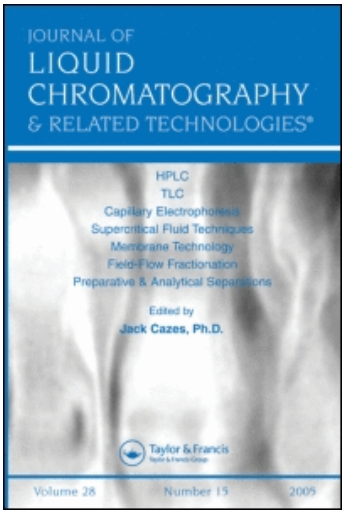

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Evaluation of Extraction Conditions and Use of HPLC-MS for the Simultaneous Determination of Acrylamide and its Primary Metabolite, N-Acetyl-S-(2-carbamoylethyl)cysteine, in Human Urine

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Abstract: Extraction conditions were evaluated for the simultaneous determination of acrylamide and its primary metabolite, N-Acetyl-S-(2-carbamoylethyl)cysteine (NACEC), in human urine. Acrylamide is an animal carcinogen and a human neurotoxicant; and it is widely used within industry. The toxicity of acrylamide makes it a health concern, and the use of its metabolite, NACEC, as a biomarker of exposure would be of value in the prevention of occupational diseases. Sample preparation studies evaluating several different types of solid-phase extraction (SPE) cartridges and different buffered or acidic matrices of standing urine samples were conducted. Measurement of acrylamide and NACEC was by reversed-phase high performance liquid chromatography (HPLC) with a mobile phase gradient. Detection for quantification was by single ion monitoring using electrospray mass spectrometry (MS). A basic method validation, using the final optimized SPE conditions, was conducted. Recovery studies of fortified urine samples at various concentration levels demonstrated good accuracy and precision; recovery varied between 97 and 108% for acrylamide and with relative standard deviations (RSD) of 7.6% or less. Recovery for the NACEC metabolite varied between 97 and 102% with RSD of 10% or less. The limit of detection (LOD) for the optimized

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procedure was found to range from 0.02 to 0.03 μg/mL for acrylamide and 0.1 to 0.2 μg/mL for NACEC in urine, using two chromatographic columns of different production lots.

Keywords: Acrylamide, N-Acetyl-S-(2-carbamoylethyl)cysteine, HPLC-MS, SPE, Urine

INTRODUCTION

Acrylamide has long been considered a potent neurotoxicant,[1] and in recent years it has been classified as a probable human carcinogen,[2–5] as well as being suspected of causing serious health effects in humans.[6] Acrylamide can be readily absorbed through the skin, and many of the reported cases of neurotoxicity have resulted from exposure by that route.[7] The formation of DNA and protein adducts by acrylamide and its reactive metabolites has been demonstrated,[8–10] and mutagenic effects from acrylamide have been detected.[7] Since acrylamide was identified and analyzed in food,[11–13] it has become an important topic in the literature, and its biochemistry and toxicology have been reviewed.[14] Low levels of acrylamide have been detected in baked, fried, and roasted foods, largely derived from heat induced Maillard reaction between asparagine and the carbonyl group of reducing sugars, such as glucose, during the process of cooking.[15–17] Leachate from packaging materials is another possible source of acrylamide in foods. Acrylamide is also present in tobacco smoke and has been attributed to the incomplete combustion or the heating of organic matter.[18] In industry, acrylamide and its various polymers are widely used. Applications of acrylamide polymers include waste water treatment, soil stabilization, and paper manufacture. Acrylamide is also a chemical intermediate, and it is used in grouting agents and in the preparation of laboratory gels for electrophoresis; therefore, acrylamide occupational exposure is of interest to this laboratory.

