UNIVERSITEIT
GENT

biblio.ugent.be

The UGent Institutional Repository is the electronic archiving and dissemination platform for all UGent research publications. Ghent University has implemented a mandate stipulating that all academic publications of UGent researchers should be deposited and archived in this repository. Except for items where current copyright restrictions apply, these papers are available in Open Access.

This item is the archived peer-reviewed author-version of:

Title: An optimized and validated SPE-LC-MS/MS method for the determination of caffeine and paraxanthine in hair

Authors: De Kesel P., Lambert W., Stove C.

In: Talanta, 144, 62-70, 2015

To refer to or to cite this work, please use the citation to the published version:

De Kesel P., Lambert W., Stove C. (2015). An optimized and validated SPE-LC-MS/MS method for the determination of caffeine and paraxanthine in hair. Talanta 144 62-70.

http://dx.doi.org/10.1016/j.talanta.2015.05.084

An optimized and validated SPE-LC-MS/MS method for the determination of caffeine and paraxanthine in hair

Pieter M.M. De Kesel, Willy E. Lambert and Christophe P. Stove*

Laboratory of Toxicology, Department of Bioanalysis, Faculty of Pharmaceutical Sciences, Ghent University, Ottergemsesteenweg 460, B-9000 Ghent, Belgium.

pieter.dekesel@ugent.be, willy.lambert@ugent.be, christophe.stove@ugent.be

*Corresponding author:

Christophe P. Stove

Laboratory of Toxicology

Department of Bioanalysis

Faculty of Pharmaceutical Sciences

Ghent University

Ottergemsesteenweg 460

B-9000 Ghent

Belgium

E-mail: christophe.stove@ugent.be

Tel.: +32 9 264 81 21

Fax: +32 9 264 81 83

Abstract

Caffeine is the probe drug of choice to assess the phenotype of the drug metabolizing enzyme CYP1A2. Typically, molar concentration ratios of paraxanthine, caffeine's major metabolite, to its precursor are determined in plasma following administration of a caffeine test dose. The aim of this study was to develop and validate an LC-MS/MS method for the determination of caffeine and paraxanthine in hair. The different steps of a hair extraction procedure were thoroughly optimized. Following a three-step decontamination procedure, caffeine and paraxanthine were extracted from 20 mg of ground hair using a solution of protease type VIII in Tris buffer (pH 7.5). Resulting hair extracts were cleaned up on Strata-X™ SPE cartridges. All samples were analyzed on a Waters Acquity UPLC® system coupled to an AB SCIEX API 4000™ triple quadrupole mass spectrometer. The final method was fully validated based on international guidelines. Linear calibration lines for caffeine and paraxanthine ranged from 20 to 500 pg/mg. Precision (%RSD) and accuracy (%bias) were below 12 and 7 %, respectively. The isotopically labeled internal standards compensated for the ion suppression observed for both compounds. Relative matrix effects were below 15 %RSD. The recovery of the sample preparation procedure was high (> 85 %) and reproducible. Caffeine and paraxanthine were stable in hair for at least 644 days. The effect of the hair decontamination procedure was evaluated as well. Finally, the applicability of the developed procedure was demonstrated by determining caffeine and paraxanthine concentrations in hair samples of ten healthy volunteers. The optimized and validated method for determination of caffeine and paraxanthine in hair proved to be reliable and may serve to evaluate the potential of hair analysis for CYP1A2 phenotyping.

1 Introduction

Numerous approaches for qualitative and quantitative analysis of low molecular weight compounds in hair have been developed in the last decades. The majority of published methods deals with the determination of drugs of abuse and markers of alcohol use. Standard protocols are not available and several procedures to extract analytes from hair, such as extraction using organic solvents or aqueous buffers, digestion of the hair with NaOH or acid solutions and enzymatic digestion, have been combined with different separation and detection techniques, mainly GC- or LC- MS(/MS) [1,2]. Hair analysis is actively applied as an alternative matrix in different areas, such as clinical and forensic toxicology, abstinence monitoring for driving license regranting or child custody cases, workplace drug testing and doping control [3,4]. It provides some distinct advantages compared to traditional samples used in bioanalysis. Once drugs are trapped within the hair, they are no longer subject to biotransformation. Many drugs were shown to be stable in hair for prolonged periods of time, allowing retrospective assessment of drug use with a window of months up to years, depending on hair length. Furthermore, collecting hair strands represents a non-invasive sampling technique, eliminating the need for specialized personnel. On the other hand, hair analysis is characterized by certain inherent limitations, mainly in the interpretation of hair results. False positive results may result from external contamination of the hair [1-4]. Effective approaches to differentiate active ingestion from external contamination are subject of an ongoing debate in the hair analysis community. Certain drugs may also be lost from the hair due to cosmetic treatments, such as perming, bleaching or dying [5]. In many cases, hair concentrations cannot be correlated with plasma or serum concentrations.

Recently, a novel application for hair analysis has been proposed in several studies, in which specific metabolites are quantified along with their parent drugs. The corresponding metabolite-to-parent drug concentration ratios are then used to assess the metabolic phenotype of drug metabolizing enzymes [6]. For example, it was found that nortriptyline/amitriptyline ratios in hair correlated with the number of functional alleles of CYP2C19 [7]. In another study, different acetylisoniazide/isoniazide ratios were found in hair samples from individuals with different arylamine N-acetyltransferase-2 (NAT-2) genotypes [8]. Metabolite-to-parent drug ratios can also be used to (retrospectively) detect changes in drug metabolism. This

approach has been applied to demonstrate altered metabolism of nicotine [9], citalopram [10] and methadone [11] during pregnancy.

The aim of our study was to develop and validate an LC-MS/MS-based procedure for the determination of caffeine and its major metabolite paraxanthine in hair. Caffeine is a generally accepted and widely used probe drug to determine the phenotype of the drug metabolizing enzyme CYP1A2. Since N3-demethylation of caffeine to paraxanthine is entirely controlled by CYP1A2, the paraxanthine/caffeine ratio provides an ideal phenotyping index for this enzyme [12,13]. Typically, paraxanthine/caffeine ratios are determined in plasma or serum 4-6 h following the administration of a defined caffeine test dose [14]. Given the generalized consumption of caffeine, intake of a test dose would no longer be required in the case of a phenotyping procedure involving hair analysis. Caffeine has been determined in hair using a variety of extraction media and analytical techniques, such as GC-MS, LC-UV, ion mobility MS, LC-QTOF-MS and LC-MS/MS [15-20], although only one published method was fully validated for caffeine [19]. To the best of our knowledge, our study is the first to provide an optimized and validated method for the combined determination of caffeine and its metabolite paraxanthine in hair. This method can be applied to evaluate the potential of hair analysis for CYP1A2 phenotyping purposes. The latter may be relevant in cases in which CYP1A2 phenotyping is desired but not possible, e.g. in postmortem cases involving an intoxication with a CYP1A2 substrate and a suspected role for aberrant CYP1A2 activity. The kinetics of incorporation of caffeine and paraxanthine in hair has not been investigated in a systematic way. Incorporation of compounds in hair is a complex process in which various factors are involved. From a physicochemical point of view, the incorporation rate of a given compound is mainly determined by its lipophilicity and basicity [1,3,4]. Given the slightly higher lipophilic and basic characteristics of caffeine compared to its demethylated metabolite paraxanthine, it can be expected that caffeine may be better incorporated into hair. In general, lipophilic compounds accumulate more in hair as they easily penetrate cell membranes through passive diffusion. In addition, given the lower pH of keratinocytes and melanocytes compared to plasma, basic compounds will be trapped in hair matrix cells. The incorporation of basic compounds is also favored by a higher affinity for binding to melanin. On the other hand, the more polar compound paraxanthine may show a better retention in hair [3]. Apart from these factors, many other variables, such as melanin content or cosmetic treatment of hair, may affect the incorporation and stability of caffeine and paraxanthine in hair. Therefore, it is difficult to accurately estimate the kinetics of the incorporation of both compounds in hair

