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Microfluidic direct injection method for analysis of urinary 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) using molecularly imprinted polymers coupled on-line with LC–MS/MS

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ABSTRACT

The work described in this paper involves development of a high-throughput on-line microfluidic sample extraction method using capillary micro-columns packed with MIP beads coupled with tandem mass spectrometry for the analysis of urinary NNAL. The method was optimized and matrix effects were evaluated and resolved. The method enabled low sample volume (200 μL) and rapid analysis of urinary NNAL by direct injection onto the microfluidic column packed with molecularly imprinted beads engineered to NNAL. The method was validated according to the FDA bioanalytical method validation guidance. The dynamic range extended from 20.0 to 2500.0 pg/mL with a percent relative error of ±5.9% and a run time of 7.00 min. The lower limit of quantitation was 20.0 pg/mL. The method was used for the analysis of NNAL and NNAL–Gluc concentrations in smokers' urine.

1. Introduction

There is a need for reducing development and analysis times of bioanalytical assays. The ability to monitor biomarker concentrations is very important in understanding disease progression and for drug development. A large number of clinical trials are conducted, increasing the number of samples requiring bioanalysis in a given timeframe. Apart from throughput requirements, most bioanalytical investigations also require analysis of biological samples at very low quantification levels [1]. An important component of bioanalytical method development is sample extraction prior to analysis. Solid phase extraction (SPE) techniques have turned out to be the most preferred techniques for extraction of analytes of interest from biological fluids prior to quantitative analysis [2]. SPE has many advantages compared to traditional liquid/liquid extraction methods. The most important advantage is the availability of a wide range of sorbents which makes this technique applicable to most classes of compounds. Moreover, SPE is easily amenable to automation also. Recently molecularly imprinted polymers (MIPs) have attracted much attention as SPE sorbents because they show promise as compound-selective or group-selective media [3].

NNAL is a metabolic product of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a potent carcinogenic nitrosamine present in tobacco products. In humans, NNK is extensively, almost entirely reduced to NNAL which is also carcinogenic [4]. NNAL–glucuronide is the most important detoxification product of the NNK–NNAL pathway [5]. Both NNAL and its glucuronide conjugate are excreted in urine. NNK by itself is not measurable in urine. Measuring urinary tobacco specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and its glucuronide conjugate may provide one of the best biomarkers of tobacco smoke lung carcinogen metabolism. The existence of differences in the extent of NNAL metabolism rates may be potentially related to an individuals' lung cancer susceptibility. In fact, findings of a recent study have directly linked NNK exposure to lung cancer development in humans [6]. Low concentrations of NNAL in smokers urine (<1 ng/mL) require sensitive and selective methods for analysis. Traditionally, this involves extensive, time-consuming sample preparation prior to using GC-thermal energy

Abbreviations: DFN, deviation from nominal; ESI, electrospray ionization; ETS, environmental tobacco smoke; Gluc, glucuronide; LOQ, lower limit of quantitation; MIP, molecularly imprinted polymer; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; QC, quality control; RSD, relative standard deviation; SD, standard deviation; SPE, solid phase extraction; TSNA, tobacco specific nitrosamine.

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analysis [7–9]. To address this problem, molecularly imprinted polymers (MIPs) have been developed for the analysis of urinary NNAL by offline cartridge extraction combined with LC–MS/MS [9–11].

However, a drawback of off-line SPE procedures is that they can be time consuming and cumbersome to perform, often requiring several steps prior to reaching a concentrated extract suitable for instrumental analysis [12]. The use of on-line SPE techniques, particularly in a miniaturized format such as a micro-capillary or a chip format, can enable faster method development by reducing the sample preparation time and thus increasing the sample throughput. Conditioning, washing and elution steps can be automated and performed online. The need for high throughput can be met to a great extent by application of an automated integrated analytical system which also includes the sample extraction step. Other important advantages of online sample extraction include decreased risk of contamination of the sample, elimination of analyte degradation in evaporation steps, and improved precision and accuracy [13]. Higher sensitivity is achievable by concentrating the sample on-column. Analysis of the entire sample leads to lower detection limits, and consequently smaller sample volumes may be sufficient to obtain sufficient sensitivity. Additionally, the on-line sample extraction configuration has been shown to reduce solvent consumption, and is thereby environmentally friendly [14]. The SPE sorbent can be regenerated and can be used multiple times unlike the single use offline SPE cartridge format [15]. Thus, online SPE procedures are particularly attractive for high-throughput, low-cost, low sample volume automated analysis. Method development for determination of drug or metabolite concentrations from urine samples can be substantially simplified with the implementation of online SPE. Because of their aqueous nature and lack of protein content, urine samples can be directly injected and extracted online.

The aim of this work was to design a capillary microfluidic system employing MIP beads specifically directed toward the tobacco specific nitrosamine NNAL. The system was developed for online sample extraction combined with mass spectrometric detection to enable fast online extraction and small volume injection with the ability to detect low pg/mL concentrations of NNAL and its glucuronide metabolite in human urine. The system was optimized and evaluated with respect to matrix effects. The method was validated as per the FDA bioanalytical method validation guidance [16] and used for analysis of urine samples from smokers.

2. Experimental

2.1. Materials and reagents

NNAL ($C_{10}H_{15}N_3O_2$, M_W = 209.25) (Fig. 1) and 4-(methyl-d_3-nitrosamino)-1-(3-pyridyl)-1-butanol (i.e. d_3-NNAL) ($C_{10}H_{12}D_3N_3O_2$, M_W = 212.16) (Fig. 1) were purchased from Toronto Research Chemicals (Toronto, ON, Canada). High purity water was obtained in-house using a Nanopure Diamond water system from Barnstead International (Dubuque, IA, USA). High purity methanol was obtained from Burdick and Jackson (Muskegon, MI, USA). Acetic acid was procured from Curtin Matheson Scientific Inc. (Houston, TX, USA). Formic acid was obtained from EMD Chemicals Inc. (Gibbstown, NJ, USA). Blank as well as smokers human urine samples were purchased from BioChemed Services (Winchester, VA, USA), Type H1 β-glucuronidase and ammonium dihydrogen phosphate were obtained from Sigma–Aldrich (St. Louis, MO, USA). SupelMIP – SPE NNAL molecularly imprinted polymeric cartridges were obtained from Supelco (Bellefonte, PA, USA).

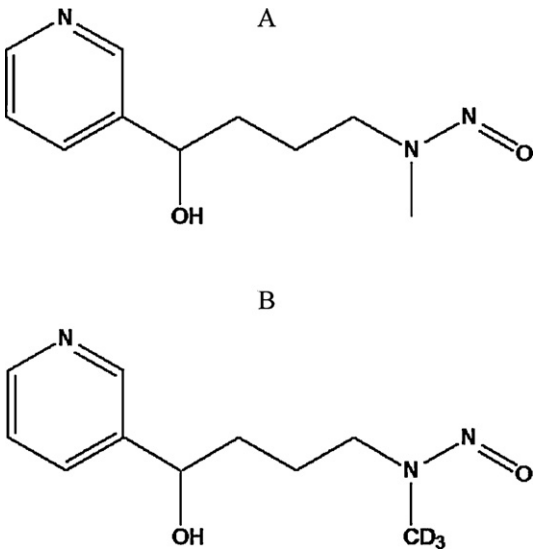

Fig. 1. Chemical structures of (A) NNAL and (B) d_3-NNAL.