Urinary testing for acrylamide and its metabolites offers the best noninvasive test procedure to determine possible exposure to this chemical. Figure 1 shows the metabolic pathway for acrylamide; N-Acetyl-S-(2-carbamoylethyl)cysteine (NACEC) is quantitatively the primary metabolite in both rodents[19–22] and humans.[23–25] In the rat, NACEC was reported to comprise 67% of the total urinary metabolites,[19] while in man, the metabolite was greater than 80%.[24] This major pathway for acrylamide biotransformation is first-order conjugation with glutathione, catalyzed by hepatic glutathione-S-transferase (GST), which leads to the formation of NACEC.[22,26] Acrylamide is catalyzed to glycidamide by cytochrome P450; the mercapturic acids of glycidamide, N-acetyl-S-(1-carbamoyl-2-hydroxyethyl)cysteine, and N-acetyl-S-(3-amino-2-hydroxy-3-oxopropyl)cysteine, as well as 2,3-dihyroxy-propionamide (see Figure 1) are quantitatively minor metabolites[23] and were not of interest for this study. NACEC is, therefore, a metabolite suitable for use as a possible biomarker of exposure to acrylamide. NACEC has been used previously

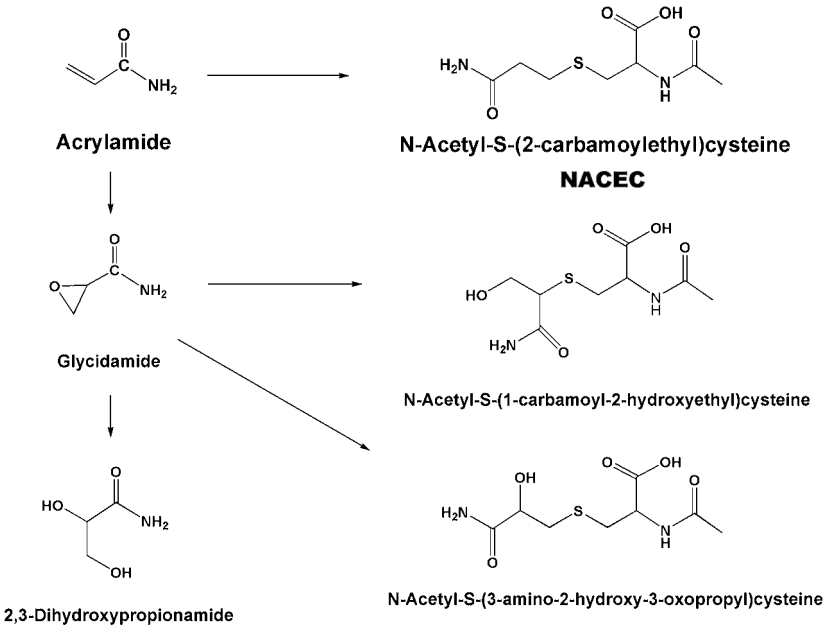

Figure 1. The metabolic pathways for acrylamide. N-Acetyl-S-(2-carbamoylethyl)-cysteine (NACEC) is a metabolic product in urine and is the focus of this study as a measurable biomarker.

as a urinary biomarker for acrylamide exposure in humans and monitored by various analytical procedures.[25,27–29] The kinetics of the elimination of these urinary acrylamide metabolites in humans has also been reported.[25]

The objective of this work was to develop a simple and effective extraction procedure and high performance liquid chromatographic (HPLC) conditions, to determine the levels of both acrylamide and NACEC in human urine for future work in their evaluation as biomarkers of exposure. It was also an objective to have validated[30] HPLC conditions in place for further studies by this laboratory. Solid-phase extraction (SPE) was chosen to remove the water soluble components of urine from the sample injected and analyzed by HPLC. SPE provided a measure of sample cleanup necessary for the analytical stage of analysis. A similar sample preparation procedure had been reported by Boettcher et al.[28] for the analysis of NACEC metabolite; however, the current methodology was intended for simultaneous quantification of both urinary acrylamide and the NACEC metabolite. Li et al.[27] reported a test procedure to quantify urinary NACEC using an online sample cleanup system and HPLC-MS. Wu et al.[31] used HPLC analysis with *o*-phthalaldehyde precolumn derivatization and fluorescence detection to analyze for NACEC in urine. Acrylamide, but not NACEC, was analyzed using HPLC and nonspecific UV detection from rat plasma

preparations.[32] Mass spectrometric detection was chosen in the current study for its improved specificity and the less likely possibility of interferences from affecting the quantification of either analytes. Precolumn derivatization was avoided to devise a less complicated analysis procedure. Deuterated NACEC was chosen as an internal standard for this chromatographic test. To the authors' knowledge, the current study has the most complete validation experimentation reported over the earlier works describing the analysis of NACEC metabolite from any sample matrix.

