Chitescu, C. L., Nitescu, V. G., Vlasceanu, A. M., and Baconi, D. L. (2023). Application of ionic liquids as mobile phase additives for simultaneous analysis of nicotine and its metabolite cotinine in human plasma by HPLC–DAD. *Molecules,* 28(4): 1563.
64. Massadeh, A. M., Gharaibeh, A. A., & Omari, K. W. (2009). A single-step extraction method for the determination of nicotine and cotinine in Jordanian smokers' blood and urine samples by RP-HPLC and GC-MS. *Journal of Chromatographic Science, 47*(2): 170-177.