2.2. Software

Data acquisition and quantitation software used was Analyst (Applied Biosystems, version 1.5).

2.3. Instrument and analytical conditions

A schematic diagram of the instrumentation is displayed in Fig. 2. A Shimadzu (Shimadzu, Kyoto, Japan) chromatographic system was used in the study. The HPLC system consisted of a Shimadzu system controller SCL-10A VP, pumps LC-10AD VP, and

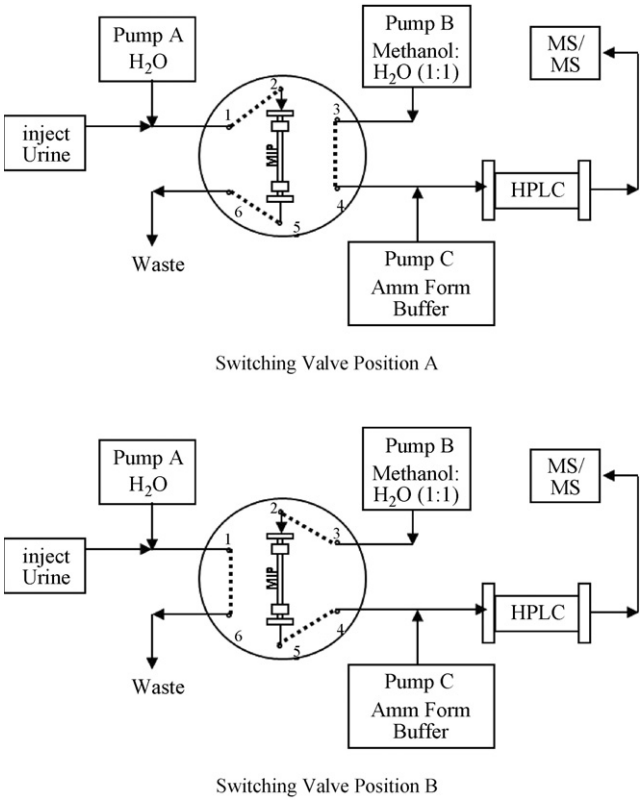

Fig. 2. Schematic diagram of on-line sample extraction.

a solvent degasser DGU14A. The auto-sampler HTS PAL from CTC Analytics (Zwingen, Switzerland) and a CH-30 column heater from Eppendorf (Westbury, NY, USA) were used. Sample extraction was performed online on a capillary micro-column packed with molecularly imprinted polymeric beads specific to NNAL (a description of the construction of the micro-column is given in Section 2.5) coupled with a Phenomenox Gemini C-18 column (100 mm × 2.0 mm ID, 5.0 μm) with incorporation of an isocratic elution mode. Mobile phase A was water with a flow rate of 0.24 mL/min. Mobile phase B was a 1:1 mixture of water and methanol with a flow rate of 0.18 mL/min. And mobile phase C was 10 mM ammonium formate buffer (pH 6.1) also with a flow rate of 0.18 mL/min. A Phenomenex C-18 guard column (4.0 mm × 2.0 mm) was used for extending the life of the analytical column. The column temperature was maintained at 60 °C. A multi-port Cheminert switching valve and a microelectric actuator obtained from Valco Instruments Co. Inc. (Houston, TX, USA) were used to divert the effluent from the micro-column during washing and elution steps. Initially, the switching valve was operated in position A, and switched to position B at 3.50 min. It was re-switched to position A at 3.70 min, and held in this position until the end of the run time of 7.00 min.

2.4. Mass spectrometric conditions

A 4000 QTrap hybrid triple quadrupole/linear ion trap mass spectrometer from Applied Biosystems (Foster City, CA, USA) was used. The mass spectrometer was operated in the positive electrospray ionization (ESI) mode employing multiple reaction monitoring (MRM). The mass spectrometer parameters were tuned and optimized to achieve maximum sensitivity. The desolvation temperature was maintained at 450 °C. The ion spray voltage and the multiplier voltage were fixed at 5.5 kV and 2.3 kV, respectively. The de-clustering, entrance and exit potentials were set at values of 49, 10 and 18, respectively. The Q1 and Q3 were set at unit mass resolution. The collision gas was set at high. The mass spectrometer was equipped with a Varian MS40+ single stage rotary vane vacuum pump from Varian Vacuum Technologies (Lexington, MA, UK). Nitrogen was generated using LCMS-5000NA TriGas generator obtained from Parker Balston (Haverhill, MA, USA). The curtain gas was set at 15, while the ion source gas 1 and gas 2 flow rates were set at 62 and 20, respectively. NNAL and NNAL-d_3 were assayed by quantifying the MRM transition of $[M+H]^+$ ion of NNAL at m/z 210.1 → 180.2 and d_3-NNAL at m/z 213.0 → 183.2.

2.5. Micro-column design

The micro-column design and construction procedure were based on a previously published method developed in our laboratory [17]. Micro-columns were constructed from polyetheretherketone (PEEK) capillary tubing of 500 μm inner diameter (ID) and 1/16 in outer diameter (OD) purchased from Upchurch Scientific (Oak Harbor, WA, USA). The choice of the PEEK tubing with 500 μm ID was governed by the 20–90 μm particle size distribution of the packing material. Also, 1/16 in OD is the most commonly used tubing size for standard, analytical LC and related techniques. The PEEK tubing has a pressure and temperature rating of 7000 psi and 100 °C, respectively. Stainless steel external column end-fittings and ferrules were purchased from Valco Instrument Co. Inc. (Houston, TX). A stainless steel 0.5 μm frit in a polymer ring (Upchurch) with dimensions 0.038 in × 0.030 in × 0.062 in was used to retain stationary phase material.

The PEEK tubing ends were cut and leveled with a PEEK tubing cutter to make them suitable for use with a HPLC system. After installing the frit, ferrule and fittings on the PEEK tubing, it was wrench-tightened. One set of end-fittings and frit was then removed, and the other open end of the micro-column was subjected to slurry packing under negative pressure (approximately 600 psi maximum) using a vacuum pump. The slurry consisted of a 10 mg/mL suspension of molecularly imprinted polymeric beads obtained from the commercially available SupelMIP – SPE NNAL molecularly imprinted polymeric cartridges in a 1:1 mixture of water and methanol. Approximately 5–10 μL drops were added each time using a microlitre pipette. To maintain the homogeneity of the slurry, it was continuously rotated on a suspension mixer. The procedure was continued until beads were seen at the top of the column. After leveling off the beads, the frit and end-fittings were reassembled by wrench tightening. The micro-column was then subjected to a 0.3 mL/min flow of a 1:1 mixture of methanol and water using the HPLC pump. This further compressed the packing material and ensured packing uniformity. Next, the fittings and frit at the end of the micro-column originally used for packing were again de-assembled. More slurry was added at the top of the column under vacuum to fill up any drop in packing level. Finally, the column fittings and frit were reinstated. Column lengths of 19 mm (smallest possible size considering end-fitting dimensions) were packed using this procedure. Approximately 100 μL of slurry added drop-wise was sufficient to pack the micro-column. This corresponded to approximately 1 mg of packing material (based on 10 mg/mL suspension of molecularly imprinted polymeric beads as stated above). Thus, a 19 mm micro-column was able to accommodate approximately 1 mg of the packing material and could be packed in approximately 15 min. Fig. 3 shows a diagram of the components of the PEEK micro-column packed with MIP beads specific to NNAL and the packing procedure for the same.

