by Patricia I. Mathias and Kenneth L. Cheever

Evaluation of Surface-Enhanced Laser Desorption Time-of-Flight Mass Spectroscopy in the Development of Biomarkers of Occupational Acrylamide Exposure

Surface-enhanced laser desorption time-of-flight mass spectroscopy (SELDI-TOF-MS) is a bioanalytical technique used for the rapid examination of intact protein or protein mixtures to exploit the biochemical or biophysical characteristics of intact molecules to separate a complex protein mixture or isolate specific protein classes. Surface-enhanced laser desorption time-of-flight mass spectroscopy allows for rapid examination of protein components of body fluids or cell lysates without extensive extraction or preparative measures. The levels and composition of proteins found in blood and urine may change after exposure to toxic agents.[1,2] Such potential changes make the proteins found in these easily obtained fluids a desirable source of new or altered proteins that indicate toxic exposure. This article describes the use of SELDI-TOF to examine urinary proteins or hemoglobin present in erythrocyte lysates.

Acrylamide is a widely used industrial chemical intermediate with many applications,[3] such as a polymerizing agent in grouts and in the preparation of laboratory gels for protein and nucleic acid electrophoresis. Low levels of acrylamide present in baked, fried, and roasted foods and in tobacco smoke are common sources of human exposure.[4] Acrylamide is a potent neurotoxin[5] and is a probable human carcinogen that makes exposure a concern for human health.[6]

Acrylamide and its metabolite glycidamide, also considered a toxicant, are reactive compounds and readily form adducts with biological macromolecules, including proteins.[7,8] Both acrylamide and glycidamide react with hemoglobin (HGB), specifically at the *N*-(2 carbamoylethyl) valine residue of the β-peptide subunit

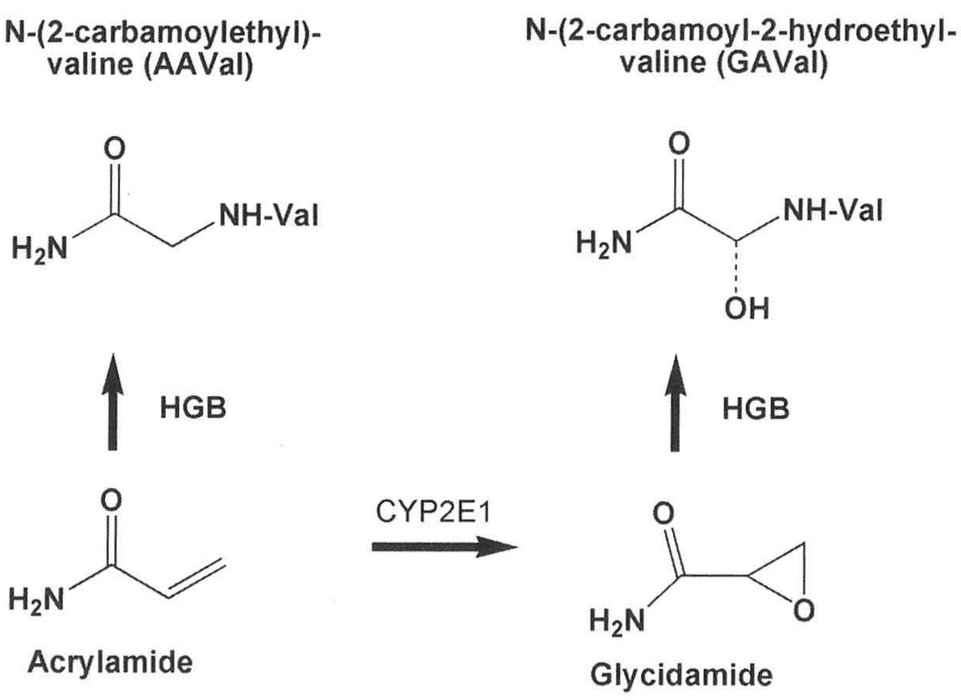

Figure 1 Acrylamide metabolism and hemoglobin adduct formation. Acrylamide is metabolized to glycidamide by a detoxifying enzyme Cytochrome P450 CYP2E1 found in liver, lung, and brain tissue.