or their potential to replace similar ratios in plasma. To evaluate the latter, ratios in both matrices need to be compared on a large scale, which was out of the scope of this study.

2 Materials and methods

2.1 Chemicals and stock solutions

Methanol, acetonitrile and methylene chloride were obtained from Biosolve (Valkenswaard, The Netherlands). A Synergy® Water Purification System (Merck Millipore, Overijse, Belgium) provided ultrapure water. Analytical standards (caffeine, caffeine-$^{13}C_3$, paraxanthine, paraxanthine-$^{13}C_4$-$^{15}N_3$, theophylline, theobromine), proteases (proteinase K, protease type VIII, protease type XIV), dithiothreitol, trishydroxymethylaminomethane (Tris) and formic acid were purchased from Sigma-Aldrich (Diegem, Belgium). HCl (1N) was obtained from Merck KGaA (Darmstadt, Germany) and liquid N_2 from Air Liquide (Brussels, Belgium). Stock solutions of caffeine and paraxanthine (1 mg/mL) and the IS (100 µg/mL) were prepared as described elsewhere [21].

2.2 Optimization of the sample preparation procedure

Hair samples from two caffeine consuming healthy volunteers obtained after regular haircuts were used for optimizing hair extraction conditions. To remove external contamination, hair (± 2 g) was washed in methylene chloride (20 mL for 2 min), followed by two washes in water (20 mL for 2 min). Two homogenization methods -manual grinding in a mortar using liquid N_2 and mechanical grinding in a ball mill- were tested along with four different extraction solvents or conditions. The latter were methanol, an aqueous buffer (acetate buffer pH 5.0), digestion of the hair matrix with an aqueous NaOH solution (0.5 mol/L) and enzymatic digestion with protease type XIV (1.4 IU/mL) in acetate buffer (pH 8.5). As enzymatic digestion was selected, solutions of three different proteases (proteinase K, protease type VIII and type XIV), corresponding to 30.0 IU/mL, were prepared in Tris buffer (50 mmol/L) at pH 7.5 and 8.5. For all conditions, 1 mL of extraction medium was added to 20 mg pulverized hair. All samples were gently shaken for 12 hours at 37 °C and 750 rpm on a Thermomixer® comfort (Eppendorf, Hamburg, Germany). Subsequently, several enzymatic digestion and extraction parameters were optimized. Evaluated extraction times ranged from 30 minutes to 12 hours and extractions at different temperatures (22, 37, 50 °C) were compared. Also the influence of the protease concentration (1.2, 6.0, 12.0, 24.0 IU/mL) and the Tris buffer concentration (25.0, 50.0, 100.0 mmol/L) was evaluated. Finally, the effect of the presence of different concentrations of dithiothreitol in the extraction medium was

evaluated. To illustrate the performance of the optimized enzymatic procedure, a side-by-side comparison with extraction in methanol was performed. Therefore, aliquots of ground hair (20 mg) from a single volunteer were extracted using the enzymatic procedure and by adding 1 mL of methanol to the ground hair samples. All samples were shaken for 1 h at 750 rpm and 37 °C.

Prior to LC-MS/MS analysis, hair extracts were cleaned-up using solid phase extraction (SPE). Four different SPE cartridges (Bond Elut Phenyl, C8, C18 and Phenomenex Strata-X™) were tested using a general protocol. The cartridges were conditioned with 3 mL of methanol and 2 mL of water. Subsequently, 1 mL of hair extract was applied to the columns. For the wash step, 2 mL of a water/methanol mixture (85/15, v/v) was used. Finally, after drying for 2 minutes, the analytes were eluted from the cartridges with 4 × 250 μL of methanol. For all four cartridges, the recovery of caffeine and paraxanthine was determined by calculating the percent ratio of the peak areas of hair samples spiked at 100 pg/mg before extraction to those of samples spiked at the same concentration after extraction. Phenomenex Strata-X™ cartridges were selected (see Results section) and the SPE protocol was further optimized by evaluating different wash solvents, being mixtures of water with methanol (85/15, v/v) or acetonitrile (90/10, v/v). Also the influence of using incremental proportions of methanol in the wash solvent was investigated. Finally, methanol, acetonitrile, methanol/acetonitrile (50/50, v/v), methanol/ HCl 1 mol/L (95/5, v/v), acetonitrile/ HCl 1 mol/L (95/5, v/v) and methanol/ammonium aceteate 5 mmol/L (70/30, v/v) were evaluated as elution solvents.

2.3   Final sample preparation procedure

In the final procedure, a three-step decontamination procedure was used. Hair samples were first immersed in methylene chloride (20 mL) for 2 minutes and then twice in the same volume of water for 2 minutes. These large volumes of wash solvents were used to make sure there was a large excess of decontamination solvent, given the very important issue of external contamination in hair analysis. Wash solvents were removed and samples were air-dried between the consecutive steps. Subsequently, samples were cut into small pieces (< 5 mm) using scissors and manually ground in a mortar with liquid N_2. Twenty mg of ground hair was weighed and 1 mL of a solution of protease type VIII (1.2 IU/mL) in Tris buffer (pH 7.5, 50 mmol/L) was added. The latter solution contained the internal standards (IS) caffeine-$^{13}C_3$ and paraxanthine-$^{13}C_4$-$^{15}N_3$ at concentrations of 5 ng/mL. This results in signals that lie around the center of the calibration curve, in particular between the 150 pg/mg and 250 pg/mg

calibrators. The samples were placed in a Thermomixer® comfort at 37 °C and 750 rpm for 1 hour and subsequently centrifuged at 10000 x g for 10 minutes. An SPE procedure using Phenomenex Strata-X™ cartridges (200 mg, 3 mL) was used for clean-up of the supernatants. The cartridges were conditioned and equilibrated with 3 mL of methanol and 2 mL of water, respectively. Following application of the hair extracts, the cartridges were washed with 2 mL of a water/methanol mixture (65/35, v/v) and dried for 2 minutes. The analytes were eluted with 4×500 µL of a methanol/acetonitrile mixture (50/50, v/v). The resulting solutions were evaporated under a stream of N_2 and redissolved in 150 µL of the mobile phase.