2.6. Stock solution preparation and stock dilution

A standard stock solution of 10 mg/mL of NNAL was prepared in methanol and stored at −20 °C. A series of standard working solutions were then obtained by appropriately diluting the standard stock solution of NNAL with water. An internal standard stock solution at 1 mg/mL was prepared in methanol and stored at −20 °C. An internal standard working solution was prepared at a concentration of 10 ng/mL by appropriately diluting the internal standard stock solution in water. Both NNAL and internal standard working solutions were prepared immediately prior to spiking into the urine.

2.7. Preparation of calibration standards and quality control samples

Six lots of analyte-free human urine (obtained from non-smokers) were thawed and pooled to provide the matrix for the study. Appropriate volumes of the working solutions of NNAL were spiked into the urine to obtain calibration curve standards over a range of 20–2500 pg/mL. Calibration standards were prepared freshly before each analytical run. In a similar fashion, quality control (QC) samples representing limit of quantitation (LOQ) QC, low (LQC), medium (MQC) and high (HQC) quality controls were prepared at 20, 60, 400 and 2000 pg/mL, respectively. A dilution QC sample above the upper limit of quantitation was prepared at a concentration of 10 ng/mL. All of the calibration standards, as well as the QC samples contained less than or equal to 5% (v/v) of the working solution in order to simulate a real sample as much as possible. For total NNAL analysis, standards and QC samples were subjected to a 24 h enzyme hydrolysis at 37 °C prior to analysis using β-glucuronidase enzyme as described in Section 2.8.

2.8. Sample extraction

Sample extraction was performed on-line by direct injection of urine samples on the molecularly imprinted polymer

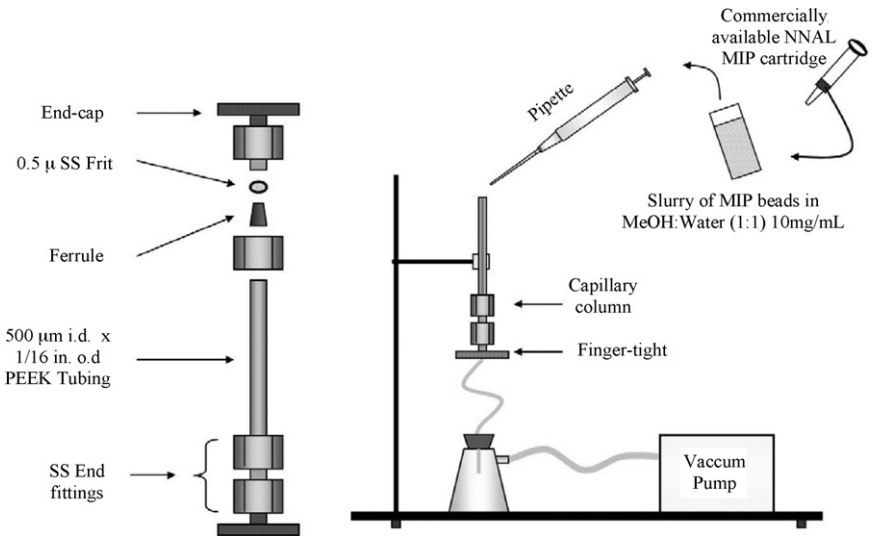

Fig. 3. Components of the packed micro-column and diagram of the packing procedure.

micro-column followed by LC–MS/MS analysis. Urine samples were thawed at room temperature. The pH of the urine was checked with pH paper, and adjusted between pH 5 and pH 7 using 10% (v/v) acetic acid solution. To a 1 mL urine aliquot, 20 μL of internal standard working solution (10 ng/mL) was added. For total NNAL analysis, the urine aliquot fortified with internal standard was mixed with 1 mL of 50 mM pH 6.4 ammonium dihydrogen phosphate buffer ($NH_4H_2PO_4 \cdot 2H_2O$). To this, 0.1 mL of 20,000 units/mL β-glucuronidase solution was added and the mixture was incubated at 37 °C for 48 h. The samples were then centrifuged at 3000 rpm for 15 min, and the clear supernatant was transferred to HPLC vials for direct injection. The injection volume was 200 μL.

2.9. Method optimization and evaluation

The purchased MIPs used in the present study were synthesized using the non-covalent approach utilizing MT101 as the template, methacrylic acid as the functional monomer, trimethylpropane trimethacrylate as the cross-linker, and progenic solvent toluene [10]. For these polymers, the MIP cavity will bind to the analyte ligands based not only on specific non-covalent interactions, but also non-specific hydrophobic interactions determined by the three dimensional structure of the polymer [18]. Kinetic parameters are also involved in the binding process [19]. Studies were performed to optimize and evaluate the system in order to obtain maximum recovery of NNAL on the MIP micro-column. These studies included wash time optimization, wash step flow rate optimization, sample pH optimization, evaluation of column loadability and injection volume optimization. These are important experimental parameters associated with the binding/extraction process.

2.9.1. Wash time optimization

To ensure that NNAL is not lost in the wash step, a wash time optimization study was performed. The matrix components were washed on the MIP micro-column by flowing water through the MIP micro-column for increasing time intervals from approximately 1.0 min to approximately 3.5 min. The wash time was optimized to allow maximum removal of matrix components without any loss of NNAL in the wash step.

2.9.2. Wash step flow rate optimization

To ensure that NNAL is not lost in the wash step, a wash step flow rate optimization study was performed. Molecularly imprinted polymers are by nature artificial immunosorbents. Thus, flow rate may have an effect on the retention of the analytes on the MIP stationary phase just as shown for immunoaffinity separations [20]. Thus, a wash step flow rate optimization study was performed in order to ensure balance between adequate recoveries of NNAL while allowing maximum removal of matrix components. A urine sample spiked with NNAL was injected on to the MIP micro-column. The MS response for different wash step flow rates (0.05, 0.1, 0.25, 0.3, 0.4, 0.5 and 0.75 mL/min) was evaluated for maximum recovery.

2.9.3. pH optimization

Recognition of NNAL molecules on a MIP column depends on interactions based on hydrogen bonding and ionic and hydrophobic effects [21]. Thus, the influence that pH may have on recovery of NNAL from the MIP micro-column was studied over a pH range of 2–10. The urine sample pH was adjusted with either 10% (v/v) acetic acid or 10% (w/v) sodium hydroxide.

2.9.4. Column loadability evaluation

The MIP micro-column loadability was evaluated in order to assess the maximum saturation binding. If the maximum saturation binding is reached before sufficient enrichment of NNAL on the MIP micro-column, the capacity of the column can be increased by packing longer columns. In order to evaluate maximum saturation binding, increasing concentrations of NNAL spiked in urine were injected on to the MIP micro-column. The column loadability was investigated for a concentration range of 50 pg/mL to 100 μg/mL.