(*Figure 1*). These valine adducts are considered a biomarker of long-term acrylamide exposure.[3] Possible reactions of acrylamide and glycidamide with other amino acids suggest that urinary proteins may be affected in acrylamide exposure.[9] One of the goals of this work was to identify proteins in human urine modified by acrylamide exposure using SELDI-TOF-MS and four types of ProteinChip® array (**Bio-Rad Laboratories**, Hercules, CA) to determine which array would be most useful for the planned analysis of urine from occupationally exposed workers. A second goal was to evaluate SELDI-TOF-MS as a low-cost, rapid screening method to demonstrate acrylamide or glycidamide adducted hemoglobin.

Methods

Urinary protein profiling

Control urine specimens were collected from healthy male volunteers working at the National Institute for Occupational Safety and Health (NIOSH) with no smoking history or occupational exposure (n = 16). End-of-work-shift samples were collected at an acrylamide production plant from nonsmoking, occupationally exposed workers (n = 34); smoking, occupationally exposed workers (n = 24); and nonsmoking, nonoccupationally exposed office workers (n = 12). Only nonsmoking office workers were selected as controls. The acrylamide workers study was approved by the NIOSH Human Subjects Review Board.

Samples collected at the production site were frozen and shipped to NIOSH and subjected to one freeze–thaw cycle prior to extraction and analysis. Urine samples were prepared and extracted using previously described methods.[10] Briefly, urine samples were prepared by centrifugation at 16,000 × g for 5 min at 4 °C to sediment cellular material prior to use. Urinary proteins were extracted from a 160-µL aliquot of each sample by mixing with 60 µL denaturing buffer (9 M urea/2% 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate (CHAPS)/50 mM Tris, pH 9.0), vortexed for 30 min at 4 °C, and then centrifuged for 1 min at 5000 × g before analysis of the supernatant in triplicate. For incubations of urine with either acrylamide or glycidamide, freshly collected urine was filtered through a 0.2-µm pore size cellulose acetate filter to remove urinary sediment and bacteria. Aliquots of urine were incubated for 24, 48, 72, and 96 hr in the presence of 10 mM acrylamide or glycidamide in sterile glass screw-cap tubes placed in an oscillating water bath at 37 °C. Samples were extracted as described above and then applied to IMAC30 ProteinChip arrays for SELDI-TOF analysis.

Table 1 ProteinChip® characteristics and preparation protocols

Chip type	NP20	CM10	H50	IMAC30
Surface chemistry	Silicon dioxide	Anionic carboxylate	Methylene C16 chains	Divalent copper
Interaction	Normal phase	Cationic exchange	Hydrophobic	Ionic
Interacting amino acids	Hydrophobic ser, thr, lys	Positively charged tyr, arg, his	Hydrophobic ala, val, phe, tyr	Negatively charged his, trp, cys
Pretreatment			50% acetonitrile (ACN) 50 µL	Copper sulfate 50 µL
Rinse	Deionized water 50 µL	Deionized water 50 µL		Deionized water 50 µL
Neutralize				Sodium acetate 50 µL
Equilibrate	Sodium acetate 2 × 100 µL	Sodium acetate 2 × 100 µL	ACN/TFA (trifluoroacetic acid) 2 × 100 µL	PBS (phosphate-buffered saline) + NaCl 75 µL 2 × 150 µL
Sample binding	Sodium acetate 75 µL Sample 25 µL	Sodium acetate 75 µL Sample 25 µL	ACN/TFA 75 µL Sample 25 µL	PBS + NaCl 75 µL Sample 25 µL
Sample wash	Sodium acetate 2 × 100 µL	Sodium acetate 2 × 100 µL	ACN/TFA 2 × 100 µL	PBS + NaCl 3 × 150 µL
Final wash	2× Deionized water	2× Deionized water	2× Deionized water	2× Deionized water