2.4 LC-MS/MS method

A Waters Acquity UPLC® system (Waters, Milford, MA, USA) and an AB SCIEX API 4000™ triple quadrupole mass spectrometer (AB SCIEX, Framingham, MA, USA) were used for all analyses. The LC-MS/MS configuration was controlled by AB SCIEX Analyst 1.5.2 and Waters Acquity console software. Identical chromatography parameters as previously described for the analysis of caffeine and paraxanthine in dried blood spots, whole blood and plasma were used [21]. Mass spectrometer parameters were slightly adapted. The ion spray voltage was set at 4000 V, gas 1 at 40 psi, gas 2 at 80 psi, the curtain gas at 10 psi and CAD vacuum at 9 (arbitrary setting). All compounds were detected in scheduled multiple reaction monitoring (sMRM) mode. Precursor to product ion transitions were identical as previously described [21].

2.5 Validation

Validation of the hair analysis procedure was based on European Medicines Agency (EMA) and U.S. Food and Drug Administration (FDA) guidelines for bioanalytical method validation [22,23]. Selectivity, carry-over, lower limit of quantification (LLOQ), linearity, precision, accuracy, matrix effect, stability and dilution integrity were evaluated. It should be noted that no blank, caffeine-free hair matrix was available. Hair samples from 4 young children ($1 - 5$ years), collected at different time points, were analyzed; however, caffeine and paraxanthine were detected in all samples. A pool of hair matrix to prepare calibrators and quality control (QC) samples was obtained by combining decontaminated and ground hair samples in which the lowest concentrations of both compounds were measured. In every analytical run, the resulting hair matrix pool (with IS added to it) was analyzed in triplicate. The mean ratio of caffeine and paraxanthine to their respective IS, obtained from this analysis of triplicates, was calculated. To correct for the background caffeine and paraxanthine, these mean ratios were subtracted from the ratios that were obtained for all calibrators and QCs. We did not consider

applying standard addition, since this would require two analyses per sample. For hair analysis in general (and certainly for a volunteer study, as is the case here), the amount of hair to be sampled remains limited because of esthetic issues. In this respect, sampling the double amount of hair does not seem appropriate.

Given the lack of blank matrix, selectivity could not be assessed. Cross-interferences with caffeine or paraxanthine were evaluated by analyzing samples spiked with caffeine, paraxanthine, theophylline or theobromine at 500 pg/mg (n = 5). To evaluate the purity of the isotopically labeled IS, non-spiked and IS-spiked hair matrix pool samples were analyzed in every sample batch. Interferences were considered unacceptable if the increase in peak areas was higher than 20 % of the peak area of the LLOQ for the analytes and 5 % for the IS [22]. Potential carry-over was assessed by applying these criteria on non-spiked samples injected after the highest calibrator as well.

Linearity was determined by constructing seven-point calibration lines (20, 40, 80, 150, 250, 350, 500 pg/mg) for caffeine and paraxanthine on four consecutive days. An F-test at the 99 % confidence level was performed and residuals were plotted against nominal concentrations to evaluate homoscedasticity of the data [24]. Unweighted and weighted ($1/x$, $1/x^2$, $1/\sqrt{x}$, $1/y$, $1/y^2$ and $1/\sqrt{y}$) linear regression were applied and slopes and intercepts of the corresponding calibration lines were calculated. The sum% relative error (RE) was calculated and %RE was plotted against nominal concentrations to select the best-fitted calibration model [24]. Mean back-calculated concentrations of the calibrators should be within ± 15 % of the nominal value in order to accept the chosen model [22]. The LLOQ was defined as the lowest concentration of caffeine and paraxanthine which could be measured with %bias and %RSD below 20 %.

On the same four consecutive days, QCs for caffeine and paraxanthine at four concentration levels were prepared and analyzed in duplicate to determine precision and accuracy. Assessment of accuracy ideally requires analysis of certified reference material (CRM); however, for caffeine and paraxanthine in hair, no CRM is available. Therefore, we opted to use spiked QCs for this purpose, as is commonly applied in hair analysis. Nominal QC concentrations were 20 (LLOQ), 60 (low), 200 (medium) and 400 (high) pg/mg. Intra- and interbatch precision (%RSD) were calculated using single factor ANOVA [25]. Accuracy (%bias) was calculated by dividing the difference between the obtained concentration and the

nominal value by the nominal value and multiplying it by 100. %RSD and %bias should be within ± 15 % or within ± 20 % for the LLOQ [22].

Determination of matrix effects and recovery was based on the approach described by Matuszewski *et al.* [26]. Hair samples originating from five different individuals, including blonde, brown, dark and black hair, were spiked at low and high concentration levels (respectively 60 and 400 pg/mg) before (C) and after (B) extraction. Standard solutions of caffeine and paraxanthine in the injection solvent at the same concentration levels (A) were prepared as well. Absolute matrix effect values were obtained by calculating the percent ratio of peak areas of (B) to those of (A), absolute recovery values by calculating the percent ratio of peak areas of (C) to those of (B). The %RSD of absolute matrix effect and recovery values represented the relative matrix effect and recovery [26], the word "relative" referring to the comparison of the values for matrix effect and recovery between different sources of hair. Relative matrix effect should not exceed 15 % [22].

Long-term stability of caffeine and paraxanthine in hair was evaluated by analyzing ground hair samples in triplicate at time point zero and after various time points of storage (i.e. 7, 61, 369 and 644 days) at ambient temperature protected from light. Processed sample stability was examined by re-injecting extracts of low and high QCs after 4 days of storage at 4 °C and 7 days at -20 °C (n = 3). All stability samples were analyzed together with freshly prepared calibrators. The obtained concentrations should be within ± 15 % of the concentration at time point zero and the nominal concentration (for QCs).

In order to safely dilute sample extracts when caffeine or paraxanthine concentrations above the ULOQ (500 pg/mg) are measured in real hair samples, dilution integrity was evaluated. Extracts of hair samples spiked at a concentration of 1000 pg/mg (n = 6) were diluted (1:5) with the injection solvent and analyzed. Using this dilution, the signals of the IS were high enough to still allow trustworthy integration of the corresponding peaks, as they were still within the linear range (20-500 pg/mg). The peak areas of the IS in the diluted samples were multiplied by 5. These peak areas were used to calculate the analyte/IS peak area ratios, from which the analyte concentrations were derived using the calibration curve. Finally, these concentrations were multiplied by 5 to obtain the actual concentrations. Accuracy and precision should be within ± 15 % [22].

2.6   Evaluation of the hair decontamination procedure

To evaluate the effect of the hair decontamination procedure, proximal 3-cm segments of hair samples collected from 6 volunteers were decontaminated using the procedure described in section 2.3. All obtained wash solvents were analyzed for caffeine and paraxanthine. Therefore, methylene chloride washes were evaporated under a stream of N_2 and redissolved in 150 µL of the mobile phase. An identical SPE procedure as used for clean-up of hair extracts was applied to the water washes. To ensure that this procedure was reliable when a higher volume (20 mL) was applied to the SPE cartridges, recovery was determined using solutions in water (20 mL, n = 3) in which absolute amounts of caffeine and paraxanthine corresponded to those present in extracts of hair samples spiked at the LLOQ concentration level.

