Automated In-Tube Solid-Phase Microextraction Coupled with HPLC for the Determination of N-Nitrosamines in Cell Cultures

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An automated in-tube solid-phase microextraction (SPME) HPLC analysis method for 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and several metabolites has been developed. NNK is one of the tobacco-specific N-nitrosamines (TSNA), which has been linked to cancers associated with the use of or exposure to tobacco products. In-tube SPME is an on-line extraction technique in which analytes are extracted and concentrated from the sample directly into a coated capillary by repeated draw/eject steps. In this study, a tailor-made polypyrrole (PPY)-coated capillary and several commercially available capillaries (capillary GC columns) were used to evaluate their extraction efficiencies for NNK and several metabolites in cell cultures. Compared with commercial capillaries that were currently used for in-tube SPME, the PPY-coated capillary showed better extraction efficiency for all of the compounds studied. After optimization of the extraction conditions, NNK and five metabolite compounds were analyzed in spiked cell cultures, confirming the applicability of the developed method. Excellent linearity was observed for all compounds (av \( R^2 = 0.9942 \)) and detection limits that ranged from 20 to 250 ng/mL. The average within-day and between day variations (% RSD) were 2.9 and 3.6%, respectively. This automated extraction and analysis method simplified the determination of the TSNA, requiring a total sample analysis time of only \(~30 \text{ min.}\)

The carcinogenic properties of the tobacco-specific nitrosamines (TSNA), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) has been widely recognized and is believed to be a causative factor for lung cancer in smokers in all species tested.¹ There is also an increased risk for cancer of the upper digestive tract in tobacco chewers,² and a recent report has confirmed the presence of TSNA in nonsmokers who had been exposed to environmental tobacco smoke, highlighting their risk for increased lung cancer.³ NNK is a procarcinogen with a complex bioactivation⁴ and metabolic pathway.⁵ Consequently, the metabolites of NNK are useful biomarkers for an individual’s ability to metabolically activate or detoxify these nitrosamines.⁶

The significance and potency of this class of compounds demands an accurate sample preparation and analysis approach. The most widely used approach for the determination of NNK and various metabolites is high performance liquid chromatography (HPLC),⁷–⁹ but gas chromatography (GC)¹²¹¹ has been used to a lesser extent. The presence and potential ingestion of over 3000 compounds in tobacco and 4000 in tobacco smoke¹¹ increases the complexity of the sample matrix and places more demands on the sample preparation and analysis techniques for biological samples. To overcome these difficulties, sample preparation techniques such as solid-phase extraction (SPE)¹² have been employed to help extract, preconcentrate, and clean up the sample prior to analysis and are often required to improve the sensitivity and selectivity of the analyte’s determination.¹³ However, these traditional off-line procedures are time-consuming and labor-intensive, may require large volumes of sample and solvent, suffer great risks of contamination and analyte loss, and require additional instrumentation to automate. Alternatively, solid-phase microextraction (SPME) has gained increasing acceptance as a simple, fast, solventless, reliable, and flexible sample preparation technique.¹₄–¹⁶ SPME is based on a thin film of appropriate

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extraction phase coated on a fused-silica fiber for extraction of the analytes.

The technique has been recently extended to include the extraction phase coated on the inner surface of an open tubular capillary and is referred to as in-tube SPME.17-19 In-tube SPME allows for the convenient and on-line HPLC automation of the extraction process for improved analysis time, accuracy, and precision relative to off-line manual techniques. However, commercial extraction phases currently used for in-tube SPME do not show high extraction abilities for polar compounds.20,21 To extend the applications of this approach, alternative extraction coatings, such as polypyrrole (PPY) conductive polymers, are being investigated. Higher extraction efficiency toward polar compounds and aromatic compounds20,21 has been demonstrated as a result of the inherent multifunctional properties of the PPY polymer, such as interactions based on acid−base, π−π, ion exchange, and hydrogen bonding.22,23

In this study, application of PPY-coated and commercially available capillaries was applied for the in-tube SPME HPLC analysis of NNK and several metabolites. The PPY capillary showed improved extraction efficiency over all others tested, and an on-line and automated in-tube SPME technique was developed for the analysis of NNK and several metabolites in cell cultures. The ability of the capillary to successfully coextract several metabolites of NNK confirmed the suitability of the approach for drug metabolism studies. This may prove important for studying the metabolism of NNK in specific cell cultures, necessary for understanding the mechanisms by which this compound induces cancerous effects.