EXPERIMENTAL

Instrumentation and Chromatographic Conditions

The chromatographic analysis was carried out using an Agilent Technologies model 1100 liquid chromatograph (Palo Alto, California, USA) equipped with the 1100 series SL mass spectrometer (MSD) and an electrospray ionization (ESI) interface. Single ion monitoring was used for quantification of the analytes; m/z 72 positive was used for acrylamide, and m/z 233 negative for NACEC, and m/z 237 negative was used for the deuterated NACEC. The analytical column was a Phenomenex Synergi 4 μ Hydro-RP 80 A (Torrance, California, USA) with dimensions of 250 × 3 mm (ID) and was used with a Phenomenex C18 SecurityGuard^{TM} 4 × 2 mm guard cartridge. The column temperature was set at 30°C. Mobile phase A contained acetonitrile/water/formic acid, 2/98/0.05% (v/v/v), and mobile phase B contained acetonitrile/water/formic acid, 80/20/0.05% (v/v/v). For each chromatographic run, the mobile phase composition was held for 3 minutes at 100% A, then a linear gradient proceeded to 100% B over a 15 minute interval. A hold at 100% mobile phase B for 7 minutes was used to remove well retained urine sample components from the column; then the system was re-equilibrated with mobile phase A. Capillary voltage was 3 kV. The drying gas flow was 10 L/min, the nebulizer pressure was 35 psig and the drying gas temperature was 350°C. Fragmentor voltage was 80 V. A 10 μL volume of chromatographic sample was injected for LC-MS analysis, and data was acquired for roughly 20 minutes after injection. The acrylamide eluted in approximately 5.5 minutes, and NACEC eluted in approximately 9 minutes using these chromatographic conditions.

Chemicals and Reagents

Standard compounds of acrylamide (Chem Service, West Chester, Pennsylvania, USA), N-acetyl-S-(2-carbamoylethyl)cysteine (NACEC, CDN Isotopes, Quebec, Canada), and the d_4-NACEC (CDN Isotopes) were commercially available. All other reagents were of analytical grade and are regularly available in a laboratory.

General Urine Sample Preparation for Recovery Efficiency Study

Urine fortified at 2 μg/mL acrylamide and NACEC was used to test various extraction conditions to determine extraction efficiencies. Standard solutions prepared in mobile phase A prepared at 0.2, 1, 4, and 10 μg/mL levels were used for the chromatographic analysis to assay the extracted solutions and standing urine samples. All urine samples used for these experiments and the method validation came from volunteers who were not knowingly exposed to acrylamide.