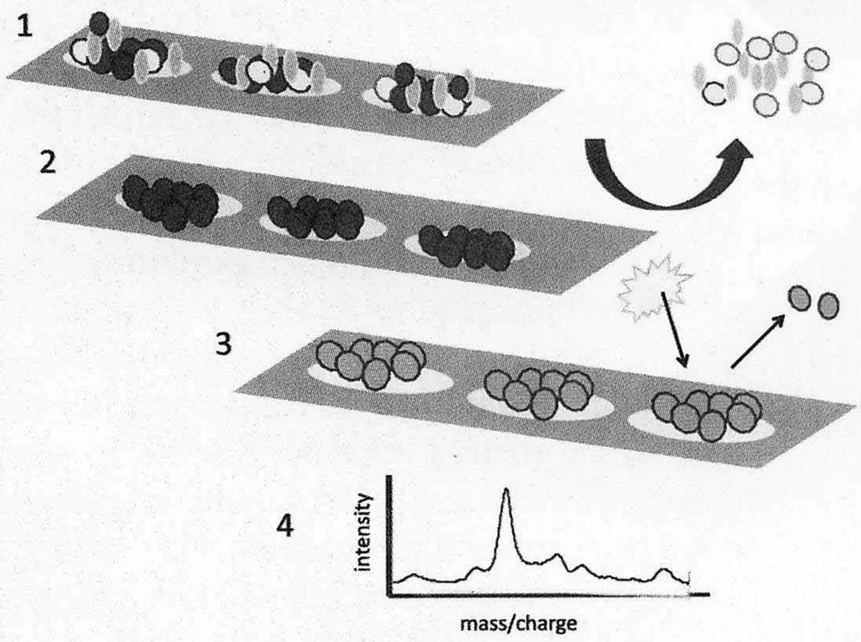

Figure 2 Process flow in SELDI-TOF-MS. 1) A complex protein mixture is applied to a ProteinChip. 2) Nonspecifically bound proteins are washed away. 3) Energy-absorbing matrix is applied to all samples, and a laser beam desorbs and ionizes the protein crystallized in the matrix. 4) Ions are captured and the mass of each protein is calculated by TOF-MS.

SELDI-TOF analysis of urinary proteins

Urinary proteins were processed on NP20, CM10, H50, and IMAC30 chip arrays (**Bio-Rad**) having normal-phase, cationic-exchange, hydrophobic, and ionic surface chemistries, as described in *Table 1*. Various factors were optimized during the development of binding protocols for each chip, including chip surface pretreatment, sample binding time, and washing methods (see Table 1). *Figure 2* illustrates sample preparation and process flow. ProteinChip arrays were analyzed by SELDI-TOF-MS with a Ciphergen PBS II Reader (**Bio-Rad**) using the following conditions in positive mode: laser intensity, 150; detector sensitivity, 9; detector voltage, 1700 V; spectra average, 240/sample; optimization range, 500–12,500 Da for urinary protein detection; calibration, external mass accuracy using a ProteinChip QC Peptide Array, MW range 7–147 kDa. Mass spectra baseline correction, normalization, peak detection, and alignment were performed using either Ciphergen ProteinChip software (version 3.0.2) or R language (version 2.0.1)[11] functions implemented in the PROcess library of the BioConductor Project (version 1.5).[12] The number of peaks and mean peak intensity varied significantly with chip type, where the number and intensity of peaks was greatest using IMAC30 > CM10 > NP20 > H50.

Erythrocyte incubation with acrylamide or glycidamide

Whole human blood specimens were collected by venipuncture into ethylenediaminetetraacetic acid (EDTA) containing Vacutainers (**Becton Dickinson**, Franklin Lakes, NJ). Blood was separated into erythrocytes and plasma by centrifugation. Erythrocytes were isolated and washed three times with physiological saline solution. To examine native hemoglobin, washed erythrocytes were lysed by adding one volume of distilled water, and the lysate was immediately processed for SELDI-TOF analysis. To produce adducted hemoglobin, washed erythrocytes were suspended in an equal volume of RPMI 1640 medium base with 1% penicillin-streptomycin solution with or without 10 mM acrylamide or glycidamide. Erythrocyte cultures were incubated at 37 °C in a 5% CO_2 incubator. Cultures were sampled at 24, 48, 72, and 96 hr incubation.

Cultured erythrocytes were washed with saline and lysed as described above. After centrifugation to remove cell membranes and cellular debris, hemoglobin samples were used immediately in SELDI-TOF analysis. Hemoglobin samples were processed on H50 ProteinChip arrays with a Ciphergen PBS II ProteinChip reader using instrument conditions similar to those described above in urinary protein analyses. SELDI-TOF analysis was performed as described above for urinary proteins using a 14–17 kDa optimization range for globin adduct detection.