**EXPERIMENTAL SECTION**

**Chemicals and Reagents.** Pyrrole (98%) (Aldrich, ON, Canada) was distilled before use. Ferric perchlorate (Fe(ClO₄)₂·6H₂O) and perchloric acid (70%) were used as received (BDH, Toronto, ON, Canada). 1-(3-Pyridyl)-1-butanone-4-carboxylic acid (PBECA), 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB), 1-(3-pyridyl)-1,4-butanediol (PBD), and 1-(3-pyridyl)-1-butanol-4-carboxylic acid (PBLCA) were purchased from Toronto Research Chemicals, Inc. (Toronto, ON, Canada). 1-(3-Pyridyl)-1-butanone-4-carboxylic acid (23) was prepared as directed by the manufacturer. All other chemicals were of HPLC grade and was prepared as directed by the manufacturer. All other chemicals were obtained from Merck KGaA (Darmstadt, Germany). All of the solvents used in this study were of HPLC grade or analytical-reagent grade from Mallinckrodt-Baker (Deventer, Holland). Water was obtained from a Barnstead/Thermolyne NANopure ultrapure water system (Dubuque, IA).

**Safety Considerations.** The tobacco-specific nitrosamines (TSNA) are believed to be carcinogenic; please handle accordingly.

**Instrument and Analytical Conditions.** Experiments were performed using an Agilent 1100 series HPLC coupled with an autosampler and variable wavelength detector (Agilent Technologies, Waldbronn, Germany). The chromatographic column was a LiChrospher 100 RP-18e (15.0 cm × 4.0 mm i.d.; 5.0 μm particle size) from Merck KGaA (Darmstadt, Germany). A LiChrospher 100 RP-18e (4.0 cm × 4.0 mm i.d.; 5.0 μm particle size) guard column from Merck KGaA (Darmstadt, Germany) was installed at the inlet of the chromatographic column. The optimized mobile-phase gradient (at flow rate = 1.0 mL/min) used was 90% PBS (pH = 7.4) and 10% methanol from 0 to 4 min, changing to 80:20 PBS/methanol (v/v) from 4 to 15 min, returning to 90:10 PBS/methanol (v/v) from 15 to 16 min, and holding this composition until 19 min. UV spectra were recorded for NNK and NNAL, and the absorbance maximum of λ = 230 nm was used for the determination of all of the compounds in this study.

**Preparation of PPY-Coated Capillary.** Polypyrrole (PPY) film was coated on the inner surface of a fused-silica capillary (60 cm long, 0.25 mm i.d.) by a chemical polymerization method described previously.24 Briefly, the PPY inner surface coating was prepared by passing first the monomer solution (pyrrole in 2-propanol, 50:50v/v) through the capillary with the aid of nitrogen gas to form a thin layer of monomer on the capillary inner surface and then allowing oxidant solution (0.2 M ferric perchlorate in 0.4 M perchloric acid) to flow through the capillary in the same manner as for the monomer. The polymer was formed by oxidative reactions when the oxidant reagent reached the monomer in the capillary. The above procedure was referred to as one PPY coating cycle, which could be repeated several times (5 times in this study) to increase the coating thickness. The capillary was first cleaned with acetone and then dried with N₂ before it was coated. During polymerization, the color of the capillary changed gradually from yellow to black, indicating the formation of PPY on the inner wall of the capillary. The PPY-coated capillary was then washed with methanol for 2 min and dried by purging with nitrogen. Finally, it was coupled to the HPLC system as outlined in the next section and conditioned with the mobile phase.