2.9.5. Evaluation of matrix effects

In order to investigate matrix effects, a post-column infusion study was conducted as suggested by Bonfiglio et al. [22]. A syringe pump from Harvard Apparatus (Holliston, MA, USA) was used for infusion of NNAL for post-column infusion studies. A 500 ng/mL solution of NNAL was prepared in methanol. It was continuously infused at 10 μL/min post HPLC column into the mass spectrometer using a “tee” connection. Upon stabilization of the baseline response, a blank urine sample was injected which was subjected to extraction in the on-line format on the MIP micro-column. The matrix effect was investigated from the resulting profile for any change in the ESI response of NNAL. A 100 ng/mL solution of NNAL in urine was injected as a reference.

A post-extraction addition experiment was also conducted to evaluate the degree of matrix effects. Because of the nature of the extraction (on-line extraction) post-extraction addition experiment was performed in an off-line format. Urine samples were spiked with equivalent final concentrations corresponding to two QC concentrations (400 and 2000 pg/mL) of NNAL post-extraction on a MIP cartridge in an offline format. The washing and elution steps in the offline extraction were mimicked as in the online format. These samples were compared with external solutions of the analyte at equivalent final concentrations prepared in 1:1 water/methanol (v/v). Triplicate measurements were made in all cases. The peak area response of the post-spiked samples and the external solutions were then compared to determine the degree of matrix effects.

2.10. Validation

The method validation was performed based on the FDA guidelines for Bioanalytical Method Validation [16]. Validation runs containing calibration standards in duplicate, blank samples, blank sample spiked with internal standard, and replicates of QC samples were run on three separate days.

2.10.1. Linearity and LLOQ

Seven calibration standards having concentrations of 20, 50, 100, 250, 500, 1000 and 2500 pg/mL were prepared in duplicate in pooled blank human urine. For the determination of NNAL concentration, a $1/x$ weighing was employed for linear regression of the ratios of the peak area responses of NNAL and internal standard versus concentration. For each calibration curve, the back-calculated standard concentrations must be within 15% deviation from the nominal value (DFN) with RSD < 15% except at the LOQ, where it can be within 20% DFN with RSD < 20%. The lower limit of quantitation (LLOQ) was the lowest calibration standard concentration of NNAL at which the analyte response was at least 5 times the signal to noise ratio of the blank response, with accuracy and precision as stated above.

2.10.2. Accuracy and precision

Accuracy and precision were determined from the QC samples for three different validation runs. The concentrations of the QC samples were calculated from the calibration curves analyzed in the same run. An acceptance criterion of ±15% of the nominal concentration was used to assess accuracy (±20% for LOQ). Precision expressed as %RSD should not exceed 15% (20% for LOQ). Both intra- and inter-assay accuracy and precision were determined. Dilution controls at a level of 10 ng/mL were prepared to evaluate the capability of accurately diluting a sample having a concentration above the upper limit of quantification (2500 pg/mL). These controls were analyzed in triplicate in each run with a 1:10 dilution in blank human urine to obtain an effective concentration of 1000 pg/mL.

To evaluate the precision of the enzyme hydrolysis step in the analysis of total NNAL, a separate experiment was conducted. Urine samples from three different smokers were pooled and subject to enzyme hydrolysis after fortifying with working internal standard as describe in Section 2.8. This experiment was performed in six replicates and the samples were analyzed. The %RSD of the ratio of total NNAL to internal standard was used to evaluate the precision of the enzyme hydrolysis.

2.10.3. Selectivity

Six different lots of blank urine obtained from six different individual donors were used to assess selectivity. Each individual lot was processed and analyzed as per the procedure described earlier. In order to establish selectivity for urine samples subjected to enzymatic hydrolysis to measure total NNAL, six individual lots of blank urine samples were incubated with β-glucuronidase enzyme and processed as described in Section 2.8. For the method to be selective, areas of peaks co-eluting with NNAL should be less than 20% of the peak area of the LOQ sample of NNAL for all the six lots of blank urine. This would ensure that the endogenous urine components do not interfere with the assay.

2.10.4. Recovery and carryover

Recovery or the extraction efficiency of NNAL from the urine after the online extraction procedure was determined as follows. Urine samples were spiked with NNAL at the two QC concentrations (400 and 2000 pg/mL) and analyzed using online extraction. These samples were compared with urine samples that were spiked with equivalent final concentrations of NNAL post-extraction on a MIP cartridge in an offline format. The washing and elution steps in the offline extraction were mimicked as in the online format. The ratio of the peak area response of the pre-spiked and the post-spiked samples were then compared to determine the recovery of NNAL from urine.

Sample carryover was assessed by injecting a reconstituted blank immediately following a HQC sample (2000 pg/mL) injection at the end of the run. The acceptance criterion for carryover was ≤20% of the LLOQ. Sample carryover during the run was also assessed by injecting a HQC sample immediately followed by a LQC sample six times and was evaluated for any bias. Lack of carryover was confirmed if the bias was within 15% of the LQC concentration.

2.10.5. Stability

The stock solution stability for NNAL was determined during storage and processing. The stock solutions were considered stable if the concentration of NNAL was within ±5% of the original concentration. Stability experiments in human urine were performed at least two concentrations (60 pg/mL and 2000 pg/mL) in triplicate. The stability in urine was assessed during storage and after three freeze–thaw cycles at −20 °C with at least 24 h in between two cycles. These freeze–thaw QC samples were then run against freshly prepared calibration standards. The bench-top stability at room temperature was assessed in urine for 7 h to cover for the processing time of the samples. The post preparative stability or the auto-sampler stability was assessed from re-injection reproducibility after storage of the samples in the auto sampler for 24 h at 6 °C. The samples were considered to be stable in urine if concentration of NNAL was within ±15% of the nominal concentration for the QC samples tested.

3. Application of method to subject samples

To demonstrate the applicability of the validated bioanalytical method, this method was applied to the analysis of free and total NNAL in urine samples from 43 smokers. The smoker's urine samples were purchased from BioChemed Services (Winchester, VA, USA). These were 24 h urine samples donated by healthy smoking volunteers with their informed consent. The demographic details of the subjects are described in Table 1. Because these urine samples may be subject to differences in dilution resulting from each individual's state of hydration, all results were normalized by expressing concentrations per milligram of urinary creatinine levels.

4. Results and discussion

4.1. Online sample extraction and analysis

The initial studies with online sample extraction were evaluated using the MIP micro-column alone, in the absence of the

Table 1
Demographic data for the smokers' urine samples.

