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Cotinine Concentration in Serum Correlates with Tobacco Smoke-Induced Emphysema in Mice

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Secondhand smoke (SHS) has been associated with a variety of adverse health outcomes in nonsmokers, including emphysema (a chronic obstructive pulmonary disease). One way to detect SHS exposure is to measure the concentration of cotinine, the primary metabolite of nicotine, in bodily fluids. We have developed a method for cotinine analysis by combining micellar electrokinetic chromatography with enrichment techniques. We employed the method to measure cotinine concentrations in serum samples of mice exposed to tobacco smoke for 12 or 24 weeks and found that it was 3.1-fold or 4.8-fold higher than those exposed to room air for the same period. Further, we investigated the morphological changes in lungs of mice and observed tobacco smoke induced emphysema. Our results indicate that the method can be used to measure cotinine and there is an association between the serum cotinine concentration and tobacco smoke-induced emphysema in mice.

Emphysema is a type of chronic obstructive pulmonary disease (COPD) and it is pathologically defined as an abnormal dilation of air spaces distal to the terminal bronchiole, accompanied by the destruction of alveolar walls¹. Tobacco smoking is the leading cause of COPD, and “more than 1,000 people are killed every day by cigarettes” in the United States². In addition to smokers, many nonsmokers suffer from respiratory infections because of involuntary exposure to secondhand smoke (SHS)². The relationship between COPD/emphysema and SHS is often studied using animal model to elucidate pathogenic mechanisms³.

SHS, also known as environmental tobacco smoke (ETS), is a mixture of side stream smoke from cigarettes and the smoke exhaled by smokers. It is designated as a known human carcinogen by the U.S. Environmental Protection Agency (EPA) and the World Health Organization (WHO)⁴. According to U.S. Surgeon General, “children exposed to secondhand smoke are at an increased risk for sudden infant death syndrome (SIDS), acute respiratory infections, ear problems, and more severe asthma;” and “exposure of adults to secondhand smoke has immediate adverse effects on the cardiovascular system and causes coronary heart disease and lung cancer”⁴. Therefore, monitoring the SHS exposure of nonsmokers including children could be of critical importance for both establishing diagnosis of pulmonary diseases and taking precautionary measures.

The methods to monitor SHS exposure are mainly relying on the analysis of poisonous gases or chemical compounds related to tobacco smoke, such as carbon monoxide and nicotine derived chemicals^{5–8}. A number of potential biomarkers has been compiled that are associated with four tobacco-related health outcomes: cancer, nonmalignant pulmonary disease, cardiovascular disease, and fetal toxicity⁹. Many SHS studies utilize cotinine, the primary metabolite of nicotine, as the biomarker of choice for assessing smoke exposure because of its specificity and longer half-life than nicotine^{7,10–17}. In general, the cotinine level in nonsmokers is at around the low ng/mL in blood. For example, Machacek and Jiang reported the mean concentration of cotinine in plasma of 31 passively exposed nonsmokers to be 2.1 ng/mL¹¹. Similarly, Baltar et al. reported the serum cotinine level to be about 1.1 ng/mL for never-smokers with passive exposure and 1.7 ng/mL for former-smokers with passive exposure¹². Therefore, the low concentration of cotinine in bodily fluids associated with SHS exposure requires the utilization/development of sensitive methods, a representative of which is high-performance liquid chromatography (HPLC) with a low detection limit (<1 ng/mL). On the other hand, low-cost and fast-analysis

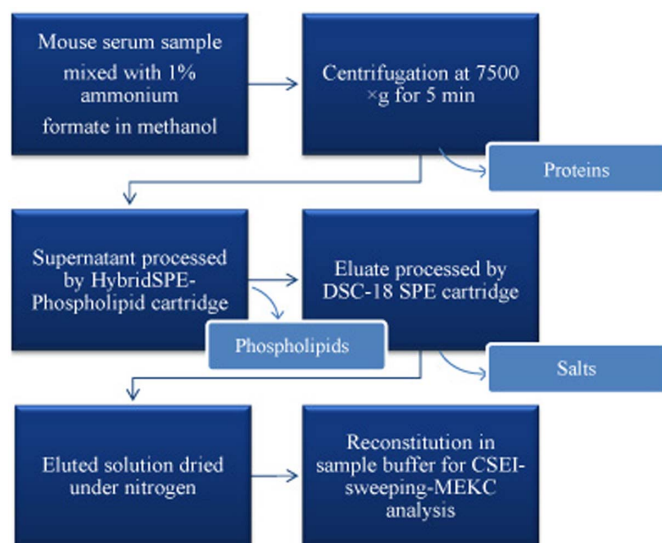


Figure 1 | Flowchart for mouse serum sample preparation before cotinine analysis using CSEI-sweeping-MEKC.

methods are desirable, which could minimize issues associated with the HPLC methods such as large reagent consumption and extensive method development.

