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SIMPLE EXTRACTION METHOD FOR DETECTING EXOGENOUS SUBSTANCES IN SCALP HAIR BY GC-MS

Karolina Rusevska[1], Zoran Zdravkovski[1]

[1]Institute of Chemistry, Faculty of Natural Sciences and Mathematics, Ss. Cyril and Methodius University, Arhimedova 5, 1000 Skopje, Republic of Macedonia

[*]e-mail: inapendovska@hotmail.com

Abstract

Hair analysis are increasingly growing in importance of being valid and reliable measure of long-term exposure that can be applied in biomonitoring of occupational exposure to xenobiotics due to the ability of hair to sequester exogenous substances into its shaft.

This article presents different extraction procedures for simultaneous determination of exogenous compounds in hair segments that are detected by gas chromatography/mass spectrometry (GC/MS).

The optimisation of the extraction strategy was performed on hair samples from adult subjects for determination on common exogenous substances such as caffeine, nicotine and its metabolite cotinine. These exogenous substances which get incorporated into hair due to repetitive exposure/consumption are associated with adverse health effects making them of interest to epidemiological studies.

As this method is simple and economical it can be applied to research studies for biomonitoring of occupational exposure to xenobiotics like pesticides, antineoplastic drugs, hard metals, polycyclic aromatic hydrocarbons.

Key words: *Hair analysis, biomonitoring, occupational exposure, xenobiotics, GC-MS method, caffeine, nicotine, cotinine.*

1. Introduction

Hair as a medium for analysis became greatly interesting in the late 1970s when a radioimmunological method was published for detection of heroin and morphine in hair of drug abusers [1]. Hair analysis is increasingly growing in importance of being valid and reliable measure of long-term exposure that can be applied in biomonitoring of occupational exposure to xenobiotics [2, 3]. Hair has advantages over specimens such as blood, urine, saliva due to the ability of hair to sequester exogenous substances into its shaft [4] where they remain unchanged for many years contrary to the traditional matrixes where in a matter of 24 hours signs of elimination or decomposition of analytes appear. Other advantages associated with hair testing are easy sampling and transportation. The sampling is non-invasive, without discomfort to the subjects, and is performed with simple tools. Transportation doesn't require special conditions, only aluminum foil and a paper envelope [5]. This is beneficial when conducting large field biomonitoring studies for assessing exposure to xenobiotic agents in occupational settings. Furthermore, hair samples are difficult to adulterate, which is beneficial in forensic cases.

Hair analysis entails few steps such as sampling, decontamination by washing of the hair, extraction of the analytes of interest and instrumental analysis. The progress in sensitivity and selectivity of the analytical procedures bring hair analysis closer to being alternative to the conventional specimens since xenobiotics are in considerable lower levels in hair than those found in blood and urine. To date many procedures have been reported. Advances in method development by gas-mass chromatography (GC-MS) and liquid-mass chromatography (LC-MS) or tandem mass spectrometry methods GC-MS/MS and LC-MS/MS only increase the pool of knowledge. Furthermore, immunoassays are being improved that serve as a screening test for target analytes, but still confirmation needs to be performed by mass spectrometric methods [6].

Various laboratory methods have been developed to access the analytes of interest in hair, namely alkali or acid hydrolysis or extraction with organic solvents (mostly used methanol, hexane, acetone) [7]. Basic extraction [8] is favorable when substances (like THC, nicotine, antidepressants) are stable in aqueous NaOH. The digestion of the hair matrix is usually performed at 80 °C for 1 h. Nakahara's group [9] reports successful acidic extraction of drugs from hair with methanol–5

M HCl (20:1) or methanol–trifluoroacetic acid (9:1) under ultrasonification for 1 h, and incubation overnight. In the analysis of pesticide metabolites in maternal hair Posecion *et al*. [10] used liquid-liquid extraction with toluene of the metabolites suspension in methanol–10 M HCl for 20 min at room temperature in an orbital shaker. Anabolic steroids like metandienone were determined in athlete's hair samples by tandem GC-MS following MSTFA derivatization [11].