2.7   Application

As a proof of concept, samples were collected from 10 healthy volunteers by cutting hair locks as close as possible to the scalp at the posterior vertex region. Caffeine and paraxanthine concentrations were determined in proximal 3-cm hair segments and corresponding paraxanthine/caffeine molar concentration ratios were calculated. This study was approved by the Ethics Committee of Ghent University Hospital (B670201111655). Written informed consent was obtained from all volunteers. No data on the participants' caffeine intake was available.

3   Results and discussion

3.1   Sample preparation

No significant differences were found between manual and mechanical grinding of hair (Figure 1). As the ball mill was not available for daily use, hair samples were manually ground in a mortar using liquid N_2. Extraction in methanol and enzymatic digestion with protease type XIV gave comparable results for both caffeine and paraxanthine (Figure 1). We opted to further optimize the enzymatic procedure as the resulting aqueous extracts were clearer compared to methanolic extracts and did not require an evaporation or dilution step prior to SPE. Solutions of three different proteases in Tris buffer at pH 7.5 and 8.5 were compared. No significant differences were found at pH 8.5 for caffeine, while slightly higher responses were seen for paraxanthine using protease type XIV. However, considerably higher responses for both compounds were obtained by using protease type VIII at pH 7.5 (Figure 2A). Hitherto, hair samples had been incubated for 12 hours at 37 °C. Different extraction

times were evaluated and decreasing responses for caffeine and paraxanthine were found when samples were extracted for 2 up to 12 hours (Figure 2B). For caffeine, no influence of the extraction temperature was observed under the evaluated conditions, while slightly higher responses for paraxanthine were obtained at 37 °C (Figure 2C). Increasing the enzyme concentration or using Tris buffer solutions at different concentrations did not influence the extraction efficiency (Figure 2D and 2E, respectively). As dithiothreitol is able to reduce disulfide bonds of proteins, it is often applied to enhance enzymatic digestion [3]. In this study, however, adding dithiothreitol to the extraction medium had no positive effect, as analyte responses slightly decreased when higher dithiothreitol concentrations were used. Especially for paraxanthine, the highest peak areas were obtained when no dithiothreitol was present during extraction (Figure 2F). In general, peak areas of paraxanthine were higher than those of caffeine in these experiments. This was partly due the fact that hair from an individual with relatively high CYP1A2 activity was used. In addition, LC and MS parameters were specifically optimized to allow a sensitive determination of paraxanthine, as it was expected that hair concentrations of this metabolite could be low in particular cases (e.g. in subjects with both low caffeine intake and reduced CYP1A2 activity). In the final procedure, 1 mL of a protease type VIII solution at 1.2 IU/mL in Tris buffer (50 mmol/L, pH 7.5) was added to 20 mg ground hair and samples were shaken for 1 hour at 37 °C and 750 rpm. To evaluate the impact of optimizing the extraction conditions, this procedure was compared with extraction in methanol. As mentioned below, hair extracts were cleaned-up using SPE. While the aqueous extracts resulting from the enzymatic procedure were directly applied to the cartridges, the methanol extracts were either evaporated under a stream of N_2 and redissolved in water or diluted 1:3 with water prior to SPE. The latter dilution step was feasible since a water/methanol mixture (65/35, v/v) is used in the SPE wash step. The results are shown in Supplementary Figure S1. Similar results were observed for both compounds when methanolic extracts were evaporated or diluted prior to SPE. The responses for caffeine and paraxanthine obtained after the enzymatic procedure were 30 %, respectively 43 – 47 % higher than the responses after extraction in methanol.

Concerning the clean-up step using SPE, the highest recovery values for caffeine and paraxanthine were achieved with Phenomenex Strata-X™ cartridges (Table 1). Therefore, an optimized SPE protocol was developed for these columns. The highest response for caffeine was obtained using water/acetonitrile (90/10, v/v) as SPE wash solvent, while water/methanol (85/15, v/v) gave the best results for paraxanthine (Figure 3A). The water/methanol mixture

was selected for further optimization because hair concentrations of paraxanthine, being a metabolite of caffeine, were expected to be lower. The response for caffeine decreased when 60 % of methanol in water was used in the wash step. Paraxanthine was increasingly lost from the cartridges when 40 % of methanol was used (Figure 3B). Finally, several elution solvents were tested. For paraxanthine, no differences between the evaluated solvents were found. For caffeine, the solvents containing acetonitrile gave the best results (Figure 3C). For the latter solvents, the effect of using higher elution volumes was investigated. For acetonitrile and methanol/acetonitrile (50/50, v/v), slightly higher responses were obtained when 4 x 500 µL was applied to the cartridges (Figure 3D). In the final SPE protocol, 2 mL of a water/methanol mixture (65/35, v/v) was used in the wash step and 4×500 µL of a methanol/acetonitrile mixture (50/50, v/v) in the elution step.

3.2 Validation

No unacceptable interferences or carry-over were observed under the evaluated conditions. Calibrator data for both caffeine and paraxanthine were heteroscedastic. Sum% RE values as well as graphical representations of %RE plotted against nominal concentrations for the unweighted linear regression and after application of the different weighting factors were highly comparable. Therefore, no weighting was applied. Resulting calibration and sensitivity data are shown in Table 2. Back-calculated concentrations of the calibrators were within 7 and 3 % of the nominal concentrations for caffeine and paraxanthine, respectively. Therefore, the selected regression models met the acceptance criteria and the calibration lines were linear. Intra- and interbatch precision and accuracy data for caffeine and paraxanthine QCs are summarized in Table 3. As %RSD and %bias lay below 12 and 7 %, respectively, the predefined acceptance criteria were fulfilled.

Ion suppression was observed for both compounds, as can be seen from the absolute matrix effect data shown in Table 4. The signal of caffeine was suppressed to a larger extent compared to its metabolite paraxanthine. However, when compensation by the IS was taken into account, matrix effects approximated 100 %, although there was a slight overcompensation for caffeine at the low concentration level. Compensation by the IS also resulted in an improvement of relative matrix effects, which for both compounds fell within 15 % limits. As matrix effects were assessed using samples from 5 different subjects with varying hair color, the latter data indicate that hair color had no apparent effect on ion suppression, or, at least, the effect is compensated for by the labeled IS, given the lower values for relative matrix effects after IS compensation. In addition, the recovery of caffeine

and paraxanthine was determined using the same 5 hair samples (Table 4). Also here, spiked samples were used and, therefore, it should be noted that these results reflect the recovery of the SPE procedure, rather than being a true measure of the actual extraction efficiency for caffeine and paraxanthine from human hair. Recovery was high (> 85 %) and found to be reproducible for both compounds, as all relative recovery values were below 15 %RSD. An additional experiment with repeated extraction [27,28] of non-spiked hair containing caffeine and paraxanthine revealed that the recovery following a single extraction was indeed high (> 73 %, data not shown), lying close to the values observed for spiked hair samples.