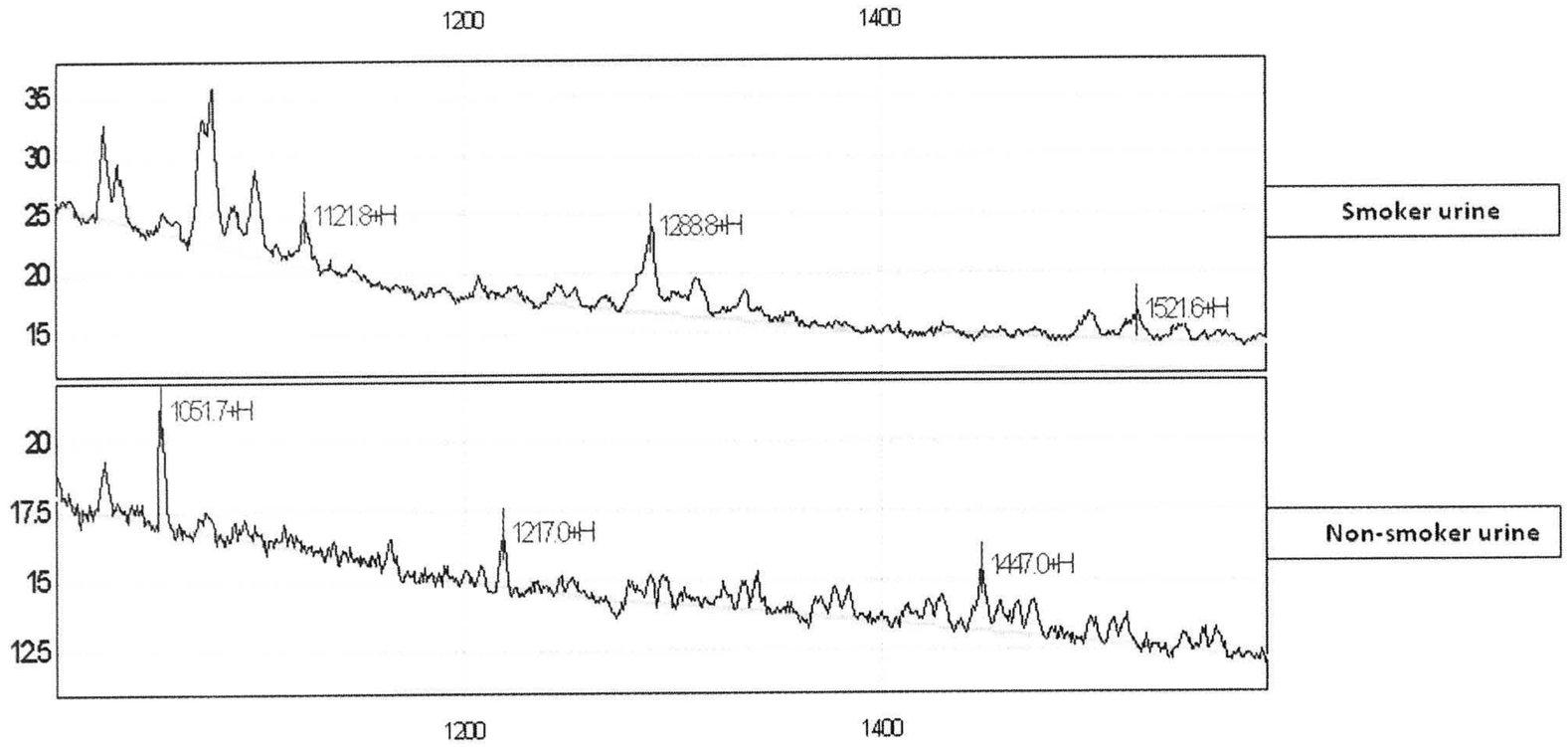

Figure 3 *Comparison of urinary proteins from a smoker and from a nonsmoker. The upper panel is a spectrum of urinary proteins from a smoker having no occupational exposure to acrylamide, and the lower spectrum of proteins is from a nonsmoker having no occupational exposure. Identified peaks in the upper panel are among 21 peaks identified in the urine of a nonoccupationally exposed nonsmoker after incubation with either acrylamide or glycidamide.*