**In-Tube Solid-Phase Microextraction.** The configuration for automated in-tube SPME has been schematically described in previous reports.17,18,19,25 Briefly, the extraction capillary (60 cm long) was used as the in-tube SPME device and was placed between the sample injection loop and the injection needle of the autosampler. The total internal volume of each capillary was ~30 μL. The autosampler was programmed to control the in-tube SPME extraction, desorption, and injection processes. Vials (2 mL) were filled with 1 mL of sample for extraction and set into the autosampler. The extraction of analytes onto the capillary coating was performed by repeated draw/eject cycles of 30 μL of sample at a flow rate of 50 μL/min with the six-port valve in the load

**References**


position. After washing the tip of the injection needle by one draw/eject cycle (2 µL) of methanol, the six-port valve was switched to the inject position, and the extracted analytes were desorbed from the capillary coating with the mobile phase flow and then transported to the HPLC column. The extraction efficacy of the PPY-coated capillary was compared to the following commercially available capillaries (from J&W, Folsom, CA): DB-210 (0.32-mm i.d., 0.25-µm film thickness), DB-1710 (0.32-mm i.d., 0.25-µm film thickness), DB-WAX (0.32-mm i.d., 0.25-µm film thickness), DB-FFAP (0.32-mm i.d., 0.25-µm film thickness), and a retention gap capillary (a polar silica tubing, 0.32-mm i.d., which was also used as the host capillary to make the PPY-coated capillary). In addition, the effects of mobile-phase composition and sample pH were investigated to optimize the sensitivity of the method.

Isolation, Cultivation, and Ultrafiltration of Hepatocytes.

All animal procedures described in this report were approved by the local authorities. The hepatocytes were isolated from a male Sprague Dawley rat, as described in Hansen et al.26 In summary, the liver was initially perfused in situ with 100 mL calcium-free Krebs-Ringer buffer (KRB) for 10 min, followed by 100 mL KRB and EDTA (1 mmol/L). Perfusion of the liver with KRB supplemented with collagenase type IV (Worthington, Lakewood, NJ) and 0.5 mM calcium chloride (Sigma, Munich, Germany) was continued for 8–10 min. The liver tissue was gently removed and the dissolved material filtered through a nylon mesh (pore size 100 µm) and washed twice with the washing buffer (1000 mL Hanks balanced salt solution (PAA Laboratories)) supplemented with 2.4 g HEPES (Sigma, Munich, Germany) and 2 g of bovine serum albumin (Sigma, Munich, Germany). The cell pellet was resuspended in Williams’ E medium including 5% fetal bovine serum (both from Biochrom, Berlin, Germany), 9.6 µg/mL of prednisolone, 0.014 µg/mL of glucagon (Novo, Mainz, Germany), 0.16 U/mL of insulin (Hoerhst, Strasbourg, France), 200 U/mL of penicillin, and 200 U/mL of streptomycin (GIBCO, Karlsruhe, Germany).

The cells were ultrafiltered using a Nanosep centrifugal microconcentrator model 3K (Pall Filtron, Northborough, MA) at 10000g for 20 min. A 500-µL aliquot of the filtrate was diluted with 500 µL of PBS (pH = 7.4) and spiked with a standards solution of NNK and the metabolites for direct analysis.

RESULTS AND DISCUSSION

HPLC Separation of NNK and Metabolites. Chart 1 illustrates the chemical structures of the compounds under study and indicates the presence of several ionizable groups on the analytes. The actual or predicted value of pKₐ for each compound was obtained using the ACD/I-lab Web service (ACD/pKₐ 5.0)27 and has been summarized in Table 1. The chemical diversity of these analytes can present several challenges for their HPLC separation. To maintain reproducible chromatographic retention times, the mobile phase was buffered at pH = 7.4 using PBS. At

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Table 1. PKa Values for NNK and Metabolites

<table>
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a. pKₐ values calculated at 25 °C in aqueous solutions. b. The site where ionization occurs on the molecule is shown in Chart 1; pKₐ₁ = 1, pKₐ₂ = 2, and pKₐ₃ = 3.

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(27) www.acdlabs.com

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this pH, the carboxylic acid groups on PBLCA and PBECA would be in their ionized form (net charge = -1), resulting in very low retention and overall analysis times. However, the lack of retention may also jeopardize the HPLC separation under these pH conditions. To compensate for this effect, an ion-pairing agent may be added to the mobile phase to enhance the retention time of these compounds. Alternatively, decreasing the pH and, hence, suppressing the ionization of the carboxylic acid group could also enhance retention time for these analytes. Improved analyte partition coefficients will be observed for neutral molecules with a neutrally charged C18 stationary phase.