Hair samples proved to be stable at ambient temperature for at least 644 days, with measured concentrations being 102.61 ± 1.05 % and 102.03 ± 6.40 % of the concentrations measured at time point zero for caffeine and paraxanthine, respectively. Processed samples were stable after 4 days of storage at 4 °C (i.e. in the autosampler) and 7 days at -20°C. Stock solutions of the analytes and the IS were stable for at least 6 months when stored at -20 °C. Finally, the dilution integrity experiment showed that extracts of hair samples could be diluted 5-fold without affecting accuracy and precision. Following dilution, caffeine and paraxanthine concentrations were measured with an accuracy (%bias) of -0.99, respectively -3.45 % and precision (%RSD) of 1.23, respectively 1.02%.

3.3 Hair decontamination procedure

At the LLOQ concentration level, the recovery of the SPE procedure was 104.44 ± 5.11 % and 101.02 ± 5.96 % for caffeine and paraxanthine, respectively, when solutions with a volume of 20 mL were applied to the cartridges. Analysis of the wash solvents of 3-cm proximal hair segments from 6 volunteers revealed that paraxanthine was not present in any of the solvents, while caffeine was detected in all cases. Peak areas were determined and all responses for caffeine were below the LLOQ. Although strictly taken no quantitative conclusions can be deduced from these data, we compared the results from the different wash solvents by expressing peak areas as percentage of the peak area of the LLOQ. Peak areas ranged from 47.7 to 79.3 % of the LLOQ (mean ± SD; 63.8 ± 11.8 %) in the methylene chloride wash, from 56.9 to 72.82 % (mean ± SD; 64.0 ± 5.2 %) in the first water wash and from 28.7 to 50.2 % (mean ± SD; 37.1 ± 7.9 %) in the second water wash. Based on these data, it seems that the presence of caffeine in the wash solvents is more likely to result from limited external contamination of the hair, rather than from premature extraction from the hair matrix. First, caffeine was detected in the first wash solvent consisting of methylene chloride, a non-protic solvent that does not make the hair swell [1,3]. Second, responses for caffeine

overall decreased from the first to the second wash step using water, a protic solvent that causes swelling of the hair and may extract analytes [1,3]. Third, caffeine's metabolite paraxanthine was not detected in any of the wash solvents, supporting limited external contamination with caffeine rather than premature extraction of the analytes. Given the intensity of the caffeine responses in the last wash solvents, the effect of its presence as a presumed contaminant on final concentrations is considered to be limited, although it may play a role in cases with hair concentrations near the LLOQ.

3.4 Application

Caffeine and paraxanthine concentrations in proximal 3-cm hair segments from 10 healthy volunteers (age 21 – 36 years; 7 women) are listed in Table 5, together with their corresponding paraxanthine/caffeine concentration ratios as potential CYP1A2 phenotyping metric. All measured concentrations fell into the linear concentration range of the developed method. A representative chromatogram of caffeine and its metabolites paraxanthine, theophylline and theobromine in a hair sample from one of the involved volunteers is shown in Figure 4. To interpret paraxanthine/caffeine ratios in hair, they need to be compared with reference CYP1A2 phenotyping indices. Therefore, these results served as the basis for a larger study in which the usefulness of paraxanthine/caffeine ratios in hair for CYP1A2 phenotyping was evaluated by comparing hair ratios with similar ratios in the reference matrix plasma, following a classical CYP1A2 phenotyping protocol [29].

4 Conclusion

In this study, an SPE-LC-MS/MS-based procedure for the determination of caffeine and paraxanthine in hair was developed and validated. By thoroughly optimizing every step of the extraction and clean-up procedure, a reliable and sensitive method was obtained. The final method was fully validated based on international guidelines and all evaluated parameters met the pre-established criteria. Special attention was paid to the evaluation of the effect of the hair decontamination procedure. Finally, the suitability of the method was demonstrated by applying it to hair samples of 10 healthy volunteers.

References

1. Vogliardi S, Tucci M, Stocchero G, Ferrara SD, Favretto D. Sample preparation methods for determination of drugs of abuse in hair samples: a review. Anal Chim Acta. 2015;857:1-27.

2. Baciu T, Borrull F, Aguilar C, Calull M. Recent trends in analytical methods and separation techniques for drugs of abuse in hair. Anal Chim Acta. 2015;856:1-26.

3. Pragst F, Balikova MA. State of the art in hair analysis for detection of drug and alcohol abuse. Clin Chim Acta. 2006;370(1-2):17-49.

4. Barbosa J, Faria J, Carvalho F, Pedro M, Queriós O, Moreira R, et al. Hair as an alternative matrix in bioanalysis. Bioanalysis. 2013;5(8):895-914.

5. Jurado C, Kintz P, Menéndez M, Repetto M. Influence of the cosmetic treatment of hair on drug testing. Int J Legal Med. 1997;110(3):159-63.

6. De Kesel PM, Lambert WE, Stove CP. The role of (bio)markers in hair analysis. In: Stove CP, editor. New sampling strategies in toxicology and therapeutic drug monitoring. Future Science, in press.

7. Thieme D, Rolf B, Sachs H, Schmid D. Correlation of inter-individual variations of amitriptyline metabolism examined in hairs with CYP2C19 and CYP2D6 polymorphisms. Int J Legal Med. 2008;122(2):149-55.

8. Eisenhut M, Thieme D, Schmid D, Fieseler S, Sachs H. Hair analysis for determination of isoniazid concentrations and acetylator phenotype during antituberculous treatment. Tuberc Res Treat. 2012;327027.

9. Koren G, Blanchette P, Lubetzky A, Kramer M. Hair nicotine:cotinine metabolic ratio in pregnant women: a new method to study metabolism in late pregnancy. Ther Drug Monit. 2008;30(2):246-8.

10. O'Brien L, Baumer C, Thieme D, Sachs H, Koren G. Changes in antidepressant metabolism in pregnancy evidenced by metabolic ratios in hair: a novel approach. Forensic Sci Int. 2010;196(1-3):93-6.

11. Himes SK, Goodwin RS, Rock CM, Jones HE, Johnson RE, Wilkins DG, et al. Methadone and metabolites in hair of methadone-assisted pregnant women and their infants. Ther Drug Monit. 2012;34(3):337-44.

12. Perera V, Gross AS, McLachlan AJ. Measurement of CYP1A2 activity: a focus on caffeine as a probe. Curr Drug Metab. 2012;13(5):667-78.

13. Kalow W, Tang BK. The use of caffeine for enzyme assays - a critical appraisal. Clin Pharmacol Ther. 1993;53(5):503-14.

14. Fuhr U, Jetter A, Kirchheiner J. Appropriate phenotyping procedures for drug metabolizing enzymes and transporters in humans and their simultaneous use in the "cocktail" approach. Clin Pharmacol Ther. 2007;81(2):270-83.

15. Mizuno A, Uematsu T, Gotoh S, Katoh E, Nakashima M. The measurement of caffeine concentration in scalp hair as an indicator of liver function. J Pharm Pharmacol. 1996;48(6):660-4.