Optimized Urine Sample Preparation for Final Recovery Study

Non-fortified urine samples and acrylamide/NACEC-fortified urine samples were treated identically. A 2.0 mL portion of the urine was placed into a screw capped polypropylene tube and acidified with 40 μL of concentrated (12 M) hydrochloric acid. A 0.5 mL volume of an 8 μg/mL d-NACEC internal standard solution was added. Next, a 0.5 mL portion of 0.1% formic acid solution in water was added. Finally, a 1.0 mL aliquot of 0.4 M ammonium formate solution (pH 2.6) was added and the sample was mixed. This solution was allowed to stand overnight at 4°C before solid-phase extraction. The samples were vortex mixed, centrifuged at 2000 rpm for 5 minutes, and 2.0 mL of the supernatant were applied to an Isolute ENV+ cartridge (200 mg, 3 mL, International Sorbent Technology, Mid Glamorgan, UK) prewashed with 2.0 mL of 94/5/1 (v/v/v) methanol/water/formic acid, conditioned twice with 2.0 mL of 0.1 M ammonium formate buffer (pH 2.6). The cartridge was washed with 1.0 mL of 0.1 M ammonium formate buffer and aspirated to dryness. Elution was performed with four 2.0 mL washes of 94/5/1 (v/v/v) methanol/water/formic acid. The solutions were combined and evaporated to dryness using a Labconco CentriVap Concentrator (Kansas City, Missouri, USA) held at 40°C. The residues were dissolved in 1.0 mL of the chromatographic mobile phase A. Acrylamide and NACEC standards for calibration were prepared at the 0.3, 0.6, 1.2, 5, 10, 20, 40, and 50 μg/mL fortified levels in control urine plus a blank 0 μg/mL level sample. Blank urine was fortified at the 2, 10, and 20 μg/mL equivalent acrylamide and NACEC levels for each analytical batch run of the primary recovery study. A secondary recovery study consisted of collecting urine from twenty non-exposed volunteers. Urine samples containing no acrylamide, NACEC or d-NACEC internal standard, and urine samples fortified at 5 μg/mL acrylamide and NACEC with 2 μg/mL internal standard were prepared for this second recovery study.

Calculations

Calculations were based on peak areas of acrylamide and NACEC peaks for the initial extraction efficiency studies, and on peak area ratios of acrylamide

or NACEC to d-NACEC for the final recovery studies. Standard calibration curves were linear within the acrylamide and NACEC ranges used; correlation coefficients were 0.97 or greater and y-intercepts approached zero for all curves generated with this method. For the final recovery studies, at least two calibration curves, at the beginning and end of the analytical run, using all the standards were collected for each set of recovery studies. Calibration curve slope drift was minimal; less than 2% was observed within any batch run. Duplicate injections were performed for all fortified samples and average values calculated for the final recovery data.

The limit of detection (LOD) was calculated as three times the standard deviation of the baseline noise level divided by the slope of the calibration curve.[24] The noise level was determined for each run by integrating noise levels in chromatograms at the retention time window for acrylamide or NACEC from the 0 μg/mL level urine samples. The slope from the calibration curve using peak height ratios of the standard levels was used for this calculation.

RESULTS AND DISCUSSION

Chromatographic Conditions

The chromatographic conditions developed for this study proved to be specific and capable for the simultaneous quantification of acrylamide and NACEC. Interferences were not detected for acrylamide and the non-deuterated NACEC metabolite. A typical single ion monitoring chromatogram of a fortified urine sample extracted under the final optimized conditions described is displayed in Figure 2. Acrylamide eluted in 5.5 minutes while NACEC was retained for 9 minutes. The chromatographic baselines displayed little drift from the gradient run, and the smaller background peaks did not interfere with the two analytes of interest. The chromatograms generated from urine of non-exposed volunteers did not show any interfering peaks for acrylamide or NACEC. The mass selective detector was useful in adding additional specificity to the analysis procedure; molecular ions were chosen for monitoring the analytes; m/z 72 (positive) for acrylamide, m/z 233 (negative) for NACEC, and m/z 237 for the deuterated NACEC internal standard. Mass m/z 55 for acrylamide was evaluated and found to have a lower response than m/z 72, so the latter was chosen for quantification for this study.

Extraction Development and Choice of the Internal Standard

Solid-phase extraction (SPE) was necessary for sample cleanup and concentration for this procedure. Liquid-liquid extraction was initially tried during