The sample preparation techniques like solid-phase extraction (SPE), headspace solid-phase microextraction (HS-SPME) often follow the extraction step when potential matrix interferences need to be eliminated. In the GC-MS assay for detection of acetaminophen [12], the pulverized hair powder mixed previously with internal standard solution, was incubated with 0,1 M HCl at 45 °C, overnight; and after neutralization with 1 M NaOH, extraction of the aqueous solution was done with SPE, Oasis HLB-cartridges, preconditioned in methanol and distilled water. The Bond Elut™ Certify cartridge conditioned with methanol and 50 mM phosphoric acid or 0.1 M phosphate buffer was the choice for SPE of cannabinoids for GC/MS/MS assays and extraction of other drugs of abuse (amphetamines, ketamine, opiates) done by Gambelunghe *et al*. and Y.-H. Wu *et al*. [13], respectively. E.S. Emidio *et al*. [14] used alkaline digestion with 1 M NaOH at 90 °C for 15 min prior to the extraction of cannabinoids from PDMS fiber (better performing was the non-polar), used in headspace mode for 40 min at 90 °C, with magnetic agitation of the system. This method is convenient since it does not involve the use of organic solvents, the hair extracts are with few interfering additions, and combined with tandem MS mode has excellent selectivity and detectability.

Several studies have demonstrated the wide range of adverse health effects of caffeine, nicotine and cotinine especially on the reproductive, cardiovascular, pulmonary, gastrointestinal, immunological systems as well as genetic toxicity in laboratory animals and humans [15, 16, 17]. Hair as a biological material has been proposed for analysis of these substances of pathogenic concern in an exposed individual.

Caffeine (1,3,7-trimethylxanthine) is the world's most widely consumed legal psychoactive drug, found in different parts of plants (leafs, seed, fruit) where it acts as a natural pesticide. Caffeine intake in humans is mostly through coffee consumption (berries of the coffee plant *Coffea arabica*) and tea infusions (leafs from tea tree *Camellia sinensis*), but also from soft drinks or energy drinks derived from the kola nut (*Cola nitida* and *Cola acuminata*) and as a constituent of many medications.

Nicotine (3-(1-methylpyrrolidin-2-yl) pyridine) is a potent phytogenic insecticide, a central nervous system drug and highly addictive substance in tobacco users. Levels of nicotine and cotinine, as the primary endogenous metabolite of nicotine, are considered important biomarkers of tobacco exposure and environmental tobacco smoke (ETS) especially in epidemiological studies [18, 19]. Nicotine is metabolized relatively fast in the liver, almost 80% to cotinine by cytochrome P450 enzyme and only 4-7% to nicotine *N*′ -oxide by flavin monooxygenaze 3 [20]. The plasma half life of nicotine is 2 h and clearance rate is 1200 mL/min, while average half life of cotinine is about 16 h and the rate of metabolism is about 45 mL/min [21]. According to Eliopoulos *et al*. [22] hair analysis is not dependent on the serum levels of these drugs due to slow hair growth rate, but differences between nicotine and cotinine incorporation rate into hair are observed, finding much lower concentrations for cotinine than nicotine [23, 24].

The present paper describes different improved extraction procedures for simultaneous determination of caffeine, nicotine and cotinine in hair samples that are detected by a GC/MS assay. These drugs were chosen for the study because caffeine and nicotine are widely consumed by the general population thus can be readily found in hair. The preliminary/clean-up procedures are time and money consuming. Following the current trend of simplification of the hair sample pre-treatment step, our extraction method overcomes these drawbacks.

2. Materials and Methods

Materials and reagents - Dichloromethane, methanol, *n*-hexane, and toluene were purchased from Aldrich, Germany. All reagents and chemicals were HPLC or ACS grade.