16. Gaillard Y, Pépin G. Screening and identification of drugs in human hair by high-performance liquid chromatography—photodiode-array UV detection and gas chromatography—mass spectrometry after solid-phase extraction. A powerful tool in forensic medicine. J Chrom A. 1997;762:251-67.

17. Dwivedi Y, Hill HH. A rapid analytical method for hair analysis using ambient pressure ion mobility mass spectrometry with electrospray ionization (ESI-IMMS). Int J Ion Mobil Spec. 2008;11:61-9.

18. Broecker S, Herre S, Pragst F. General unknown screening in hair by liquid chromatography–hybrid quadrupole time-of-flight mass spectrometry (LC–QTOF-MS). Forensic Sci Int. 2012;218:68-81.

19. Montesano C, Johansen SS, Nielsen MKK. Validation of a method for the targeted analysis of 96 drugs in hair by UPLC–MS/MS. J Pharm Biomed Anal. 2014;88:295-306.

20. Dussy F, Carson N, Hangartner S, Briellmann T. Is one hair lock really representative? Drug Test Anal. 2014;6(1):5-8.

21. De Kesel PM, Lambert WE, Stove CP. CYP1A2 phenotyping in dried blood spots and microvolumes of whole blood and plasma. Bioanalysis 2014;6(22):3011-24.

22. European Medicines Agency. Guideline on bioanalytical method validation. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf (Accessed Dec 2014).

23. U.S. Department of Health and Human Services. Food and Drug Administration. Center for Drug Evaluation and Research. Center for Veterinary Medicine. Draft Guidance for Industry. Bioanalytical Method Validation. http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm368107.pdf (Accessed Dec 2014).

24. Almeida AM, Castel-Branco MM, Falcao AC. Linear regression for calibration lines revisited: weighting schemes for bioanalytical methods. J Chromatogr B: Analyt Technol Biomed Life Sci. 2002;774(2):215-22.

25. Wille SMR, Peters FT, Di Fazio V, Samyn N. Practical aspects concerning validation and quality control for forensic and clinical bioanalytical quantitative methods. Accred Qual Assur. 2011;16(6):279-92.

26. Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. Anal Chem. 2003;75(13):3019-30.

27. Rothe M, Pragst F. Solvent optimization for the direct extraction of opiates from hair samples. J Anal Toxicol. 1995;19(4):236-40.

28. Kim SR, Wipfli H, Avila-Tang E, Samet JM, Breysse PN. Method validation for measurement of hair nicotine level in nonsmokers. Biomed Chromatogr. 2009;23(3):273-9.

29. De Kesel PM, Lambert WE, Stove CP. Paraxanthine/caffeine concentration ratios in hair: an alternative for plasma-based phenotyping of cytochrome P450 1A2? Clin Pharmacokinet. 2015;in press. DOI 10.1007/s40262-015-0237-7.

Tables

Table 1. Recovery data (n=3) for caffeine and paraxanthine spiked to hair samples at 100 pg/mg using four different SPE cartridges. An identical SPE protocol was used for all cartridges.

SPE cartridge	Absolute recovery (mean ± SD, %)	
	Caffeine	Paraxanthine
Bond Elut C18	59 ± 15	5 ± 2
Bond Elut Phenyl	83 ± 11	50 ± 7
Bond Elut C8	103 ± 18	61 ± 1
Phenomenex Strata-X™	98 ± 8	75 ± 5

Table 2. Calibration and sensitivity data for the determination of caffeine and paraxanthine in hair using LC-MS/MS (n=4).

	Slope, mean ± SD [95% CI]	Intercept, mean ± SD [95% CI]	R^2	Standard deviation of residuals	LLOQ (pg/mg)	ULOQ (pg/mg)
caffeine	3.4 ± 0.3 [3.1 – 3.6]	-0.004 ± 0.008 [-0.01 – 0.004]	0.999	0.03	20	500
paraxanthine	4.4 ± 0.3 [4.0 – 4.7]	-0.01 ± 0.02 [-0.03 – 0.008]	1.000	0.04	20	500

Table 3. Intra- and interbatch precision and accuracy (n=4x2) for QCs of caffeine and paraxanthine prepared at four concentration levels in hair.

QC	Nominal concentration (pg/mg)	Intrabatch precision (%RSD)		Interbatch precision (%RSD)		Accuracy (%bias)	
		caffeine	paraxanthine	caffeine	paraxanthine	caffeine	paraxanthine
LLOQ	20	6	12	12	12	6	5
low	60	2	3	5	5	7	4
medium	200	2	2	5	3	1	-1
high	400	2	3	2	4	2	1

Table 4. Absolute and relative matrix effect and recovery data (n=5) for caffeine and paraxanthine at two concentration levels in hair samples originating from five different individuals.

Caffeine

QC	Absolute matrix effect (mean ± SD, %)		Relative matrix effect (%RSD)		Absolute recovery (mean ± SD, %)		Relative recovery (%RSD)	
	without IS	with IS	without IS	with IS	without IS	with IS	without IS	with IS
low	66 ± 14	111 ± 13	20	12	88 ± 13	111 ± 9	15	8
high	57 ± 7	97 ± 4	13	5	89 ± 8	107 ± 7	9	6

Paraxanthine

QC	Absolute matrix effect (mean ± SD, %)		Relative matrix effect (%RSD)		Absolute recovery (mean ± SD, %)		Relative recovery (%RSD)	
	without IS	with IS	without IS	with IS	without IS	with IS	without IS	with IS
low	80 ± 5	96 ± 2	6	3	96 ± 6	121 ± 7	6	6
high	81 ± 5	100 ± 2	6	2	88 ± 4	107 ± 2	5	2

Table 5. Caffeine and paraxanthine concentrations and corresponding paraxanthine/caffeine molar concentration ratios determined in proximal 3-cm hair segments of 10 healthy volunteers.