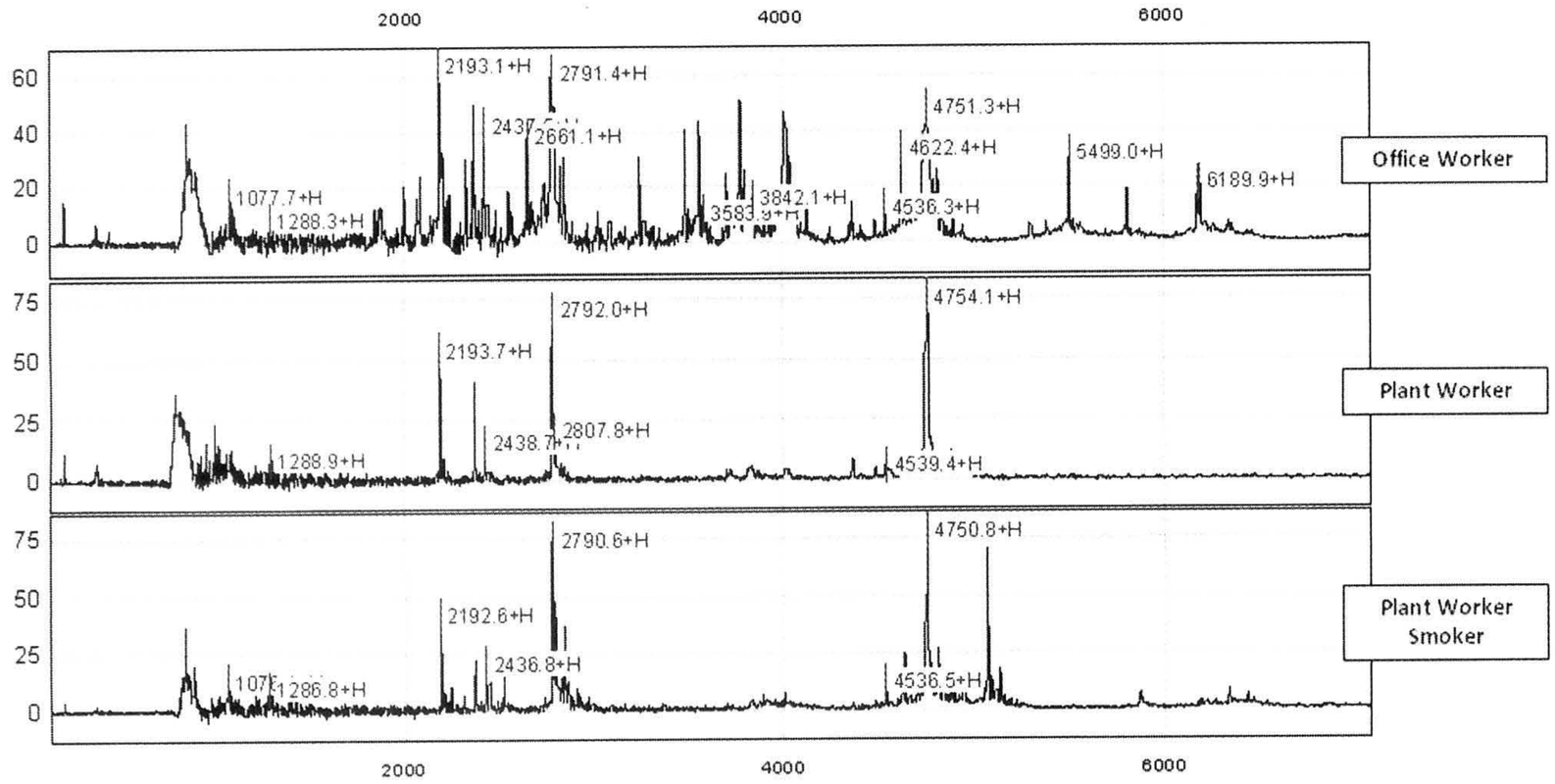

Figure 4 *Comparison of urinary protein profiles. The upper spectrum is a partial profile of proteins found in the urine of a nonexposed office worker, while the middle spectrum is a similar partial profile from the urine of a nonsmoking occupationally exposed plant worker. The lower panel is a profile from an occupationally exposed smoker. The identified peaks are among those resulting from in vitro incubation of urine of a nonoccupationally exposed nonsmoker with acrylamide or glycidamide.*

Results and discussion

Initial SELDI-TOF analyses of human urine showed that urine specimens collected from healthy male volunteers working at NIOSH with no smoking history or occupational exposure produce complex and highly variable SELDI spectra. The variations observed in these spectra, possibly due to individual dietary habits including coffee and fried food consumption, complicated the interpretation of spectra in a search for an indicator of acrylamide exposure for use in evaluating occupational exposure. Subsequent SELDI-TOF analysis of urine collected from nonoccupationally exposed, nonsmoking office workers; occupationally exposed acrylamide production plant workers; and occupationally exposed smokers also yielded equally complex and variable spectra.

Exposure to acrylamide dust in production plants may occur through intact skin or by breathing the dust produced by handling and packaging of product pellets or dust formed by sublimation of the solid into dust vapor. Because acrylamide may be metabolized into glycidamide in lung tissue, it was necessary to investigate the possible contribution of smoking to the alteration of urinary proteins. To assist in identifying urinary proteins altered by acrylamide exposure via tobacco smoke, urine collected from a healthy nonsmoking control volunteer with no occupational exposure was examined and compared to replicate urine incubated with acrylamide or glycidamide. In the same manner, replicate urine samples from a nonoccupationally exposed smoker were incubated with acrylamide or glycidamide.