Sample preparation - The analytical method for analysis of exogenous substances in hair was adapted with modifications from the method presented by Cirimele *et al*. [25]. The hair samples (in sufficient amounts for the whole analysis) for the optimization of the method were collected from a volunteer with regular intake of coffee and or nicotine. The hair samples were collected following the guidelines of the Society of hair testing [5]. Hair tuft were cut near to the scalp in the vertex posterior region where hair growth pattern is mainly uniform [26]. The whole lock of hair was analyzed in order to estimate the total exposure to exogenous substances. After washing with two 5 mL portions of dicholoromethane and drying of the hair between two paper towels, 30 mg hair was cut into small pieces and were extracted in 1 mL methanol at 45 °C for 24 h. The obtained extract was centrifuged, concentrated to 25 µL, transferred to a vial of which 1 µL was injected into the GC-MS.

Modifications to the method - We focused on evaluating the effects of the hair condition prior to the extraction steps and the concentration steps as a part of the methods' optimization parameters. In order to improve on the extraction step hair segments were manually milled / homogenized in an agate or marble mortar with pestle. The pulverization can be greatly enhanced by using a few drops of organic solvent which swells the hair and makes it easier to break. A combination of organic solvents was evaluated for the extraction process, namely methanol and toluene in different proportions. The hair powder (20 mg) was extracted at 45 °C for 1 h in a stirring silicon oil bath in 2 ml: methanol-toluene (1:1, *v/v*); methanol-toluene (1:5, *v/v*) and methanol-toluene (5:1, *v/v*). Additionally, different organic solvents were assessed for extraction of analytes at different temperatures. The hair powder was extracted in 2 mL of toluene at 75 °C for 24 h and in 2 mL of *n*-hexane at 35 °C for 24 h in a stirring silicon oil bath. In all cases the obtained extract was centrifuged at 10 000 rpm for 15 minutes. The supernatant was concentrated to 50 µL in an oven at 60 °C, transferred to a vial with glass insert and finally 1 µL was injected into the GC-MS.

Ultrasonication prior to extraction - Also, as an option we considered ultrasonication of the hair powder prior to the extraction in the silicon oil bath. The hair powder (weighed 20 mg) was transferred in a glass vial and 2 mL of methanol was added. The pre-extraction step was performed in an ultrasonic bath at room temperature for 1 h and afterwards the extraction was continued in the silicon oil bath at 45 °C for 24 h. The hair extract was centrifuged at 10 000 rpm for 15 minutes. The organic layer was concentrated to 50 µL under dry hot air at 60 °C. The final solution was transferred to a glass insert mounted in an autosampler vial and 1 µL was injected into the GC-MS for analysis.

Concentration step - Additionally we compared the concentration procedures by using nitrogen for evaporation besides the standard dry hot air evaporation. After the decontamination process, the hair powder was extracted in 2 mL of methanol at 45 °C for 24 h. The obtained extracts were centrifuged at 10 000 rpm for 15 minutes and the organic phase was collected in two separate vials to be concentrated by dry hot air and nitrogen flow. We used dry hot air evaporation in an oven at 60 °C to concentrate the supernatant to 50 µL and we performed evaporation to dryness by gentle flow of nitrogen (room temperature), after which the sample was reconstituted to 50 µL with methanol. All samples were transferred to a vial with glass insert and 1 µL was injected into the GC-MS.

Instrumentation - The instrument used was Agilent GC 6890N with autosampler and an Agilent mass spectrometer 5975B EI/CI. The column used was HP-5MS, length 30 m, internal diameter 250 µm, thickness 0,25 µm. The samples were injected in splitless mode at an injector temperature of 200 °C. The initial column temperature was 60 °C, maintained for 1 min, raised to 200 °C at a rate of 20 °C/min, then to 250 °C at a rate of 10 °C/min, and finally to 295 °C, at a rate of 5 °C/min for 10 min. The whole analysis time was 32 min. The temperature of the auxiliary line to the MS was 280 °C and electron ionization was used at 70 eV. The quadruple mass spectrometer was operated in total ion chromatogram (TIC) full scan (scan range 50 – 450 u). The obtained chromatograms and mass spectra were analyzed by AMDIS – Automated Mass Deconvolution and Identification System with the EPA/NIST built-in library.