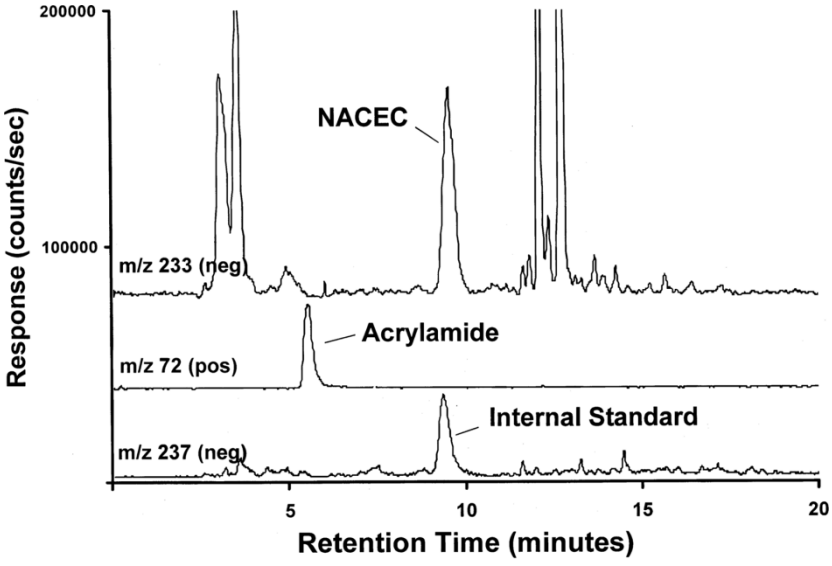

Figure 2. Single ion monitoring (SIM) chromatogram of a fortified urine sample using the optimized extraction conditions described. The urine sample was fortified at the 5 μg/mL level of acrylamide and NACEC and 2 μg/mL of the deuterated analog of NACEC used as an internal standard.

the earlier stages of this study. Although acrylamide was slightly extractable by LLE [30% efficiency by ethyl acetate], NACEC was highly water soluble and not readily extractable from the aqueous urine matrix. Also, both acrylamide and NACEC are reactive compounds. In order to obtain reasonable extraction yields of free compounds from urine and not adducted molecules, acidification and the storage of fortified urine samples overnight was necessary. Urine samples fortified at the 2 μg/mL levels with acrylamide and NACEC were allowed to stand overnight at 4°C with various pH/acidified-buffering conditions as listed in Table 1. It was found that a buffering system at pH 2.6 using 0.1 M ammonium formate gave the highest yield combination of both acrylamide and NACEC from non-extracted standing urine. Various SPE media were evaluated during this evaluation including Bond Elut C18 and Focus SPE cartridges (Varian, Inc., Palo Alto, California) and the Isolute ENV+ cartridges (International Sorbent Technology) finally used under the optimized conditions. Yields with the other SPE cartridges never exceeded the 50% range, while the Isolute ENV+ achieved 90% extraction efficiency using the optimized conditions (See Table 2). The 200 mg bed of the Isolute ENV+ SPE was determined to be optimal with 2 mL of urine/buffer solution as is shown in Table 2. The data displayed in Table 2 were obtained using the same prewash and

Table 1. Assay levels of free acrylamide and NACEC in standing urine solutions[a]

pH/Acidification or buffer	Acrylamide yield (%)[b]	NACEC yield (%)[b]
Untreated Urine[c]	63	47
1.5/Hydrochloric Acid[d]	88	54
2.6/Ammonium Formate[d]	86	76
5.0/Ammonium Acetate[d]	56	77

Notes: [a]The standing urine solutions were not extracted; the assay results are of the actual solutions.

[b]Percent (%) yield is percent of the 2 μg/mL theoretical or starting concentration of the fortified urine.

[c]Average of three fortified urine samples (n = 3).

[d]Average of six fortified urine samples (n = 6).

wash cycle described in the experimental section with 2, 4, and 8 mL volumes of urine/ammonium formate buffer solutions. Samples required overnight standing; this was verified by repeating the 2 mL urine/ammonium formate solution without allowing standing time after spiking the urine. Recovery of acrylamide dropped below 50%, while the NACEC recovery dropped slightly. Boettcher et al.[23,24] reported a similar extraction procedure for acrylamide metabolites using a much smaller bed Isolute ENV+ SPE; however, when this procedure was attempted in our laboratory, NACEC recoveries were much lower than for the optimized procedure described in the current work.

The choice of the internal standard was fairly straight forward; a deuterated analog of NACEC would not be present in a worker exposed to acrylamide.