Recently, we developed a capillary electrophoresis (CE) technique for the cotinine measurement, which is based on combining cation-selective exhaustive injection (CSEI) with micellar electrokinetic chromatography (MEKC)¹⁸. The CE method offers advantages such as smaller sample/reagent consumption and faster analysis time than HPLC. CSEI is a concentration technique that enables to sweep a long plug of sample injected and enriches them into a short band before MEKC separation¹⁹. Using the optimized parameters, CSEI-sweeping-MEKC provides enhancement of sensitivity by about 5000-fold compared to the one without the concentration step¹⁸. The enrichment reproducibility has been demonstrated and parameter optimization has been carried out¹⁸. The limit of detection for cotinine was found to be 0.2 ng/mL, which is comparable to the

HPLC methods. In addition, we demonstrated multiplexed biomarker detection by simultaneous detection of both cotinine and 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), another biomarker for exposure to SHS²⁰.

In this work we applied this CE method to determine the cotinine level in serum samples of mice exposed to room air and tobacco smoke. We found that the serum cotinine level increased in the SHS groups compared to control groups regardless of smoke exposure periods (12 or 24 weeks). More importantly, the cotinine concentration in serum samples was found to link with tobacco smoke-induced emphysema in mice.

Results

Cotinine analysis. For SHS studies, cotinine has been analyzed in a variety of biological matrices including blood serum/plasma, urine and saliva^{10–17}. Among these matrices, blood serum/plasma has been considered a reliable specimen of choice to measure the cotinine level because it could remain at near steady-state condition over regular smoke exposure⁷. However, the blood serum is a complex sample matrix that contains hundreds of components such as proteins, phospholipids and electrolytes. Both proteins and phospholipids would generate large interfering signals with the UV absorbance detector used in this study. To address the complexity of blood matrix for cotinine analysis, we employed precipitation and two commercially available solid phase extraction (SPE) cartridges to purify mouse serum samples prior to CE analysis. As shown in Figure 1, a protein precipitation procedure was first carried out to remove the abundant protein components in the serum. The collected supernatant was then processed by a HybridSPE cartridge to eliminate the serum phospholipids. Finally, we used a C-18 SPE cartridge to remove electrolytes from the eluate, which would be important for the subsequent electrokinetic injection for sample stacking during CE analysis.

The processed samples were analyzed using the CSEI-sweeping-MEKC method described in the Methods section. The detailed experimental conditions and parameter optimization have been previously reported¹⁸. Figure 2 shows the electropherograms of serum samples of mice exposed to tobacco smoke and room air. Multiple CE peaks were found for the mouse serum samples, suggesting that several substances from the serum matrix still remain