Incubation of control urine from a healthy nonsmoker with either 10 mM acrylamide or glycidamide produced a profile of 21 proteins that were not detected in freshly collected or incubated control urine. The approximate molecular weight (m/z) of three proteins corresponded with the approximate m/z of three protein peaks identified in freshly collected and incubated control urine plus the weight of acrylamide and glycidamide, 71.08 and 78.08, respectively. The three control protein/adducted protein pairs have approximate m/z values of 1051:1121, 1217:1288, and 1447:1520. Partial spectra presented in *Figure 3* provide a comparison illustrating the relationship between unaltered protein peaks found in control urine and those found in the freshly collected urine of a smoker, establishing that proteins normally present in urine can be altered by exposure to acrylamide found in cigarette smoke.

Figure 4 is a comparison of partial urinary protein profiles from a nonoccupationally exposed, nonsmoking office worker; a nonsmoking, occupationally exposed acrylamide production plant worker; and an occupationally exposed smoker. The identified peaks in all three profiles are among those 21 proteins identified by acrylamide or glycidamide incubation of control urine, or those found in the freshly collected urine from a nonoccupationally exposed smoker. Most of these acrylamide incubation-associated peaks are found between 800 and 6000 m/z in the urinary spectra examined in this work. Protein profiles from control volunteers; nonexposed office workers; and both smoking and nonsmoking, occupationally exposed plant workers contained a complex and variable mixture of protein peaks, showing peaks identified in freshly extracted control urine appearing with others identified in urine from a nonoccupationally exposed smoker. Inspection