Hair sample analysis - We applied our optimized parameters in hair analysis of collected hair samples from adult subjects who stated that they regularly drink coffee (normal daily amounts) and are active tobacco smokers. Questionnaires were made with requested information on smoking history, packs/day and use of other tobacco products and the frequency of drinking coffee, tea, along with characteristics about hair treatments. The collected hair strands from the surface of the skin in the vertex posterior region were kept in an aluminum foil and stored in paper envelopes at room temperature until analyzed. The extraction step of the hair powder that was previously passed through marble mortar was done with 2 mL of methanol at 45 °C overnight in a silicon oil bath. The obtained extract was centrifuged; the supernatant was removed after centrifugation, evaporated to dryness under nitrogen stream and reconstituted to 50 µL using methanol. The sample was transferred to a vial with glass insert and 1 µL was injected into the GC-MS system.

3. Results and Discussion

The method developed by Cirimele *et al*. [25] proved to be successful in determination of pesticides in hair of vine growers. As in all analytical methods for determination of drugs of abuse or other xenobiotics present in hair in minute quantities (pg/mg), the most essential part of the testing procedure is the initial pre-treatment step. Our method employed modifications of the existing method [25] in the sample preparation step and the incubation phase. The washing procedure remained 2 minutes with dichloromethane with reduced amount of solvent (2 x 2,5 mL) because our initial findings showed that following this procedure incorporated analytes were not removed by dichloromethane due to the small, but sufficient amount of solvent used to wash away only the impurities such as sebum, sweat, hair care products. The washing step ensures decontamination of the hair sample, with exclusion of false positive results due to passive environmental exposure [27].

The sample preparation goes further with procedures to disintegrate the hair matrix. This can be achieved either by extraction (basic, acidic) or by enzymatic digestion [28]. When using organic solvent incubation, methanol as a protic solvent delivers satisfactory results, especially when used with utrasonication [29]. This is achieved by swelling of the hair matrix and liberation of the incorporated analytes by diffusion.

Release of drugs from the hair matrix can be achieved in many ways, but optimization of the extraction procedure is needed in order to assess methods' efficiency in terms of good recovery and analyte stability. Extraction procedures in methanol according to many researchers prove best in terms of minimizing hydrolysis of labile drugs and achieve good recoveries. We established this in our optimization procedure when comparing TIC of the hair powder extracts in methanol, proportions of methanol and toluene and in the extractions in pure solvents toluene and *n*-hexane. Many impurities coming from the hair matrix like different hair lipids were present in the chromatograms of the extractions in toluene and *n*-hexane, decreasing the caffeine signal. Therefore we chose to use methanol as our extraction solvent in an overnight incubation in a silicon oil bath; used instead of water bath to avoid excessive evaporation of the water. We used magnetic stirrer bars in the glass vial with the hair sample and solvent to ensure good homogenization of the sample and solvent. We noticed also that the milling procedure was eased when we applied drops of methanol to the hair segments, which was not the case with *n*-hexane and toluene.

Hair powder proved to give better TIC, with well separated analytes compared to hair segments extractions, probably due to better access of the extraction solvent in the hair structures. Ultrasonication as a means to disintegrate the hair matrix was applied for 1 hour prior the extraction in organic solvent [30]. We did not notice significant changes in the TIC of the samples of ultrasonicated sample of hair powder and hair powder sample directly placed for extraction. Therefore we chose to apply hair grinding with methanol using mortar and pestle and methanolic extraction in silicon oil bath on real samples of hair from individuals who stated to be regular tobacco users and coffee drinkers.