Subject	Gender	Race	Age (years)	Cig/day	Subject	Gender	Race	Age (years)	Cig/day
1	Male	Black	48	28	**23**	Male	Black	49	25
2	Male	Black	42	50	**24**	Male	Black	23	25
3	Male	Black	44	20	**25**	Male	Black	19	25
4	Male	White	31	20	**26**	Male	Black	35	25
5	Male	White	32	30	**27**	Female	Black	43	25
6	Male	White	39	28	**28**	Male	Black	21	25
7	Male	Black	35	30	**29**	Male	Black	55	25
8	Male	Black	45	30	**30**	Female	White	42	25
9	Male	Black	48	40	**31**	Male	Black	45	25
10	Male	Black	54	50	**32**	Female	White	21	25
11	Male	Black	40	25	**33**	Female	Black	29	25
12	Male	Black	25	25	**34**	Female	White	46	25
13	Male	Black	47	25	**35**	Female	Black	18	25
14	Female	Black	42	25	**36**	Female	White	26	25
15	Male	Black	32	25	**37**	Female	White	52	25
16	Male	Black	57	25	**38**	Female	Black	31	25
17	Male	Black	46	25	**39**	Female	Black	19	25
18	Male	Black	33	25	**40**	Female	Black	24	25
19	Male	Black	55	25	**41**	Female	Black	30	25
20	Male	Black	27	25	**42**	Female	Black	45	25
21	Male	Black	46	25	**43**	Female	Black	32	25
22	Male	Black	56	25					

analytical HPLC column. The results demonstrated good selectivity with respect to interferences. The MS response of a 100% matrix sample spiked with NNAL was three orders of magnitude lower than a neat solution at a similar concentration, however. Subsequently, post column infusion studies revealed the presence of ion suppression at the retention time of the analyte. Ion suppression occurred immediately as the multi-port valve was switched from the washing state to the elution state. This resulted in more than a 90% drop in analyte response (results not shown). Although molecularly imprinted polymers are highly selective sample extraction sorbents, non-specific binding of matrix components to the molecularly imprinted polymer cavities can be a potential problem causing matrix effects.

To address the ion suppression problem, a reversed phase HPLC column was introduced in-line after the MIP micro-column to separate matrix components, preventing them from co-eluting with the analyte of interest. The schematic diagram of the extraction procedure after incorporation of the HPLC column is depicted in Fig. 2. NNAL is a moderately polar organic compound. The relatively moderate polarity of NNAL would facilitate retention on a reversed phase HPLC column as compared to the more polar matrix species. This was the mechanism of separation that would potentially result in a reduced impact of ion suppression matrix effects commonly encountered in electrospray ionization of biological extracts.

4.2. Optimization studies

4.2.1. Wash time optimization

For the wash time optimization study, the MS response was measured as a function of changing wash times. The results of this study indicated no significant decrease in response with increasing wash times from approximately 1.0 to 3.5 min. Thus, a wash time of 3.5 min was selected as an appropriate balance between an acceptable run time, and an opportunity to wash off the maximum amount of matrix components without loss of NNAL recovery. After 3.5 min, the multi-port valve was switched from the wash step to the elution step.

4.2.2. Wash step flow rate optimization

The MS response was measured as a function of changing wash step flow rates. At flow-rates lower than 0.3 mL per minute, the NNAL recovery was higher. The recovery decreased by approximately 50% at wash-step flow rates above 0.3 mL per minute. This is possibly due to a slower mass transfer of NNAL on the molecularly imprinted polymer at the lower flow rates, resulting in a better retention of NNAL. Thus, molecularly imprinted polymer micro-column performance can be improved by decreasing the wash step flow rate. However, at very low flow rates (<0.2 mL/min), it was observed that the variability in response was higher (%RSD > 15%). This may be possibly due to the limitations of the HPLC pump to perform accurately at the lower flow rates. These observations were in line with other published results employing molecularly imprinted polymers [23]. Thus, a wash step flow rate of 0.25 mL/min was selected to maintain an adequate balance between sufficient recovery and consistency of results.

4.2.3. pH optimization

The MS response was measured as a function of changing pH. Greatest recoveries were obtained between pH 5 and 7. These results suggest an electrostatic interaction between NNAL and the functional groups on the molecularly imprinted polymer in this pH range. The molecularly imprinted polymer for NNAL has an acidic nature due to the presence of a carboxylic acid functional group arising from the use of methacrylic acid as the functional monomer [10] during the synthetic process. The pK_a of this acid function in the polymeric complex is estimated to be about 6.5 according to previously published studies [24]. NNAL is a basic compound having pK_a = 4.9 (calculated using pK_a prediction software [25]), suggesting that it is ionizable in the pH range of 5–7. Consequently, an ion exchange mechanism can take place between the hydrogen of the carboxylic acid functionality of the molecularly imprinted polymer and the basic functionality of NNAL. The maximum recovery between pH 5 and 7 suggests that this is the pH region where both NNAL and the carboxylic acid functional groups are simultaneously ionized leading to the development of the electrostatic interaction favoring NNAL binding to the molecularly imprinted polymer cavities. Therefore, prior to analysis, the urine samples were checked to see if their pH values were within 5–7 using pH paper. If the measured pH was outside this range, buffered urine was adjusted appropriately using either 10% (v/v) acetic acid or 10% (w/v) aqueous solution of sodium hydroxide.

4.2.4. Column loadability

A three-parameter curve characterizing saturation binding was used to determine the binding capacity of the column. The model followed the equation $Y = B_{max}X^h/(K_d{}^h + X^h)$ and was sigmoidal in

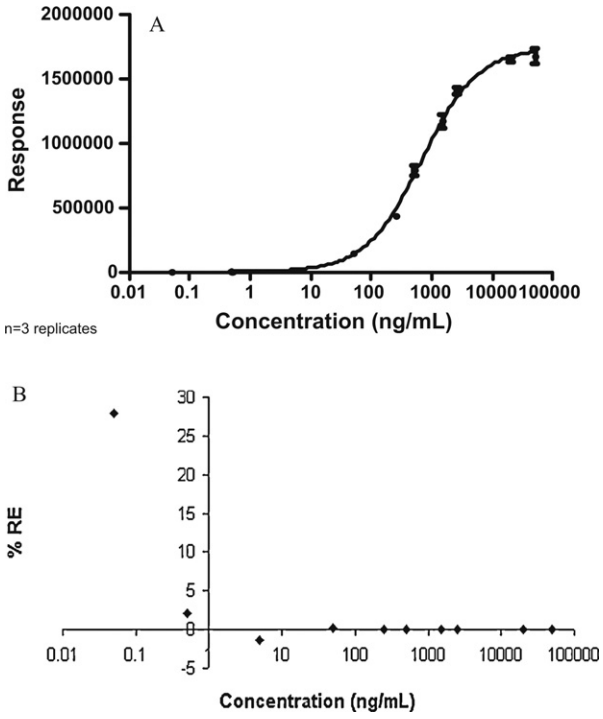

Fig. 4. (A) Saturation binding study and (B) relative error plot of NNAL spiked in urine.

nature. The model used a logarithmic *x*-axis scale as shown in Fig. 4A. In the above equation, *X* corresponds to the logarithmic concentration of free NNAL measured in ng/mL and *Y* represents the corresponding response. 'B_{max}' is the maximum specific binding (i.e. maximum number of binding sites), 'K_d' is the concentration required to achieve half-maximum binding and '*h*' is the slope. A weighting factor of $(1/Y^2)$ was used to fit the data. The parameter estimates with standard error were $B_{max} = (1.75 \pm 0.05) \times 10^6$, $K_d = 695 \pm 48$, $h = 0.939 \pm 0.007$. The percent relative error (%RE) of each calibration point was plotted against the NNAL concentration as shown in Fig. 4B. The difference between back-calculated values and nominal values was divided by the nominal values and multiplied by 100% to provide percent relative errors (%RE). The %RE data was less that 5% at all concentrations except at the lowest concentration of 50 pg/mL where it was less than 30%.