The pH of the mobile phase had an opposite effect on the retention time with the HPB and NNAL compounds, because the retention times of these compounds were shortened with decreasing mobile phase pH. It has been previously reported that the interaction of the carbonil group with the unoxidized pyridine nitrogen on these molecules will be disrupted by the protonation of the nitrogen at pH < 5.5. Ideally, a pH gradient could be employed to maximize the separation of the compounds based on their different $pK_a$ values; however, to maintain simple and fast analysis times, the mobile phase was maintained at a pH value of 7.4 during the HPLC separation.

The type and effect of organic modifier used in the mobile phase was evaluated using various combinations of acetonitrile or methanol mixed with PBS. The percentage of acetonitrile in PBS ranged from 5 to 30% (v/v). Although improvements in separation were observed with decreasing acetonitrile percentage, the presence of even 5% acetonitrile in the mobile phase caused the PBLCA and PBECA compounds to elute fairly rapidly from the column, resulting in incomplete separation. In addition, poor peak shapes were observed for the late-eluting analytes. The organic solvent was switched to methanol, and the separations experiments were repeated. Under starting conditions of 90:10 PBS/methanol (v/v), successful separation of all of the compounds with good peak shape was observed, as shown in Figure 1. The run time was enhanced with a gradient elution, and when compared to a previously published HPLC method using similar column chemistry and dimensions, it provided a much improved fast analysis times, the mobile phase was maintained at a pH value of 7.4 during the HPLC separation.

Although methanol has a lower polarity index ($P' = 5.1$) than acetonitrile ($P' = 5.8$), it is a weaker elution solvent for reversed-phase HPLC as a result of its protic nature. Methanol is able to participate in hydrogen bonding, and this interaction between the charged carboxylic acid groups and methanol may have partially neutralized the negative charge on the oxygen and may allow for better retention of these molecules.

**In-Tube Solid-Phase Microextraction.** To optimize the extraction conditions for in-tube SPME, several parameters, such as the stationary phase of the capillary, the extraction time profile (the number of draw/eject cycles), and the sample matrix pH were evaluated by coupling in-tube SPME with HPLC. The results obtained from this study on the effect of capillary length were similar to previous studies, and therefore, they will not be discussed in detail. In general, a capillary 60 cm long is optimal for extraction. Below this level, extraction efficiency will be reduced, and above this level, peak broadening will be observed. Temperature may also affect the extraction efficiency; however, since the design of the autosampler did not allow for changes in the sample temperature, this effect was not evaluated. These effects have been previously discussed in the literature.

Several commercial capillaries having a range of stationary phase polarities were compared to the PPY capillary for their extraction efficacy toward the NNK compounds. A 1.0 $\mu$g/mL standard mixture of the compounds was subjected to 10 draw/eject cycles with each capillary, followed by desorption of the analytes from the extraction coating by switching the six-port valve to the inject position, which redirected the mobile phase through the capillary for transport of the extracted analytes to the analytical column. For each capillary, the extraction was repeated a minimum of three times and a percent RSD was calculated for the compound's HPLC peak area. As illustrated in Figure 2, the PPY-coated capillary gave the best extraction efficiency for all the compounds among all of the evaluated capillaries. Among the commercial capillaries tested, the DB-FFAP (containing a nitroterephthalic-acid-modified poly(ethylene glycol)) showed slightly higher extraction efficiency than the compounds studied. Although this result was somewhat expected, since the stationary phase was designed for gas chromatography of highly polar compounds, the capillary was still unable to provide any significant amount of analyte preconcentration. In contrast, the PPY capillary was able to increase in the amount of analyte extracted by 49–343% when compared to the host silica capillary (no extraction coating). As outlined in the Introduction Section, the PPY material is able to participate in a wide range of noncovalent interactions and was better able to extract polar compounds, such as NNK and its metabolites.