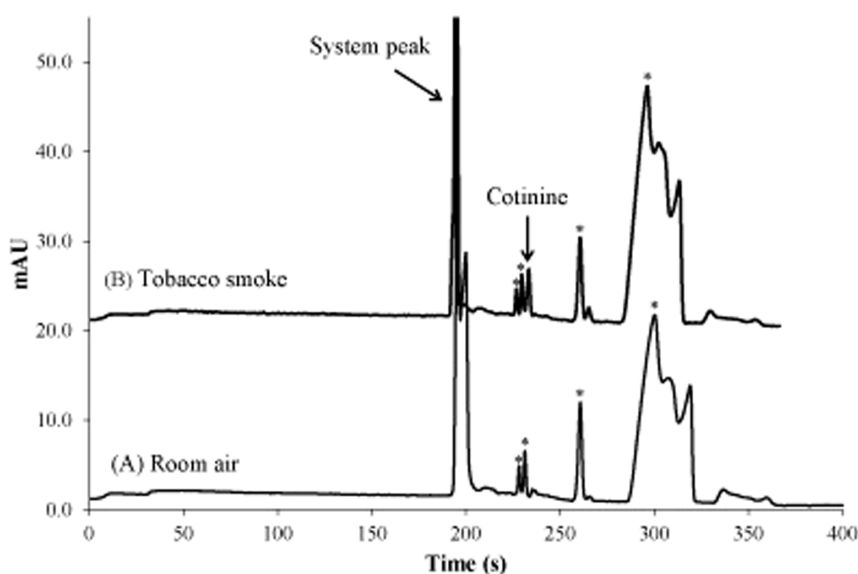


Figure 2 | CSEI-sweeping-MEKC analysis of blood serum samples of mouse exposed to (A) room air and (B) tobacco smoke. The cotinine peak is indicated, so are the system peak and other unknown substances from the serum matrix labeled with *. The experimental conditions were as follows. Micellar BGE: 50 mM phosphoric acid/75 mM SDS with 30% MeOH (pH 2.5); non-micellar BGE: 50 mM phosphoric buffer (pH 2.5); HCB: 75 mM phosphoric buffer (pH 2.5), 8 mm; water plug: 1 mm; electrokinetic injection: 10 kV for 180 s; and MEKC separation: –12 kV.



after the SPE treatment. These species, however, would not cause negative effect on cotinine measurement provided that complete separation of cotinine from these interferences is obtained with a good resolution as in Figure 2. The system peak in Figure 2 was observed in all electropherograms even when a water or buffer sample was used as previously reported¹⁸. The identification of the cotinine peak in the serum samples exposed to tobacco smoke was confirmed by spiking known amount of cotinine into a sample and observing higher peak height¹⁸.

We also observed a very small peak of cotinine for the serum sample of mice exposed to room air, and it represents the baseline cotinine level for non-SHS-exposure. This tiny peak was not observed in the blank samples¹⁸. The results in Figure 2 demonstrate the capability of the CSEI-sweeping-MEKC method for cotinine analysis in mouse serum samples associated with SHS.

Using CSEI-sweeping-MEKC, a calibration curve for cotinine was established based on the peak areas over a range of concentrations (Figure 3). Cotinine standards in a buffer were used, though a blood serum spiked with 10 ng/mL of cotinine was analyzed by the CE method and the result confirmed the validity of the established standard curve. Separation of cotinine from other components essentially eliminated the matrix effect. The relative standard deviation (RSD) for the measured signal of the 10 ng/mL of cotinine spiked in a blood serum was 10% after the SPE method and CE analysis. The results in Figure 3 present a linear range of 1–100 ng/mL for cotinine and a good linearity with correlation coefficient (R^2) of 0.998.

Mouse serum with SHS exposure. As a tobacco-specific chemical, nicotine exists in both mainstream and sidestream smokes. Seventy-five percent or more of the nicotine emitted from a cigarette is released into the air as sidestream smoke, thus contributing significantly to SHS⁷. After nicotine in the smoke is inhaled by nonsmokers, it would enter the bloodstream and reach the liver, where a majority of nicotine is metabolized to cotinine. As a result, the blood cotinine level could be indicative of exposure to tobacco smoke.

We investigated the effect of tobacco smoke on the mouse serum cotinine level under various conditions. Mice were exposed to room air or tobacco smoke for 1 h/day, 5 days/week for 12 or 24 weeks. The concentration of cotinine was calculated from the peak area in electropherograms by comparing it with the calibration curve we obtained (Figure 3). As shown in Figure 4, the serum cotinine level

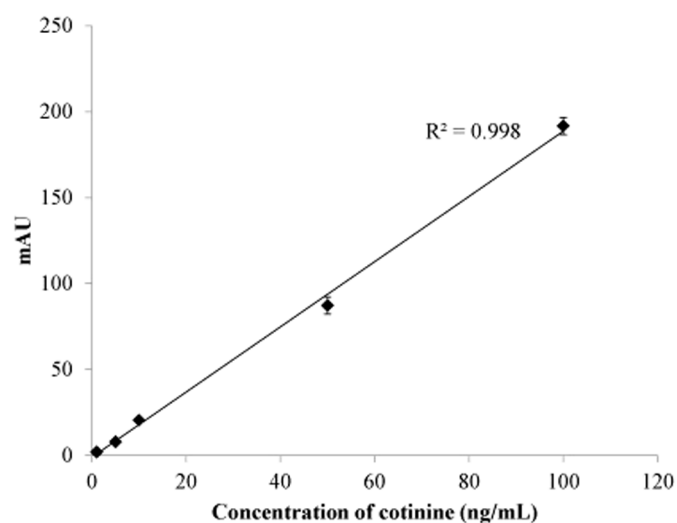


Figure 3 | Standard calibration curve for measurement of cotinine concentration. Each data point is an average of three experiments and the error bars represent one standard deviation.