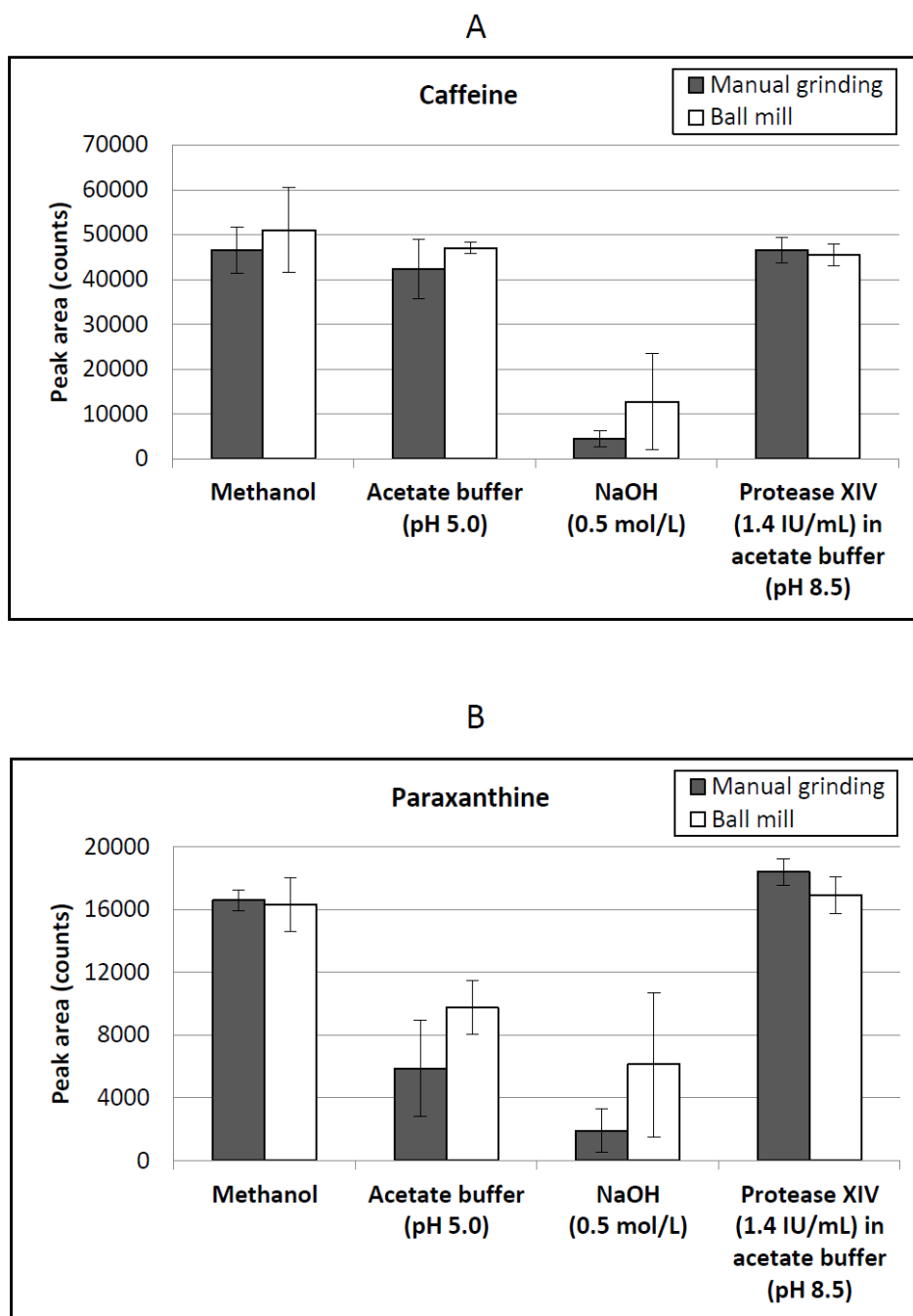| Volunteer	Caffeine (pg/mg)	Paraxanthine (pg/mg)	Paraxanthine/caffeine molar concentration ratio
1	174	88	0.54
2	343	95	0.30
3	384	74	0.21
4	366	221	0.65
5	254	144	0.61
6	285	33	0.13
7	410	135	0.35
8	205	71	0.37
9	311	92	0.32
10	389	269	0.75