Since nicotine and caffeine are lipid soluble drugs and are stable substances, methanolic hair extracts of these substances yielded satisfactory GC-MS recoveries and well separated chromatograms without further clean up from impurities [31] (Figure 1). The caffeine was well separated with good peak shape. This is also valid for the TIC of the real hair samples shown in Fig. 6. We observed well separated analytes: caffeine, nicotine and cotinine in the TIC presented in Fig. 6. This was not observed with the modifications in the proportions of organic solvents methanol and toluene, neither with the pure solvents toluene and *n*-hexane. The extractions conducted at selected temperatures below the boiling point of the solvents toluene and *n*-hexane did not yield satisfactory results in terms of caffeine detection. This procedure produced dirty samples, with high matrix interferences and required further clean up with liquid-liquid extraction or solid-phase extraction.

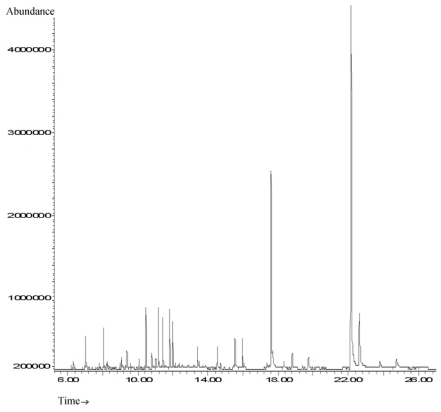


Figure 1. Total ion chromatogram of a volunteer hair sample obtained after milling the hair in marble mortar and methanolic extraction

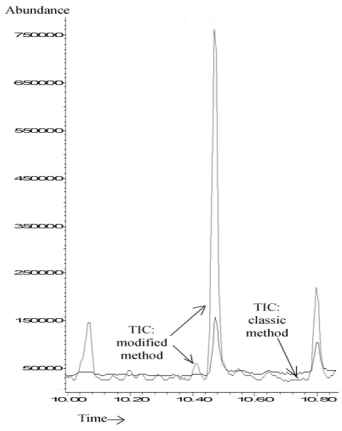


Figure 2. Overlay of chromatograms of the modified method and the classic method

We considered particle size reduction of the hair sample could improve the detection sensitivity of the drugs and lower the risk of missing analytes [32, 33] therefore our modification of the classical method was focused on pulverizing the hair snippets into fine powder using mortar and pestle. This is inexpensive equipment contrary to a mechanical pulverizer (ball mill, bead mill, stainless steel bullet) and still enables faster pulverization of the hair sample compared to using scissors. Decreasing of the particle size of the

hair strands allows greater exposure contact of the hair matrix (target compounds) and the extraction solvent, which increases the extraction efficiency as seen in Fig. 2. As presented in the chromatogram, there is a several fold increase in the sensitivity which is very important when analyzing substances with very low concentrations. It is worth noting that the extraction performed by classical method of Cirimelle *et al.* [25] was unable to detect the presence of caffeine. Furthermore, the caffeine area under the peak in the modification using pulverized hair was 70752, while in the modification using ultrasonication the caffeine area under the peak was 15218. The other modifications different solvents and different proportions of solvents did not detect the caffeine and had poor TIC.

Caffeine in the volunteers' hair, as an exogenous substance that is repetitively ingested, was evaluated based on the analysis of the analyte with a peak at 10,41 minutes (Figure 3).