in mice exposed to tobacco smoke for 12 weeks is 3.1-fold higher than those exposed to room air for the same period, suggesting that tobacco smoke exposure for 12 weeks caused greater nicotine inhalation and blood cotinine retention. Furthermore, a larger increase in the serum cotinine level was found in mice exposed to tobacco smoke for 24 weeks, which is 4.8-fold higher than those exposed to room air for the same period. These results are in accordance with previous reports in which the cotinine concentration in the serum/plasma samples of passive smokers (i.e., exposed to SHS) was in the level of less than 10 ng/mL and about 2 to 6-fold larger than those with no SHS exposure¹². These findings suggest that mice exposed to tobacco smoke provide a valuable animal model for SHS studies.

Emphysema in SHS-exposed lungs. With an intention to correlate the serum cotinine level with emphysema resulted from tobacco smoke, we investigated the morphological changes in lungs of mice exposed to SHS. Figure 5A shows representative images of lung sections of mice exposed to room air or tobacco smoke for 12 and 24 weeks. The clear morphological changes from the control mice to SHS-exposed-mice indicate alveolar enlargement, suggesting that tobacco smoke induced emphysema. The difference in the images between 12- and 24-week SHS exposure suggest that more dilation took place in lungs of mice exposed to tobacco smoke for 24 weeks.

These images were quantified as described in the Methods section. As shown in Figure 5B, the mean linear intercept in the lungs of mice exposed to smoke for 12 weeks is much larger than those exposed to room air. Similarly, there is a significant increase in the mean linear intercept from the control mice to those with 24 weeks of SHS exposure.

Discussion

We demonstrated in Figure 2 that the cotinine concentration in serum samples could be detected by using a sensitive capillary electrophoresis method, in which micellar electrokinetic chromatography (MEKC) was combined with on-line concentration techniques, cation-selective exhaustive injection (CSEI) and sweeping. In addition to sensitivity, it should be noted that the separation time in Figure 2 is about 5 min, which is much shorter than analysis time of HPLC (typically in ~30 minutes). HPLC separation time has been shortened to a few minutes in some work in the literature, but CE separation was demonstrated in seconds²¹. Rapid analysis could be advantageous, so is higher separation efficiency offered by CE.

We employed the CE method to study the effects of tobacco smoke by measuring the cotinine concentration in serum samples of mice exposed to room air or tobacco smoke for 1 h/day, 5 days/week for 12 or 24 weeks. As shown in Figure 4, the serum cotinine level in mice exposed to tobacco smoke for 12 weeks is 3.1-fold higher than those exposed to room air for the same period, and the serum cotinine level

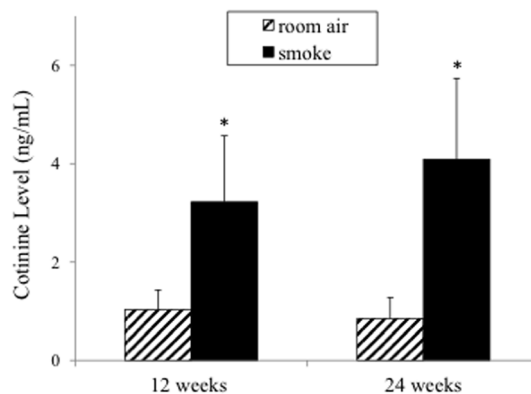


Figure 4 | Serum cotinine concentrations in mice exposed to room air and tobacco smoke for 12 or 24 weeks. Results are expressed as mean \pm SE ($n = 3-6$). * indicates $p < 0.05$ versus room air groups.