Figures

Figure 1. Evaluation of different hair homogenization methods and extraction media to extract caffeine (A) and paraxanthine (B) from hair. Mean (n=3) peak areas of caffeine and paraxanthine in hair extracts are shown, with standard deviations.
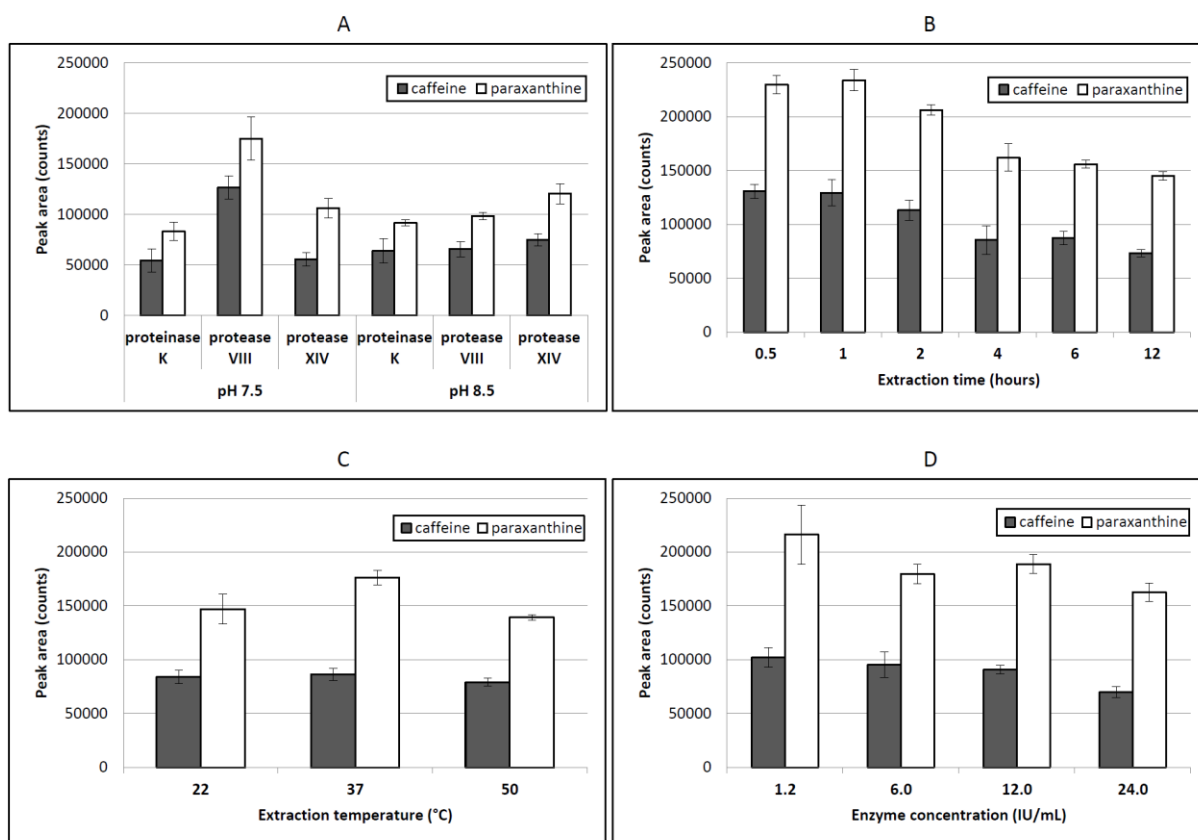
Figure 2. Optimization of enzymatic digestion and extraction parameters for the determination of caffeine and paraxanthine in hair. (A) Evaluation of solutions of three different proteases (30.0 IU/mL) in Tris buffer (50 mmol/mL, pH 7.5 and 8.5) for enzymatic digestion of hair. (B) Evaluation of different extraction times using 1 mL of protease type VIII (1.2 IU/mL) in Tris buffer (50 mmol/L, pH 7.5). (C) Evaluation of different extraction temperatures using 1 mL of protease type VIII (1.2 IU/mL) in Tris buffer (50 mmol/L, pH 7.5). (D) Evaluation of different concentrations of protease type VIII in Tris buffer (50 mmol/L, pH 7.5). (E) Evaluation of different concentrations of Tris buffer (pH 7.5) as solvent for protease type VIII (1.2 IU/mL). (F) Evaluation of different concentrations of dithiothreitol added to a solution of protease type VIII (1.2IU/mL) in Tris buffer (50 mmol/L, pH 7.5). In all panels, mean (n=3) peak areas of caffeine and paraxanthine in hair extracts are shown, with standard deviations.
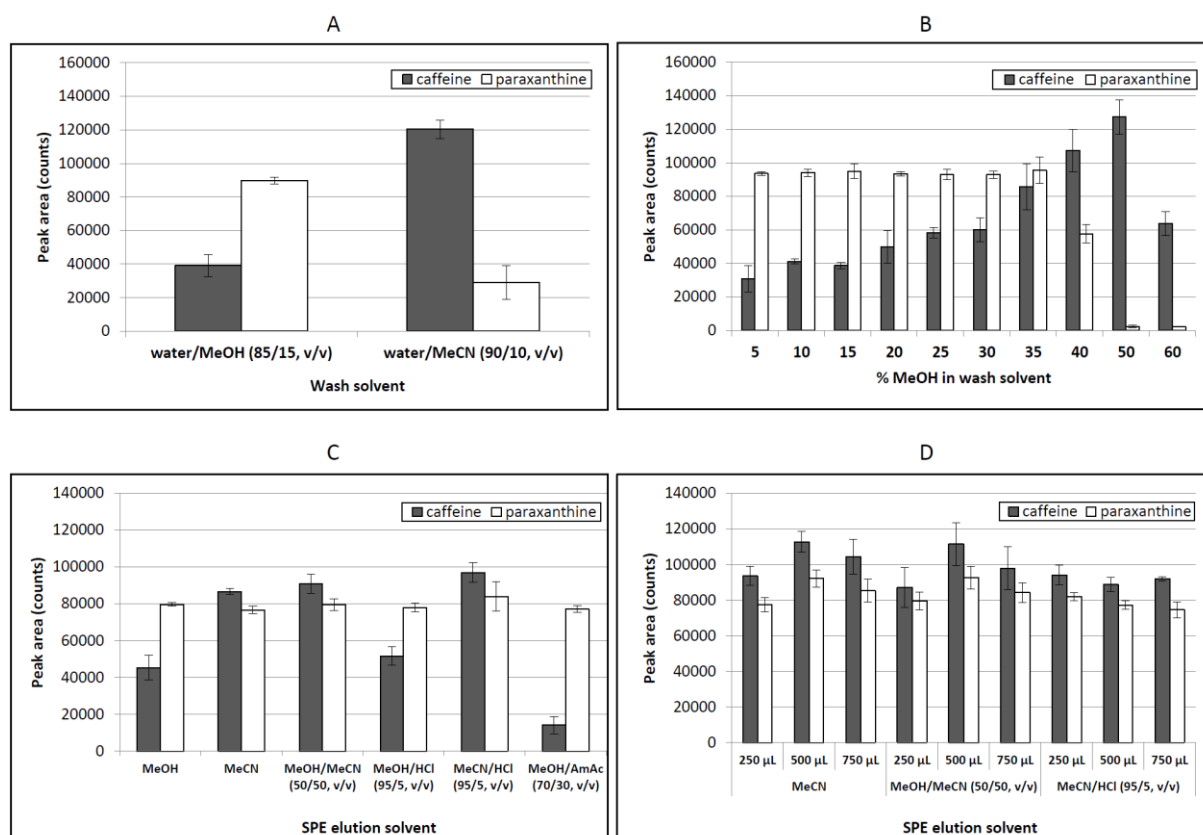
Figure 3. Optimization of the wash and elution step of an SPE protocol using Phenomenex Strata-X™ cartridges. (A) Evaluation of different SPE wash solvents. (B) Evaluation of the addition of incremental proportions of methanol to the SPE wash solvent (water). (C) Evaluation of different SPE elution solvents. (D) Evaluation of different volumes of SPE elution solvents. In all panels, mean (n=3) peak areas of caffeine and paraxanthine in hair extracts are shown, with standard deviations.
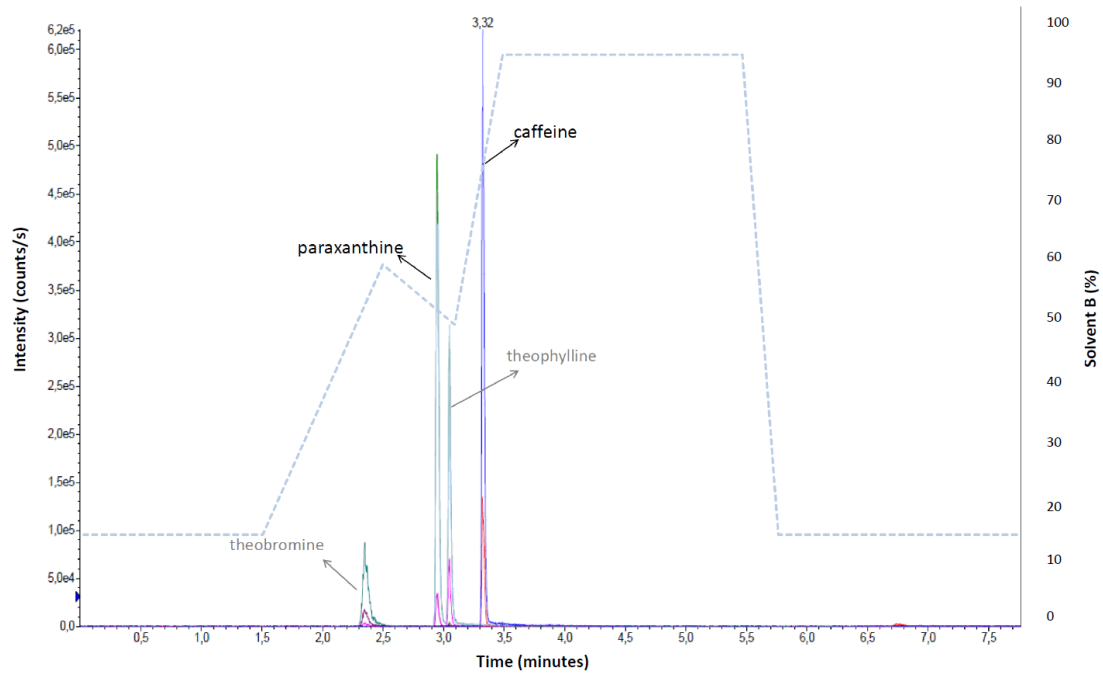
Figure 4. Chromatogram of paraxanthine, caffeine, theobromine and theophylline in a hair sample from a healthy volunteer. The dashed line indicates the proportion of solvent B (methanol containing 0.01 % formic acid) in the mobile phase over the chromatographic run.