Table 2. Urine/buffer volume optimization for 200 mg bed isolute ENV + solid-phase extraction cartridge

Urine/buffer volume (mL)	Acrylamide recovery (%)	NACEC recovery (%)
2	92	90
4	46	72
8	8	25

Notes: The urine was fortified at 2 μg/mL with acrylamide and NACEC metabolite; the urine was buffered with an equal volume of 0.4 M ammonium formate, pH 2.6. The recoveries are the mean of three fortified urine samples (n = 3).

Generally, an internal standard compensates for changes in solvent volume; however, the use of the d-NACEC as a procedural standard reduced analysis variation to acceptable levels. Variability caused by differences in extraction can be compensated for by the use of the deuterated analog added to the initial urine sample. During the early stages of this work, other general internal standard compounds were evaluated including the deuterated analog of phenylmercapturic acid, N-acetyl-S-(2-hydroxyethyl)-cysteine, and N-acetyl-S-(3-hydroxypropyl)-cysteine; none extracted or performed as well as d-NACEC. Good calibration curves were obtained using this internal standard in the final optimized condition recovery study, and the precision of the recovery date implies a reproducible extraction of both analytes. Additionally, the use of an internal standard increases the precision of chromatographic injections. Five replicate injections of the 5 μg/mL standard sample gave relative standard deviations (RSD) of the peak area ratios of acrylamide with day-to-day ranges of 2.1 to 7.1%; the RSD of the peak area ratios of NACEC ranged from 2.0 to 6.4% during the final recovery study.

Precision and Accuracy of the Optimized Conditions

A primary recovery study using the optimized procedure and blank urine fortified with acrylamide and NACEC was performed over three separate analytical batch runs to demonstrate the accuracy and precision of the procedure. These data are presented in Table 3; average recovery ranged from 100 to 108% for the three levels of acrylamide investigated and from 99 to 102% for the three levels of the NACEC metabolite investigated. For each analytical run, the experimental trial consisted of three urine samples prepared at three concentration levels. The 2 μg/mL level had the lowest

Table 3. Recovery study from fortified urine samples

Spike level (μg/mL)	Mean recovery (μg/mL, n = 9)	Average recovery (%)	Standard deviation (μg/mL)	RSD (%)
A. Acrylamide				
2	2.00	100	0.08	4.0
10	10.8	108	0.55	5.1
20	21.0	105	0.42	2.1
B. NACEC				
2	1.98	99	0.20	10.0
10	10.1	101	0.32	3.1
20	20.3	102	0.89	4.4

Notes: Three different fortified samples were prepared at three concentration levels and chromatographed during three separate analytical batch runs. % RSD = percent relative standard deviation.

recovery for both the acrylamide and NACEC, 100 and 99%, respectively. The recovery results for both analytes are within the statistical expectations and do not appear to have any bias. The highest relative standard deviation (RSD) was 10% for the 2 μg/mL level NACEC samples, which was considered acceptable for a test procedure to assay a metabolic product in urine. Most of the variation on recovery would be attributed to the extraction step within the sample preparation of this test procedure. A secondary recovery study used urine samples from 20 non-work-exposed volunteers again demonstrating that the procedure was both accurate and precise (See Table 4). The 5 μg/mL fortified samples showed a recovery of 97% for both acrylamide and NACEC. The lowest individual sample recovery was 4.05 μg/mL for acrylamide and 4.26 μg/mL for NACEC. The highest recovery was 5.45 and 5.35 μg/mL for acrylamide and NACEC, respectively. The second recovery study also indicated that differences in individual urine samples did not cause matrix variation, which would significantly affect recovery of the analytes; the RSD for acrylamide recovery was 7.6%, and the RSD for NACEC metabolite was 5.1% (See Table 4). Urine from the twenty volunteers showed no detectable level of acrylamide in any individual urine. Two individuals showed NACEC levels at 0.2 and 0.3 μg/mL which is not unusual, since acrylamide comes from many other sources including processed foods. Exposure in a work environment would likely show higher levels of the metabolite.