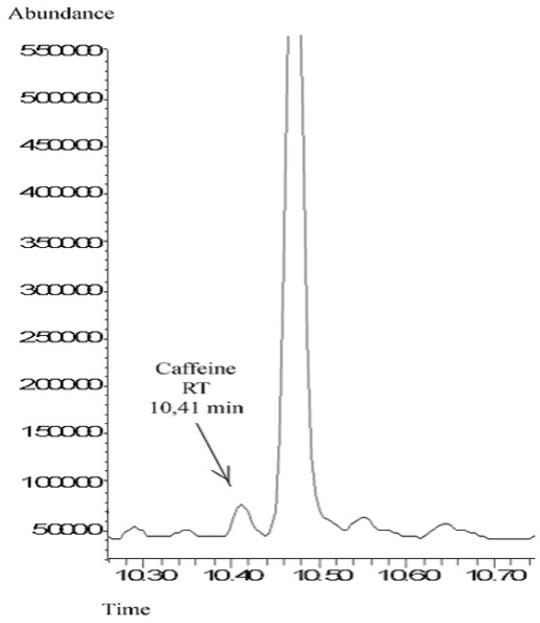


Figure 3. GC chromatogram of volunteers' hair sample obtained by modification with marble mortar zoomed at the peak at RT 10,41 min

The mass spectrum of this chromatographic peak revealed several characteristic ions, the most prominent being at *m/z* 194 and *m/z* 109. Analysis of this mass spectrum shows that it represents caffeine, the most intensive 194 peak corresponding to the molecular ion and the second most intensive 109 peak to the 4-substituted imidazole moiety as given in Fig. 4.

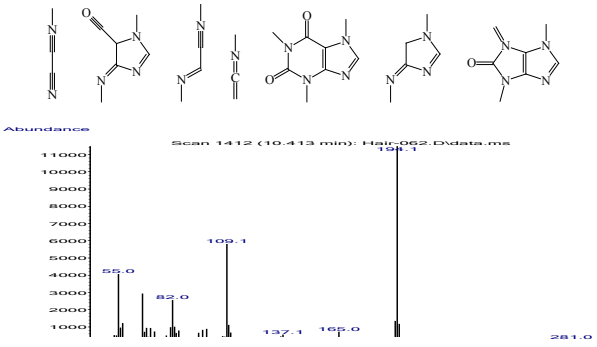


Figure 4. Mass spectrum of analyte obtained at 10.41 min and the corresponding fragments of caffeine

Comparison of this mass spectrum with the mass spectra from the NIST library confirmed the identity of caffeine with a very high degree of reliability (Figure 5) [34].

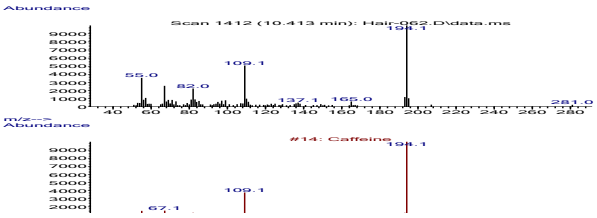


Figure 5. Comparison of mass spectrum at 10.41 min and the NIST library spectrum of caffeine

The applicability of the modified and improved method was examined on real samples of human hair from smokers (Figure 6).