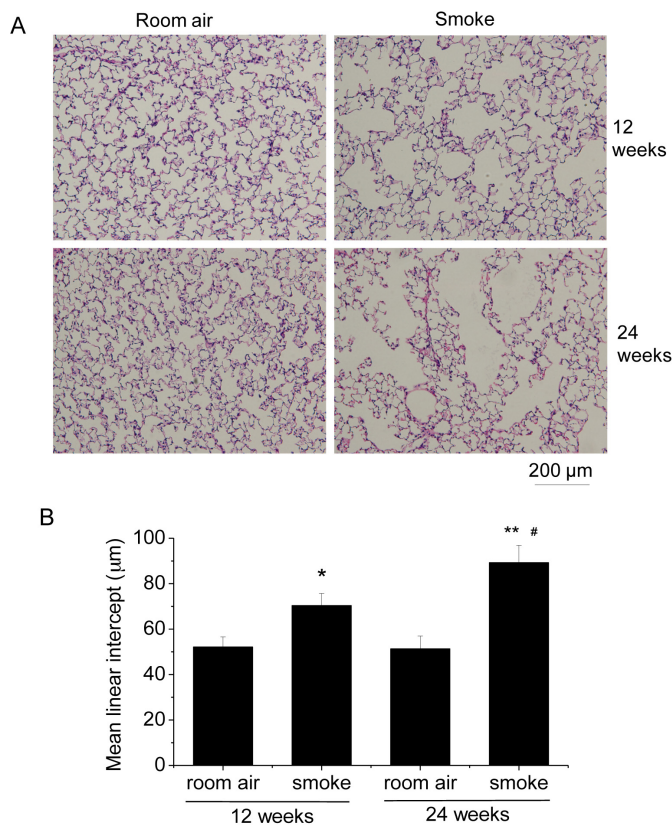


Figure 5 | Tobacco smoke-induced emphysema in lungs of mice. (A) Representative images of lung sections of mice exposed to room air or tobacco smoke for 12 and 24 weeks. (B) Comparison in the mean linear intercept in the lung sections between mice exposed to room air and mice exposed to tobacco smoke for 12 and 24 weeks. Results are expressed as mean \pm SE ($n = 6$ experiments). * $P < 0.05$ vs. room air group for 12 weeks; ** $P < 0.05$ vs. room air group for 24 weeks. # $P < 0.05$ vs. smoke group for 12 weeks.

in mice exposed to tobacco smoke for 24 weeks is 4.8-fold higher than those exposed to room air for the same period.

It should be noted that there is no statistically significant difference in the serum cotinine level between mice exposed to tobacco smoke for 12 and those for 24 weeks, although the average cotinine concentration for mice with 24 weeks of exposure is higher than those with 12 weeks of exposure. This result can be partially explained by the fact that the half-life of cotinine is ~ 17 hours in human and ~ 1 hour in mice^{7,22}. In other words, the cotinine level in the blood circulation falls throughout the day after an intermittent exposure. These results suggest (1) any delay in sampling blood from mice after SHS exposure should be minimized as much as possible and the delay time should be about the same among different samples for the data precision; and (2) the serum cotinine level does signify the accumulated SHS exposures, but it will not increase linearly with the exposure time for long-term inhalation of tobacco smoke due to continuous metabolism of cotinine.

Further, we investigated the morphological changes in lungs of mice and found that the mean linear intercept in the lungs of mice exposed to smoke for 12 weeks is much larger than those exposed to room air, suggesting that tobacco smoke induced emphysema (Figure 5). The increase in the mean linear intercept in mouse lungs exposed to tobacco smoke for 24 weeks was higher than those for 12 weeks, indicating that longer exposure to SHS resulted in worse lung damage.

It should be noted that there is statistically significant difference in the mean linear intercepts between mice exposed to smoke for 12

weeks and those for 24 weeks. This is in contrast to the cotinine results above, in which there is no statistically significant difference in the serum cotinine level between mice exposed to tobacco smoke for 12 and those for 24 weeks. Cotinine went through further breakdown through metabolism during the intermission between SHS exposure (~ 1 hour half-life of cotinine in mice). However, the damaged alveolar did not have sufficient time to recover or repair before tobacco smoke exposure in the following day. Therefore, a higher mean linear intercept in mouse lungs exposed to tobacco smoke for 24 weeks than those for 12 weeks indicates that longer exposure to SHS resulted in worse lung damage, even though the serum cotinine level showed only marginally accumulated effects of chronic SHS exposures.

Methods

Reagents. Ammonium formate, methanol, phosphoric acid, sodium dodecyl sulfate (SDS) and sodium hydroxide were obtained from Fisher Scientific (Fair Lawn, NJ). Cotinine was purchased from Alfa Aesar (Ward Hill, MA) and a stock solution (1 mg/mL) was prepared in methanol and stored at -20°C . The micellar background electrolyte (BGE) contained 50 mM phosphoric acid and 75 mM SDS (pH 2.5). The non-micellar BGE was 50 mM phosphoric buffer (pH 2.5). The high-conductivity buffer (HCB) was 75 mM phosphoric buffer (pH 2.5). The sample matrix solution for CE analysis was 0.1 mM phosphoric acid. Purified water from a Millipore Nanopure water system (Barnstead, Dubuque, IA) was used to prepare all solutions.