Method Reproducibility, Limit of Detection, and Analyte Stability

Two Phenomenex Synergi 4 μ Hydro-RP 80 A columns of different production lots were used during the recovery studies; these results are, therefore, expected to be consistent and reproducible with HPLC columns from this manufacturer. This would indicate robustness for the chromatography used in the chromatographic procedure. The limit of detection (LOD) was found to range from 0.02 to 0.03 μg/mL for acrylamide and 0.1 to 0.2 μg/mL for NACEC

Table 4. Recovery of 5 μg/mL acrylamide and NACEC fortified urine samples from 20 non-exposed volunteers

Analyte	Mean recovery (μg/mL)	Average recovery (%)	Lowest value (μg/mL)	Highest value (μg/mL)	RSD (%)
Acrylamide	4.84	97	4.05	5.46	7.6
NACEC	4.85	97	4.26	5.35	5.1

Notes: Ten volunteer sample solutions were analyzed during one analytical batch run; two analytical runs were used for all twenty volunteer samples. % RSD = percent relative standard deviation.

equivalent levels in urine. Day-to-day variation in the MS detector noise and the differences in the two analytical columns used for this study accounted for this range. Boettcher and Angerer[29] reported a detection limit of 0.045 μg/mL for NACEC using a more sensitive MS-MS detector with HPLC and individual smoker levels of 0.3 μg/mL. This procedure's LOD, therefore, is consistent with the reported literature.[28,29] A seven day stability study was conducted on the final chromatographic sample solution. Acrylamide appeared to be stable for seven days when refrigerated (4°C) in darkness, when at room temperature in darkness, and when at room temperature exposed to light. NACEC appeared to be stable when refrigerated in darkness, but appeared to loose nearly 30% of its initial level after seven days at room temperature in ambient room light, including sunlight from a window. NACEC loss at three days in darkness was minimal [statistically not measurable]; however, the loss was nearly 20% from standing in darkness at room temperature after seven days. As the internal standard is the deuterated analog of NACEC, the use of light protected vials and a cooled autosampler would be prudent for long chromatographic runs, when sample standing time would be more than several days.

Linearity and Other Procedural Considerations

As briefly mentioned in the experimental section, all curves generated in the concentration range of 0.1 to 50 μg/mL for acrylamide and in the range of 0.3 to 50 μg/mL for NACEC were linear. Correlation coefficients were 0.97 or greater for the numerous calibration curves generated during the recovery studies using the optimized conditions of the procedure. Although this method appears to be valid, improved sensitivity by the use of tandem mass spectrometry (MS-MS) would be a possible future goal. Tandem MS would give added specificity of any analysis method, and MS-MS has been commonly used in many analysis methods in the literature for acrylamide metabolites. Also, improvement in this procedure's sensitivity would be likely with MS-MS. This is a topic for future research within this laboratory and is not within the scope of the presented work. Additionally, the recent work by Fennell et al.[25] has suggested that the sulfoxide analog of NACEC is another possible metabolite or degradation product of acrylamide exposure. This compound was not analyzed for during the stability study, but will be the subject of the future investigation. The extraction conditions, as well as basic chromatographic conditions, have been optimized for this study; they have been demonstrated to be valid for the simultaneous analysis of free acrylamide and its metabolite, NACEC, in human urine.

CONCLUSIONS

An accurate and precise extraction and chromatographic procedure to monitor the levels of acrylamide and its primary metabolite NACEC has been

developed and validated. Optimized solid-phase extraction conditions have been developed; both analytes gave the best extraction yield upon standing in urine buffered with ammonium formate at pH 2.6. The quantification of the analytes was completed using a reversed-phase gradient high performance liquid chromatography procedure with detection by mass spectrometry. Average recovery of known acrylamide and NACEC fortified urine samples using the optimized conditions were between 97 and 108%, with relative standard deviations as high as 10%; analyte concentrations of 2, 5, 10, and 20 μg/mL were used. Standard curves generated linear responses in the range of 0.1 to 50 μg/mL for acrylamide and 0.3 to 50 μg/mL for NACEC. The limit of detection was found to be approximately 0.02 μg/mL for acrylamide and 0.2 μg/mL for NACEC. This procedure has been demonstrated to be applicable for the simultaneous quantification of free acrylamide and NACEC in human urine.

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