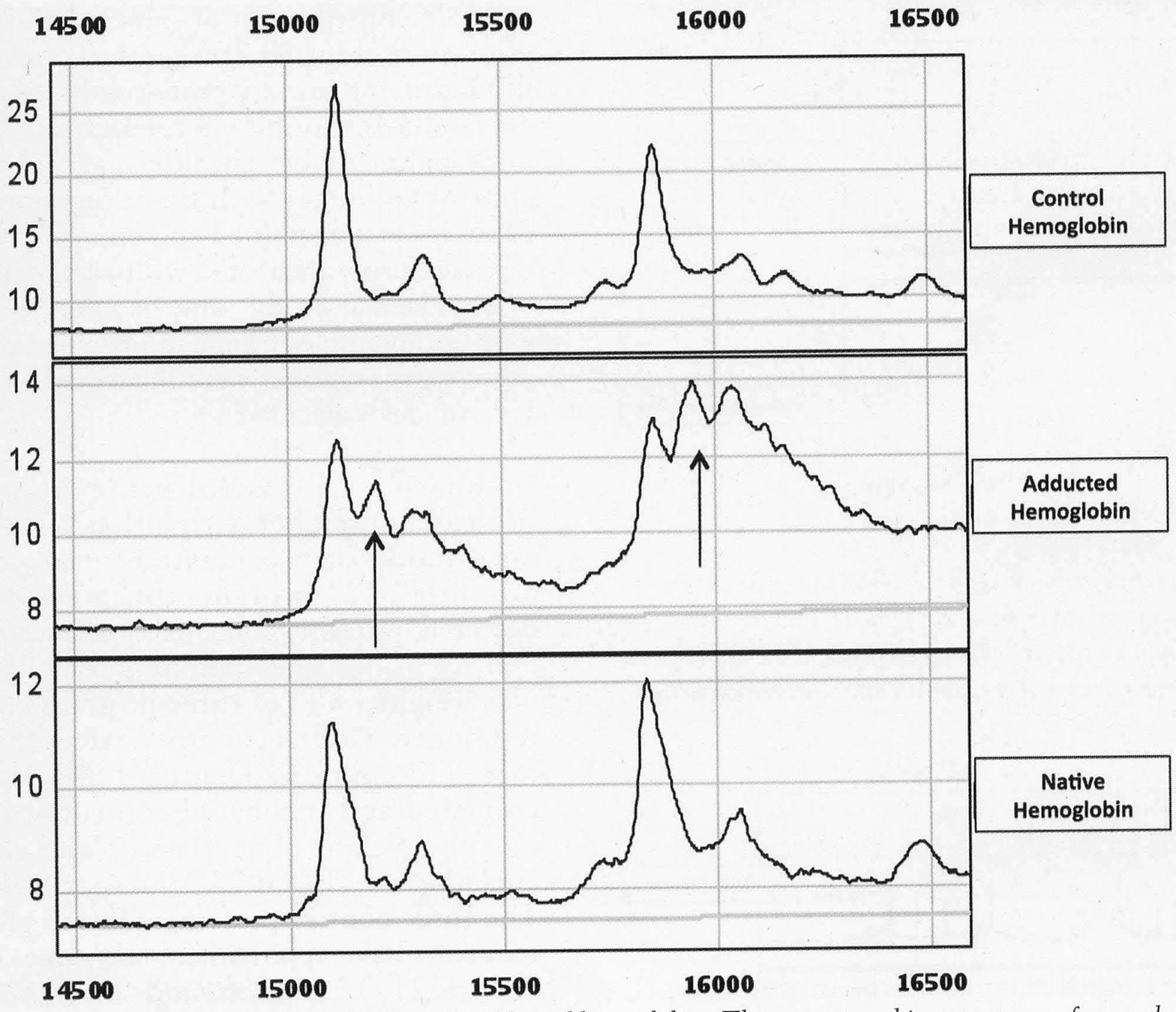

Figure 5 Spectra of in vitro glycidamide adducted hemoglobin. The upper panel is a spectrum of control hemoglobin from intact erythrocytes incubated for 72 hr. The middle spectrum is that of hemoglobin from intact erythrocytes incubated 72 hr in the presence of 10 mM glycidamide. Two arrows indicate the location of presumptive glycidamide adducts having approximate molecular weights of 15195 and 15938, respectively. The lower panel is a spectrum of hemoglobin isolated from erythrocytes removed from the control culture at the beginning of incubation, indicating the native α- and β-globin subunits with approximate molecular weights of 15115 and 15855, respectively.

of all spectra examined in this work for the presence of control protein/adducted protein pairs 1051:1121, 1217:1288, and 1447:1520 revealed peaks at the upper *m/z* values either absent or present as minor peaks having intensity values less than three times the baseline intensity.

Interestingly, the most complex and variable spectra were produced by urine from control male volunteers and unexposed, nonsmoking office workers, including proteins having the same *m/z* values as those 21 peaks identified by acrylamide/glycidamide incubation, suggesting that dietary exposure is a confounding variable in this work. In typical proteomic analyses, proteins of interest are identified by subtracting peaks found in control specimens from those appearing in spectra obtained from treatment or investigational specimens. In this study, peaks found in the urine collected from unexposed, nonsmoking office workers were subtracted from the peaks found in urine collected from nonsmoking, exposed acrylamide production plant workers. This technique proved uninformative. Protein peaks identified either by incubation of control urine with acrylamide or glycidamide or those identified in the urine of a nonoccupationally exposed smoker were found in the majority of the investigational urine samples examined. The abundance of these peaks in both control office workers and acrylamide production workers indicates that both smoking and dietary exposure are confounders in identifying a urinary protein or a protein profile characteristic of occupational acrylamide exposure using SELDI-TOF-MS.

A second goal of these studies was to evaluate the suitability of SELDI-TOF-MS to demonstrate acrylamide or glycidamide adducted hemoglobin as a low-cost, rapid screening alternative to the currently used Edman degradation-based determination. To examine the utility of SELDI-TOF-MS to detect acrylamide or glycidamide adducted blood protein, hemoglobin from erythrocytes collected from nonsmoking, nonoccupationally exposed volunteers was compared with replicate hemoglobin samples from erythrocytes incubated with acrylamide or glycidamide. Similar comparisons were performed examining hemoglobin from the erythrocytes of a nonoccupationally exposed smoker after incubation with acrylamide or glycidamide.

Erythrocytes incubation demonstrated adduct formation (*Figure* 5) at *m/z* values 15195 and 15938 with an increase in molecular weight of approximately 80 and 83, respectively (glycidamide MW = 78.08; Figure 5, upper panel). This represents the α- and β-globin adducted subunits. Spectra obtained using control conditions (Figure 5, upper and lower panels) demonstrated no interferences or artifacts from incubation conditions. In some replicate incubations, hemoglobin adducts were not detected or were unresolved. Sub-

sequent studies using erythrocytes from a smoker incubated with acrylamide or glycidamide produced either unresolved broadened peaks or broadened peaks with unresolved shoulders.