Based on the parameter estimates, the concentration required to achieve 90% binding was ~76,000 ng/mL, which corresponds to an on-column capacity of 1.52 μg of NNAL/mg of packing material. Thus, the on-column capacity was more than five orders of magnitude greater than the expected levels of NNAL in urine indicating a great potential to concentrate the sample on-column without saturating the micro-column.

4.3. Matrix effects

A post column infusion study was performed in order to evaluate the presence of matrix effect. Results of the post column infusion study after integration of the analytical HPLC column are shown in Fig. 5. It can be observed that the region of ion suppression is chromatographically resolved from the NNAL retention time.

On-column concentration was accomplished by increasing the injection volumes in order to detect low concentration of urinary NNAL. However, the effectiveness of increasing injection volume was limited because of an expected decrease in the resolution of NNAL from the ion suppression region due to the increased amount of matrix components injected. Lack of adequate resolution from the region of ion suppression may in turn compromise assay precision. Thus, maintenance of an adequate balance between resolution of NNAL from the region of ion suppression (baseline resolution of at least 1.2) and the optimum injection volume required to achieve the necessary on-column concentration (target concentration of at least 20 pg/mL) was essential. On-column concentration was measured in terms of NNAL response. The graph in Fig. 6 shows the relationship between the change in injection volume as a function of analyte response (measured in terms of signal-to-noise ratio) and the resolution of the analyte from the ion suppression region. As the injection volume was increased, the S/N increased up to about

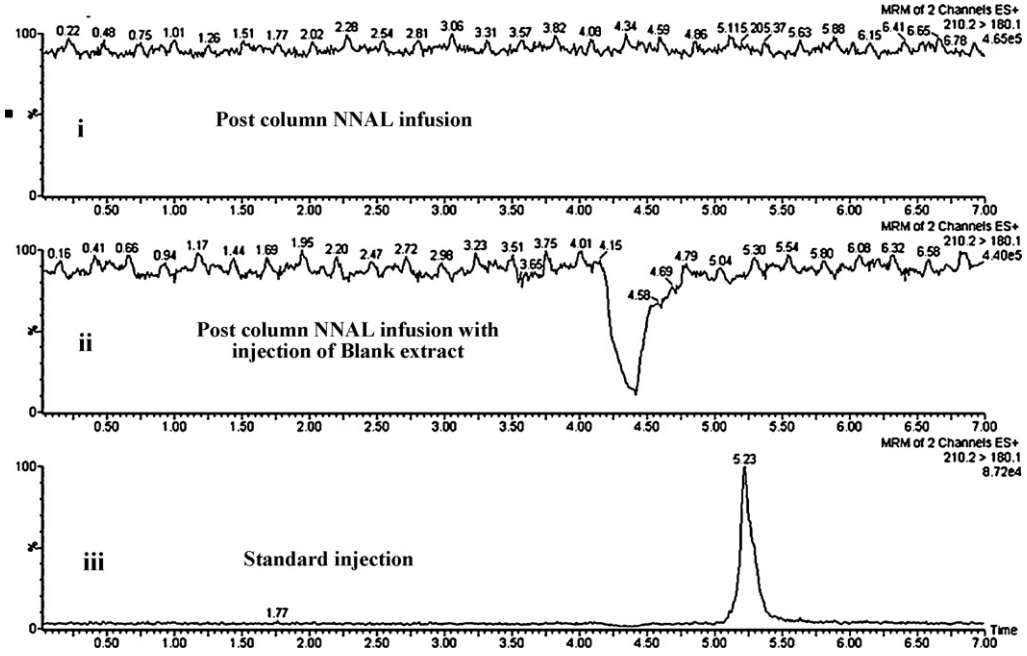

Fig. 5. Results of the post-column infusion study for online extraction of NNAL on a MIP micro-column after integration of the analytical column. (i) Ion profile when a 500 ng/mL solution of NNAL is infused into the mass spectrometer; (ii) ion profile upon injecting a processed blank sample; and (iii) representative chromatogram showing retention time of the analyte.

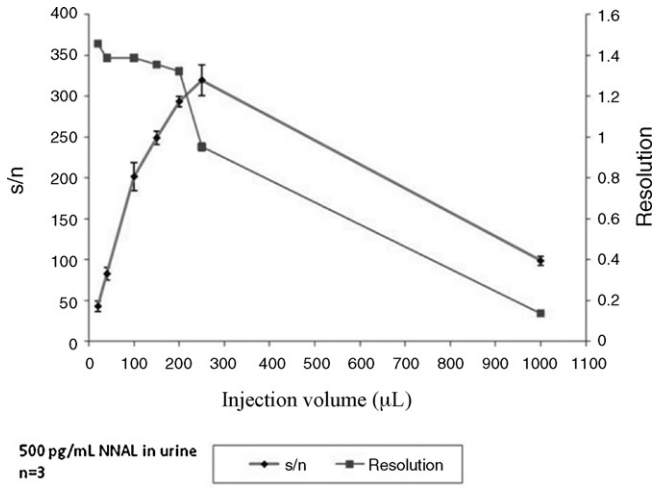

Fig. 6. S/N and chromatographic resolution from the region of ion suppression after integration of the HPLC column with the MIP micro-column as a function of injection volume.

300 μL injection volume, and then began to drop. Similarly, sufficient resolution of about 1.4 was observed between the region of ion suppression and the peak of interest up to about 200 μL injection volume. Further increases in injection volume compromise resolution drastically. So, to maintain sufficient balance between the two parameters, an injection volume of 200 μL was chosen. This volume was sufficient for an on-column concentration to achieve a target LOQ level of 20 pg/mL.

Post-extraction addition experiments revealed mean matrix effects of 92.3% and 96.8% for the QC concentrations at 400 and 2000 pg/mL, respectively. These results corresponded well with the results observed from the post-column infusion study after integration of the analytical HPLC column, indicating minimal matrix effects.

4.4. Validation results

4.4.1. Linearity

The peak area ratio of NNAL to internal standard in human urine was linear as a function of concentration over the range 20–2500 pg/mL. The calibration curves were well described with a mean correlation coefficient ≥0.999. A weighing factor of $1/x$ was used since the back-calculated residuals demonstrated heteroscedasticity with a proportional change in residuals as the concentration changed. Accuracy calculated in terms of the percent deviation from nominal (%DFN) for the mean back-calculated values of the calibration standards ranged from −3.5% to 5.9%, while precision measured in terms of percent relative standard deviation ranged from 0.95% to 7.39%. The LLOQ was established at 20 pg/mL of NNAL in human urine. The LLOQ was reproducible with accuracy and precision within the FDA guidance acceptance criteria with a S/N ratio of 10.