The preparation of the PPY coating also produced a very porous structure. The enhancement in surface area may have

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also improved the binding capacity of the capillary, providing an improved sensitivity. An increase in porosity will result in a larger number of active binding sites for adsorption of the analytes. The equilibrium amount of analyte extracted (extraction efficacy) via adsorption in the porous PPY coating is directly proportional to the partition coefficient ($K_p$), volumes of the sample solution ($V_s$) and coating ($V_t$); the initial analyte concentration in the sample ($C_0$) is the maximum concentration of active sites on the coating ($C_{fmax}$) and the equilibrium concentration of the analyte on the coating ($C_{fA}$), as shown by eq 1 \(^{13}\)

$$n_A = \frac{K_A V_t V_s C_0^0 (C_{fmax} - C_{fA})}{[V_s + K_A V_t (C_{fmax} - C_{fA})]} \tag{1}$$

Since it is difficult to use the above equations to obtain $n_A$ because some of the terms, such as $K_A$, $C_{fmax}$, $C_{fA}$, and $V_t$ are often unknown or difficult to measure, the amount of analyte extracted ($n_A$) by a SPM E coating can be easily obtained by experimental measurements using the following equation,

$$n_A = F \times A = (m/A_d) \times A \tag{2}$$

where $n_A$ is the amount (mass) of analyte extracted by SPM E, $F$ is the detector response factor that can be calculated by comparing the amount of analyte ($m$) injected and the area counts ($A_d$) obtained by liquid injection ($F = m/A_d$), and $A$ is the response (area counts) obtained by SPM E. From a practical standpoint, the extraction efficiencies of different coatings for the same analyte can therefore be evaluated by comparing the $n_A$ values obtained by SPM E with different coatings under the same extraction conditions.

For in-tube SPM E, the amounts of analytes extracted (as indicated by the corresponding HPLC peak areas) increase greatly when the number of extraction cycles (draw/eject cycles) increases. This trend will continue until the equilibrium extraction value is reached, where the maximum sensitivity is achieved. The extraction time profile of a 1 $\mu$g/mL standard mixture was constructed by increasing the number of draw/eject cycles prior to desorption. As shown in Figure 3, a detectable amount of analyte was extracted only after 2 cycles, and the equilibrium was not reached, even after 12 draw/eject cycles. The trend continued up to the maximum number of cycles programmable by the autosampler (40 cycles). Therefore, further increases in the number of extraction cycles will increase the amount of analyte extracted, but it will also increase the total analysis time. The number of cycles required to reach equilibrium extraction can be theoretically decreased by increasing the sample volume that is drawn/ejected; however, previous studies have shown peak broadening to be associated with large sampling volume.\(^{25}\) Alternatively, decreasing the tube inner diameter will increase the linear velocity of the sample traveling though the capillary and reduce the total time required to reach equilibrium extraction. However, changing the inner diameter of the host capillary will not affect the total amount extracted at equilibrium (as indicated by eq 1).

The thickness of the PPY coating will also influence the amount of analyte extracted at equilibrium and can be adjusted, as outlined in the Experimental Section. As shown in eq 1, increasing the coating thickness will result in a larger coating volume ($V_t$) and concentration of active sites on the coating ($C_{fA}$), leading to an increase in the extracted amount. To ensure adequate sensitivity and time considerations, 12 draw/eject cycles was used in all subsequent experiments.

The pH of the extraction mixture is important for compounds that contain a pH-dependent dissociable group. As indicated in Table 1, adjustment of the sample pH could affect the net charge of the analytes and their extraction efficacy. This effect was evaluated with pH values of 4.0 and 7.4. As discussed previously, at pH 7.4, the carboxylic acid group on PBLCA and PBECA would be in the ionized form (net charge of $-1$), but all other analytes would have a net charge of zero. The PPY extraction film was formed in its oxidized form, carrying approximately one positive charge in every three to four pyrrole rings.\(^{34}\) However, this charge is counter-balanced by an anion taken from the electrolyte solution during polymerization to yield a neutral coating. Therefore,\(^{33}\)

![Figure 2](image1.png) **Figure 2.** Comparison of PPY and commercial capillaries for extraction efficacy of NNK and metabolites. In-tube SPME conditions: sample concentration, 1.0 $\mu$g/mL (in H$_2$O); sample draw/eject volume, 30 $\mu$L; no. draw/eject cycles, 10; draw/eject speed, 50 $\mu$L/min; HPLC conditions as in Figure 1.