Additional efforts were made to demonstrate adducted hemoglobin by extracting globin protein from incubated erythrocyte lysates using accepted methods before applying protein to the chip array, and by performing a second extraction of isolated globin using urea-based denaturants before analysis. After these additional measures failed to demonstrate hemoglobin adduction in the blood of a smoker, it was concluded that the levels of acrylamide and glycidamide hemoglobin adducts formed in vivo by physiologic levels of acrylamide and glycidamide found in a smoker, demonstrable by Edman degradation based methods, are too low to be demonstrated by SELDI-TOF-MS in the conditions of this study.

Conclusion

The SELDI-TOF proteomic method used in this study to identify modified proteins in urine demonstrated 21 proteins associated with incubation of urine with acrylamide or glycidamide. These same peaks were also found in the urine of acrylamide production plant workers. However, the presence of these peaks in the spectra of nonoccupationally exposed individuals and smokers excludes use of these proteins/peaks as indicators of occupational exposure or in construction of a SELDI-TOF protein profile indicative of occupational acrylamide exposure. It is possible that the quantity of one or more of these proteins may vary with the level of exposure to acrylamide. However, SELDI-TOF-MS is a qualitative tool and cannot be used to investigate this possibility.

Similarly, SELDI-TOF-MS under the conditions of this study lacks the resolving power to detect the physiologic levels of hemoglobin adducts formed in a nonoccupationally exposed smoker. It was concluded that SELDI-TOF cannot, without some modification to select or concentrate adducted hemoglobin from the volume of whole blood typically collected in exposure monitoring methods, provide an alternative to the currently used Edman degradation-based method.

References

1. Sun, W.; Li, F. et al. Human urine proteome analysis by three separation approaches. *Proteomics* **2005**, *5*(18), 4994–5001.
2. Schaub, S.; Wilkins, J. et al. Urine protein profiling with surface-enhanced laser-desorption/ionization time-of-flight mass spectrometry. *Kidney Int.* **2004**, *65*, 323–32.
3. Agency for Toxic Substances and Disease Registry. Toxicological Profile for Acrylamide. Division of Environmental Toxicology and Environmental Medicine. U.S. Department of Health and Human Services, U.S. Public Health Service, Atlanta, Georgia, 2009.
4. Dybing, E.; Sanner, T. Risk assessment of acrylamide in foods. *Toxicol. Sci.* **2003**, *75*, 7–15.
5. LoPachin, R.M. The changing view of acrylamide neurotoxicity. *Neurotoxicology* **2004**, *25*(4), 617–30.
6. Tornqvist, M. Acrylamide in Food: The Discovery and Its Implications. In: *Chemistry and Safety of Acrylamide in Food. Advances in Experimental Medicine and Biology Series*; Friedman, M.; Mottram, D., Eds.; Springer: New York, NY, 2005; Vol. 56; pp 1–20.
7. Fennel, T.R.; Sumner, S.C.J. et al. Metabolism and adduct formation in humans. *Toxicol. Sci.* **2005**, *85*, 447–59.
8. Calelman, C.-J.; Bergmark, E. et al. Acrylamide is metabolized to glycidamide in the rat: evidence from hemoglobin adduct formation. *Chem. Res. Toxicol.* **1990**, *3*, 406–12.
9. Friedman, M. Chemistry, biochemistry, and safety of acrylamide: a review. *J. Agric. Food Chem.* **2003**, *51*(16), 4505–26.
10. Ciphergen ProteinChip® System Users Guide. Ciphergen Biosystems, Inc.: Fremont, CA, 2000.
11. Gentleman, R.; Carey, V.J. et al. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.* **2004**, *5*(10), R80.
12. R Development Core Team. 2005. R: A language and environment for statistical computing. R Foundation for Statistical Computing: Vienna, Austria, ISBN 3-900051-07-0, 2004.

The authors are with the U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health, Division of Applied Science and Technology, Biomonitoring and Health Assessment Branch, Robert A. Taft Laboratories, 4676 Columbia Parkway, Cincinnati, OH 45226, U.S.A.; tel.: 513-533-8216; e-mail: pmathias@cdc.gov.

The authors wish to thank Clayton B'Hymer, Gary P. Carlson, John Lipscomb, and Dennis W. Lynch for their assistance as reviewers in the preparation of this manuscript.

Disclaimers: The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health (NIOSH) or the Centers for Disease Control and Prevention (CDC). Mention of company names and/or products does not constitute an endorsement by NIOSH.