4.4.2. Accuracy and precision

The intra- and inter-run precision and accuracy data are summarized in Table 2. The intra- and inter-run accuracies and precision were determined at LOQ QC, LQC, MQC and HQC. The intra-run accuracy was within ±14.8% (maximum RSD of 11.3%) for all the concentrations including the LLOQ. Also, the inter-run accuracy was within ±9.6% (maximum RSD of 11.4%) for all concentrations. The method was both accurate and precise according to established acceptance criteria. Analysis of the dilution QC samples revealed that concentrations above the upper limit of quantitation could be accurately and precisely diluted up to 10-fold. The dilution QC prepared at a concentration of 10,000 pg/mL when diluted 10-fold demonstrated an intra-run accuracy of −0.24% DFN and a precision of 3.47% %RSD.

The %RSD of the ratio of total NNAL to internal standard for the smokers urine pool subjected to the enzyme hydrolysis step was 8.1% indicating uniformity in the enzymatic hydrolysis step.

4.4.3. Selectivity

The selectivity of the method with regard to endogenous components in urine was evaluated in six different lots of blank human urine. No endogenous peaks at the retention time of NNAL and the internal standard were observed for any of the urine lots. Fig. 7 demonstrates the selectivity results with representative chromatograms of (A) blank urine, (B) blank urine spiked with internal standard, and (C) analyte at the LLOQ level (20 pg/mL). The method also showed good selectivity for urine samples spiked with β-glucuronidase enzyme.

4.4.4. Recovery and carryover

The mean recoveries of NNAL from human urine determined at the MQC and HQC concentrations were 30.3%, and 31.7%, respectively. The mean extraction recovery is 31.0% for the current extraction protocol. No detectable carryover in the analysis of NNAL was observed.

4.4.5. Stability

The stability tests performed indicated no significant degradation under the conditions of freeze–thaw test, bench-top stability and post-preparative stability. The accuracy was within ±11.6% (maximum RSD of 10.1%) for all conditions evaluated which was within the FDA prescribed limits for accuracy and precision. This confirmed the overall stability of NNAL in the urine matrix under frozen conditions, assay processing and freeze–thaw conditions. The long term storage stability experiments were not performed as it is reported in the literature that NNAL is stable in frozen urine samples stored at −20 °C for up to four years as demonstrated by Yuan et al. [6].

4.5. Capillary micro-column ruggedness

After validation of the method, the capillary micro-column packing reproducibility was evaluated. Three capillary micro-columns were packed on separate occasions and tested at low, medium and high QC concentrations. Additionally, three different lots of MIP beads were packed in capillary micro-columns and evaluated at similar concentrations. The results of the column packing uniformity as well as the lot-to-lot uniformity study are shown in Fig. 8. Excellent column packing reproducibility was obtained at all QC concentrations as seen in Fig. 8A. The lot-to-lot recovery was slightly lower for one lot of MIPs at the high QC concentration as

Table 2
Intra- and inter-run accuracy and precision data for NNAL.

Concentration	NNAL concentration (pg/mL)			
	2000	400	60	20
Intra-run[a]				
Mean	1822.5	341.0	52.1	19.7
%RSD	2.4	5.9	3.1	11.3
%DFN	−8.9	−14.8	−13.2	−1.8
Inter-run[b]				
Mean	1807.5	379.3	54.8	19.4
%RSD	1.9	11.4	5.3	10.2
%DFN	−9.6	−5.2	−8.7	−2.8

DFN, deviation from nominal; RSD, relative standard deviation.
[a] Mean of six replicates observed at each concentration.
[b] Mean of 12 replicate observations over three different analytical runs.

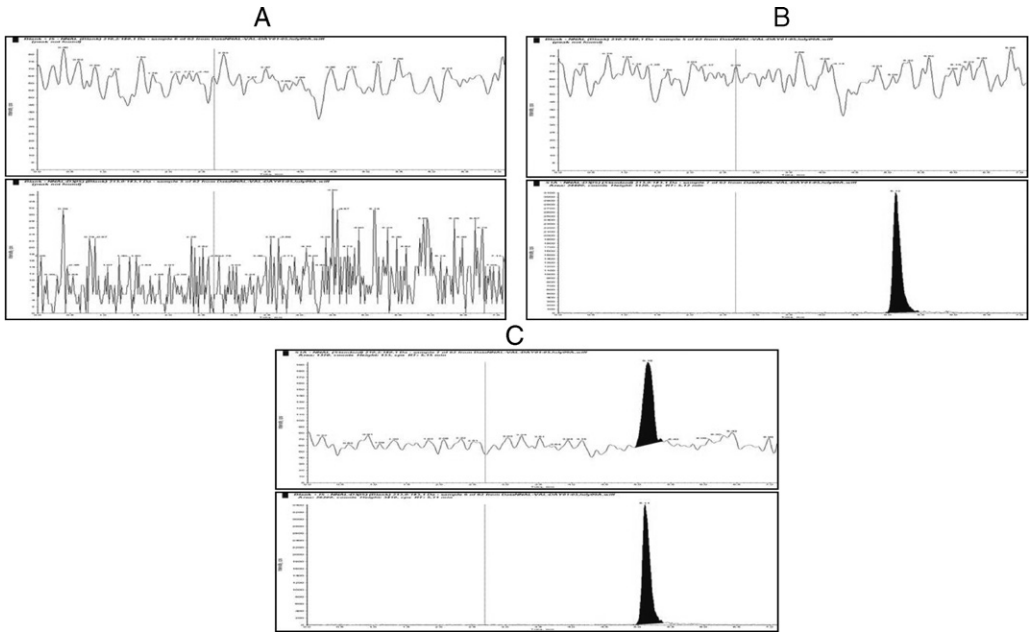

Fig. 7. Representative chromatograms of (A) blank urine (B) blank urine spiked with IS at 250 pg/mL and (C) urine spiked with NNAL at LLOQ of 20 pg/mL and IS at 200 pg/mL. MRM transitions 210.1 → 180.2 (upper channel) and 213 → 183.1 (lower channel) corresponds to NNAL and NNAL-d_3, respectively.

seen in Fig. 8B. This may potentially be due to a reduced number of binding sites for that particular lot. A significant advantage of the online capillary micro-column was the ability to re-use the micro-column for multiple injections. More than 300 injections were obtained on a single micro-column without loss of performance. There was no significant changse in the retention time and peak shape, and the column back-pressure remained almost constant. The recovery also remained adequate to achieve the low pg/mL levels. However, keeping in mind the highly time efficient column packing procedure, it was our standard of practice to replace the MIP micro-column with a newly packed column after about every 100–150 injections.