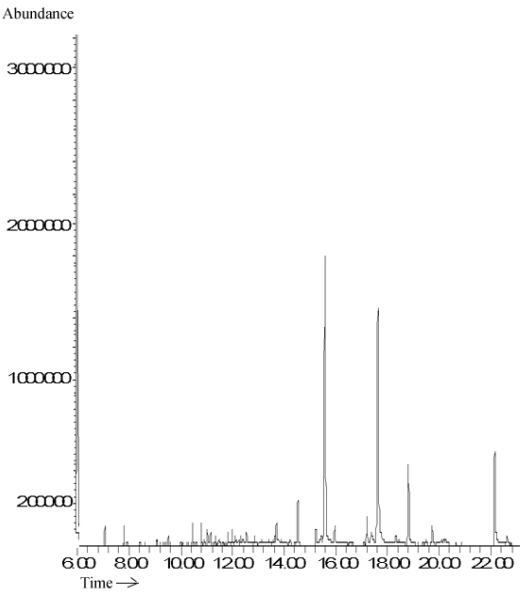


Figure 6. GC chromatogram of a hair sample of a smoker

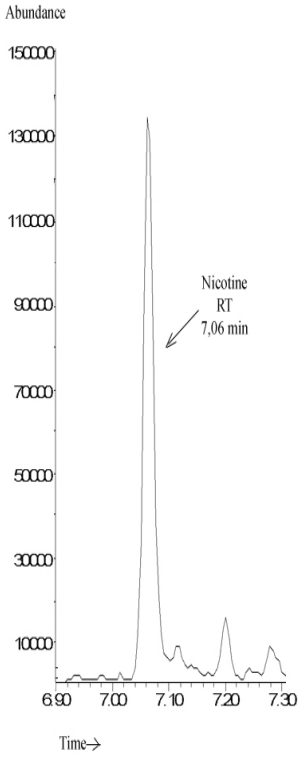


Figure 7. Chromatogram of smoker zoomed at the peak of nicotine at retention time 7,06 min

Nicotine, its primary metabolite cotinine and caffeine as exogenous substances that get incorporated into the hair matrix after long term exposure (daily intake) were tested positive in all samples. Figure 7 presents TIC of hair sample of a smoker presenting nicotine at 7,06 min., while Figure 8 and Figure 9 show TIC of cotinine (retention time 9,45 min) and caffeine (retention time 10,41 min), respectively.

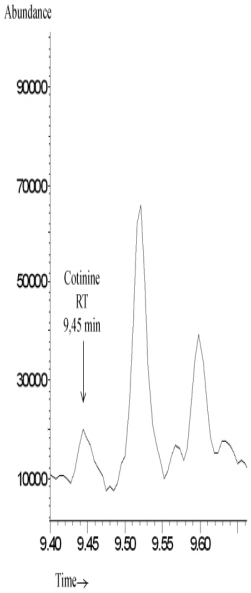


Figure 8. Chromatogram of smoker zoomed at the peak of cotinine at retention time 9,45 min

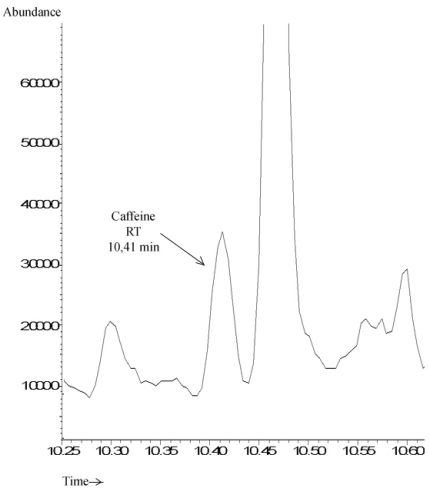


Figure 9. Chromatogram of smoker with zoom at the peak of caffeine at retention time 10,41 min

Cotinine levels are lower than those of nicotine, which complements the different metabolism rates of these compounds [35]. Extracted mass spectra of nicotine, cotinine and caffeine were analyzed by the Chem Station software (Figures 10, 11 and Figures 12, 13).

In the mass spectrum of nicotine the most prominent peak is at $m/z = 84$ corresponding to the pyridine fragment as given in Fig. 10. The secon most prominet peak at 133 is from the fragment obtained by loss of a $-CH_2CH_2-$ group from the nicotine molecule.

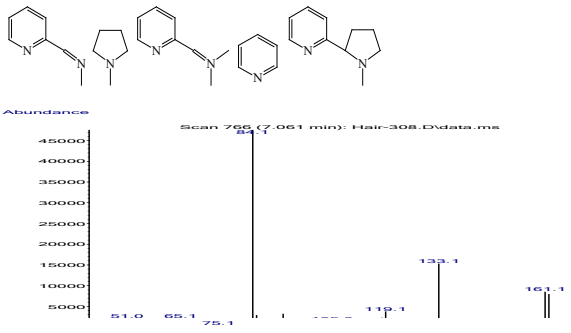


Figure 10. Mass spectrum of analyte at 7.06 min and corresponding fragmentation of nicotine