![Figure 3](image2.png) **Figure 3.** Extraction time profile of NNK and comparison of PPY and commercial capillaries for extraction efficacy of NNK and metabolites. In-tube SPME conditions: sample concentration, 1.0 $\mu$g/mL (in H$_2$O); other conditions outlined in Figure 2.
improved extraction efficacies are expected with nonionic analytes. As shown in Figure 4, this result was observed as the amount extracted was improved at pH = 7.4 for all analytes except for the carboxylic-acid-containing compounds of PBLCA and PBECA. Although beyond the scope of this report, it may have been possible to improve the extraction efficacy of these ionized compounds with the addition of an ion-pairing reagent.35 “Ion-pair SPME” could increase the magnitude of the partition coefficient between a charged analyte and a neutral extraction phase, thereby improving the recovery of the analyte from the matrix. Regardless, since most analytes had optimal extraction efficacies at higher pH values, all samples were buffered at pH 7.4 to ensure a reproducible and sensitive analysis.

Cell Cultures. The use of cell cultures has proved to be a very convenient way to study organ-specific metabolism.36 This is especially critical for carcinogenic compounds such as NNK, the metabolic profile of which has been shown to be organ specific.37 Therefore, the ability to successfully extract compounds of interest from this potentially complex and crucial matrix is an important task.

Application of the developed in-tube SPME HPLC method to cell cultures for the determination of NNK and metabolites required minimal sample preparation. After simple ultrafiltration of the sample, the sample was ready for direct extraction and analysis. The online and automated analysis approach required no sample manipulation between the preconcentration and the analysis step, which ensured no loss of the analyte and minimized the risk of contamination. An in-tube SPME HPLC blank cell culture chromatogram is shown in Figure 5A. Although some initial background compounds are observed eluting at an early time, they do not interfere significantly with NNK or any of the initial background compounds are observed eluting at an early time, they do not interfere significantly with NNK or any of the analytes for desorption and separation by the mobile phase. An additional peak was observed in both the blank and sample chromatograms at a run time of ~3.1 min. Since it did not coelute with any of the analytes, no measures were taken to identify this compound. However, the presence of antibiotics in the cell culture, such as streptomycin or penicillin was likely the source of the additional peak.

As summarized in Table 2, the spiked cell culture samples were run under a concentration range of 0.01–10 μg/mL with excellent linearity (avg. R² = 0.9942). To examine the precision of the method, 5 replicate analyses of the spiked cell culture (containing each analyte at 0.5 μg/mL) on different days were performed. The within-day and between-day variations (%RSD) were 2.5–3.6% (avg. 2.9%) and 2.5–4.8% (avg. 3.6%), respectively. The between-day precision, limit of detection (LOD), and limit of quantification (LOQ) were calculated using a 0.50 μg/mL sample and are summarized in Table 2. The LOD was determined at the analyte’s concentration where the peak height was a factor of 3 larger than the maximum baseline height of the blank (measured at the approximate retention time of the corresponding analyte’s peak). Similarly, the LOQ was determined at a signal-to-noise ratio of 10. It was possible to further preconcentrate the sample for enhanced detection limits by increasing the number of draw/eject steps (extraction cycles). Moreover, a further reduction of the LOQ (with simultaneous increase in the selectivity) can be

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achieved by using HPLC/MS (in the reaction monitoring mode), although in this case, the buffer has to be replaced by a volatile buffer. The optimized on-line and automated in-tube SPME HPLC method required 18 min for sample extraction and 12 min for HPLC determination for a total sample analysis time of 30 min. During the method development, the PPY capillary proved to be very stable and robust as >200 injections were performed over a period of 6 months with little change in the extraction efficiency. All of these properties clearly demonstrate the exceptional suitability and robustness of this capillary and the developed approach for use in the extraction and determination of NNK and metabolites in cell cultures.

CONCLUSIONS

In-tube SPME is an efficient on-line extraction technique because of its fast operation, easy automation, reduced solvent requirement, and low cost. The applications of PPY coating for in-tube SPME of NNK and several metabolites in cell cultures was demonstrated in this work. Compared with the commercial capillaries for in-tube SPME, the PPY coating has shown higher extraction efficiency for all of the compounds studied, and highly sensitive and selective determinations have been achieved by coupling a PPY-coated capillary to in-tube SPME HPLC. Optimizing several experimental parameters, such as the number of draw/eject cycles, sample pH, and the mobile phase conditions for HPLC separation provides an improved understanding of the extraction mechanisms responsible for the isolation and analysis of this important class of compounds and confirmed the applicability of the method for use in drug metabolism studies.

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