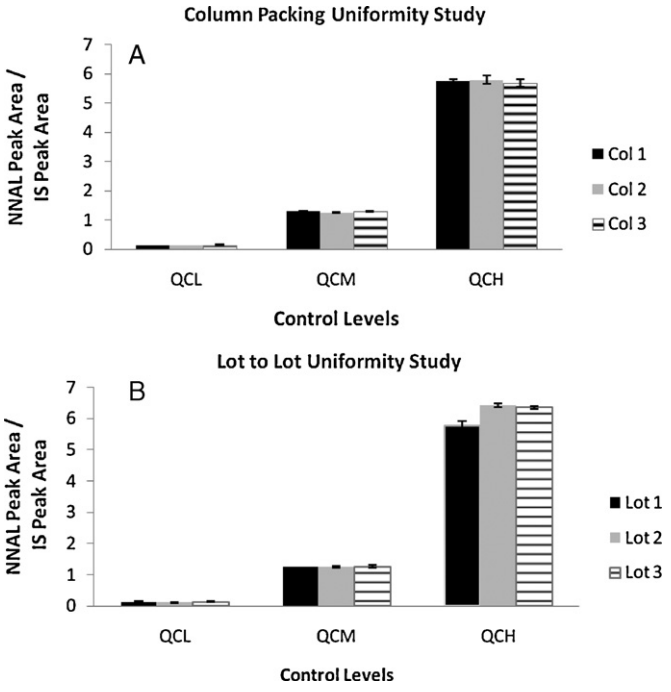

Fig. 8. Capillary micro-column reproducibility study. (A) Column packing reproducibility study ($n = 3$, error bars represent SD). (B) Lot-to-lot reproducibility study ($n = 3$, error bars represent SD).

5. Real sample analysis

Urine samples were stored at −20 °C until analysis. The concentrations of free NNAL that were measured ranged from BLOQ to 1.26 pmol/mg of creatinine while NNAL–Gluc concentrations ranged from BLOQ to 0.50 pmol/mg of creatinine. Table 3 shows the concentrations of NNAL and NNAL–Gluc measured in smokers. The ratios of NNAL–Gluc concentrations to NNAL concentrations are also depicted in Table 3. Although the total NNAL concentrations are lower compared to some of the previous reports [26,27], the difference may be attributable to several factors which may play a role in NNAL metabolism/concentrations such as age, gender, race, the type of diet, brand and type of cigarette smoked, active years of smoking, other disease conditions or impairments, concurrent medication, 24 h urine collection versus spot collection, urine creatinine correction, etc.

As described earlier, NNAL and its glucuronide conjugate may provide one of the best biomarkers of tobacco smoke lung carcinogen metabolism. Although not everyone who has a high level of NNAL is going to develop lung cancer, it would be helpful to be able to classify and/or to assign a number to a risk that was hard to quantify earlier. The benefit of knowing whether a smoker is at an increased risk for lung cancer or no allows the doctor to screen the person more regularly for abnormalities, in the hopes of diagnosing the cancer early. The method of principal component analysis (PCA) is one of the most widely used techniques in chemometrics for the transformation and classification of multi dimensional data sets in multivariate data analysis [28]. In our opinion, presenting the data in a principal component analysis form would present a valuable "tool" for such a risk classification. By conducting a controlled clinical investigation, patient follow-up, and monitoring disease progression, a "tool" based on PCA may be developed and validated for lung cancer risk classification. Amongst the several features that may be selected for the development of such a classification tool, levels of urinary NNAL and NNAL glucuronides, as well as their ratios would form key aspects. In this regards, the

Table 3
Concentrations of NNAL and NNAL–Gluc measured in urine of smokers.

Subject ID	NNAL concentrations	NNAL–Gluc concentrations	Ratio	Subject ID	NNAL concentrations	NNAL–Gluc concentrations	Ratio
1	0.194	0.080	0.412	**23**	0.378	0.353	0.934
2	0.153	0.096	0.630	**24**	0.311	0.187	0.603
3	0.120	0.176	1.466	**25**	0.319	0.155	0.485
4	0.389	0.229	0.589	**26**	0.574	0.399	0.695
5	0.350	0.166	0.474	**27**	1.259	0.165	0.131
6	0.235	0.054	0.229	**28**	0.310	0.113	0.363
7	BLQ	0.221	NA	**29**	0.326	0.188	0.576
8	0.205	0.344	1.682	**30**	0.465	0.291	0.626
9	0.000	0.113	0.000	**31**	0.289	0.275	0.952
10	0.230	0.157	0.684	**32**	0.386	0.207	0.536
11	0.112	0.233	2.091	**33**	0.694	0.501	0.722
12	0.236	0.148	0.626	**34**	BLOQ	BLOQ	NA
13	BLOQ	0.125	NA	**35**	0.360	0.132	0.368
14	0.243	0.100	0.410	**36**	0.327	0.300	0.917
15	0.445	0.213	0.479	**37**	BLQ	BLOQ	NA
16	0.396	0.198	0.500	**38**	0.210	0.113	0.540
17	0.307	0.283	0.921	**39**	BLOQ	BLOQ	NA
18	0.472	0.401	0.849	**40**	BLOQ	BLOQ	NA
19	0.384	0.192	0.501	**41**	0.250	0.414	1.651
20	0.168	0.072	0.429	**42**	0.379	0.355	0.935
21	0.234	0.411	1.754	**43**	1.054	0.234	0.222
22	BLQ	BLOQ	NA				

Note: Abbreviations: BLOQ (below limit of quantitation); NA (not applicable).
NNAL and NNAL–Gluc concentrations are reported as pmol/mg creatinine.
NNAL–Gluc concentrations determined from difference between free and total NNAL concentrations.
Ratio is the ratio of NNAL–Gluc to NNAL concentrations.

analytical method presented in the current work provides a rapid, economical, sensitive and reliable method for the quantification of these nitrosamine compounds. Such a tool can be made even more robust by inclusion of several other relevant features that may play a role in cancer susceptibility of smokers such as the type and brand of cigarette smoked, active years of smoking, age, gender, diet, etc. With the limited set of subject data evaluated in the current work, application of PCA as a means to classify lung cancer risk may not provide the most robust lung cancer classification tool. Nevertheless, this may be an interesting approach for a potential future clinical investigation with a larger controlled data set and well-defined features and end-points.

6. Conclusions

The determination of NNAL in human urine using on-line extraction on a microfluidic capillary column packed with MIPs coupled with LC–MS/MS has been accomplished. The method was optimized to achieve maximum analyte recovery. Matrix effects were avoided in the current method, and the method was validated as per the FDA bioanalytical method validation recommendations. The validation data demonstrated excellent precision, accuracy and stability. This method can be used for routine quantitative analysis of low concentrations of free and total urinary NNAL in smokers. A distinct advantage of the current on-line format compared with the off-line format as published previously [10,11] is micro-column re-usability. While the MIP cartridges used for off-line extraction are only single-use cartridges, as many as 300 urine samples were successfully analyzed on a single MIP capillary micro-column in the on-line format. The capillary micro-column contains approximately 1 mg of the MIP sorbent beads compared to a 25 mg MIP bed-weight for the off-line cartridges. Considering this, a cost saving of approximately 7500-fold is achievable by substituting the cartridge format with the capillary micro-column format. However, it should also be realized that an extra HPLC pump would be required to perform the extraction in the on-line format, which adds to the cost of the analysis. It may also be argued that utilization of this approach may be difficult for applications requiring greater sensitivity (e.g. studies for environmental exposures). However, this limitation may be easily addressed with the adoption of more sensitive mass spectrometers. Also, depending on the sensitivity needs, a longer MIP capillary micro-column can easily be packed to enable larger injection volumes. Chromatographic conditions can be modified, and a study similar to that described in Fig. 6 can be conducted to study the relationship between S/N and resolution of matrix effects as functions of injection volume. Finally, this method can be used for routine assay of low concentrations of free and total urinary NNAL.

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