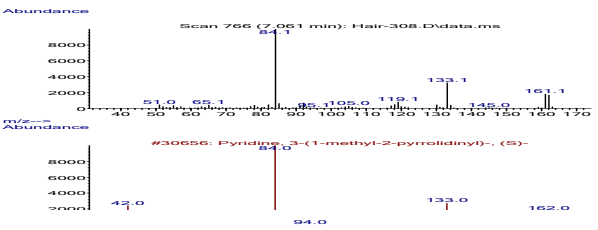
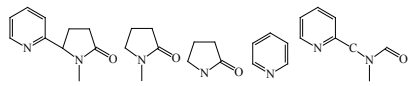


Figure 11. Comparison of mass spectrum of analyte at 7.06 min with mass spectrum from NIST library for nicotine

In the mass spectrum of cotinine the molecular peak is second in intensity while the most prominent peak is at *m/z* = 98 corresponding to the *N*-methylpyrrilidinone moiety.



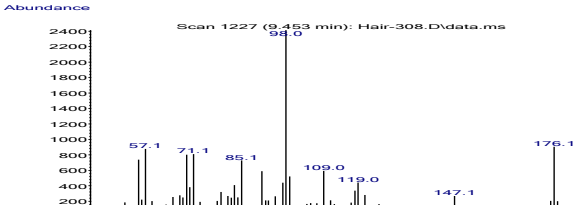


Figure 12. Mass spectrum of analyte at 9.45 min and fragmentation pattern of cotinine

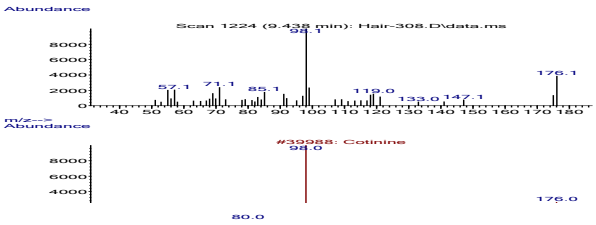


Figure 13. Compared mass spectrum of the analyzed sample with the library spectrum of cotinine

The substances were not quantified as the intention was to perform this pilot study in order to investigate if detection can be made in the most affordable way for a research laboratory. The method validation is a subject of further study efforts.

Additionally preliminary research on pesticides in hair of vineyard workers (collected after the spraying period) was performed with the modified method, because presence of agrochemicals in tissues provide information for human exposure to these xenobiotics. Our study (which was not a controlled study with controlled doses of exposure), did not yield positive results. We find this not to be surprising considering pesticides accumulate in hair in low measurable quantities in the pg/mg level [36]. The results can be attributed to the small amounts of available hair specimen (20 mg of hair) because our subjects were male with scarce head hair [30]. Furthermore, uncertainty of dosages ingested by the subjects, stability of pesticides in hair, variations of uptake of the pesticides from blood to hair, rate of sweating [37] could further explain our negative findings. Women were not included in the study due to variations that arise from chemical treatments of hair such as dyeing and permanent waving [38, 39].

4. Conclusions

- In the past several decades over a hundred organic substances have been investigated in hair by chromatographic procedures. In contrast to conventional biological samples, like serum and urine, hair fibers have proven to provide long term information concerning exposure to organic substances due to the entrapment during hair fiber formation and the absence of metabolism in the hair fiber.
- Due to its structure and individual differences, hair analysis continues to be a challenge in the investigation of trace levels of organic substances. Novel and highly sophisticated analytical procedures continuously emerge in the field of hair analysis. Our modified method detected exogenous compounds caffeine and nicotine (cotinine) in human hair, which were not detected by the previous reported method, at the same time being simple and economical GC-MS method that can be adopted by routine or research GC-MS laboratory.
- Further research is warranted on hair specimens as a marker for exposure history of people to organic pollutants like pesticides. Pooled samples of hair (100–200 mg) to increase the detection limits need to be tested for trace pollutants [40]. Additionally, further exploration is merited for the possible hair color effects on the concentration of the xenobiotics in hair [41].

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