Mice and SHS exposure. The procedures for exposing mice to SHS have been reported previously²³. Briefly, male C57BL/6 mice with ages of 8 weeks were purchased from the Jackson Laboratory (Bar Harbor, ME). All experiments were performed in accordance with the guiding principles of the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee (IACUC) of the Georgia Regents University. A whole-body smoke inhalation system (Scireq Inc., Phoenix, AZ) was used for tobacco smoke exposure. Research cigarettes 3R4F (University of Kentucky) were smoked at 1 puff/min and 10 puffs/cigarette. The mice were exposed to side stream tobacco smoke at a schedule of 1 h per day, 5 days per week for 12 or 24 weeks. The concentration of total particles in the exposure chamber was continuously monitored. The particle numbers ranged from 450 to 550 mg/m³ that are similar to what was reported previously²⁴.

After mice were anesthetized (ketamine, 90 mg/kg, xylazine 5 mg/kg, intraperitoneal), 1 ml of blood was drawn through abdominal vein. Serum samples were obtained by allowing each blood sample to clot, followed by centrifugation and collection of the supernatant.

To study emphysema, lungs from anesthetized mice were removed and filled with 4% paraformaldehyde solution with 0.5% agarose at a pressure of 25 cm H₂O and fixed for 24 h. The fixed lungs were then sliced midsagittally and embedded in paraffin. The 7- μm -thick slides were stained with hematoxylin and eosin for morphometric analysis. 10 slides of each lung were examined by Olympus BX41 microscope and images were acquired by Olympus DP72 digital camera, followed by image analysis using ImageJ software (available from <http://rsbweb.nih.gov/ij/>).

To quantify emphysema, alveolar enlargement and destruction were evaluated by the mean linear intercept (Lm) in 10 randomly selected fields per slide. The mean linear intercept was obtained by dividing the total length of lines drawn across the lung section by the total number of intercepts encountered in 80 lines per each mouse lung as described previously²⁵. Other signs of emphysema such as the change in right ventricular weight between the control and smoked mice have been reported²³.

Serum sample preparation. The process flow of preparing mouse serum samples is illustrated in Figure 1. The detailed protocols can be found in the previous report¹⁸. In brief, an ammonium formate solution (1% in methanol) was added to the mouse serum sample and mixed thoroughly. The precipitation was pelleted down by centrifugation at $7500 \times g$ for 5 min, and the precipitated proteins were removed. The supernatant was then transferred into a HybridSPE-Phospholipid cartridge (Sigma, St. Louis, MO) to get rid of phospholipids. The eluate was reconstituted with water and then loaded into a DSC-18 SPE cartridge (Sigma, St. Louis, MO) that had been conditioned with methanol and water. After washing with water, organic molecules on the cartridge column were eluted out using methanol. The final eluate in methanol was dried under nitrogen stream and reconstituted in the sample matrix solution designed for CSEI-sweeping-MEKC analysis.

CSEI-sweeping-MEKC analysis. The experimental conditions for CSEI-sweeping-MEKC have been detailed in the previous report¹⁸. Briefly, a CE instrument (ProteomLab PA800) from Beckman Coulter Inc. was used, with UV detection at 200 nm. CE separations were carried out in a fused silica capillary with an internal diameter of 50 μm , outside diameter of 365 μm , and effective separation length of 20 cm. To carry out CSEI-sweeping-MEKC, the capillary was conditioned with non-micellar background electrolyte (BGE), followed by hydrodynamic injection of a zone of the high-conductivity buffer (HCB) and a water plug. A sample was then electrokinetically injected for 180 s by applying 10 kV to the capillary. After sample



injection, a separation voltage of -12 kV was applied to load micellar BGE into the capillary and initiate the sweeping process as well as subsequent MEKC separation.

Statistical analysis. The results from CE analysis were processed and presented as mean \pm standard error (SE) in the text. Similarly, the degree of emphysema was indicated by the mean linear intercept of alveolar enlargement, also in mean \pm standard error. The t-test was used to determine the statistical significance of differences between two groups, with p value of < 0.05 considered statistically significant.

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Author contributions

X.X., Y.S. and Z.H.F. designed research, X.X. and Y.S. performed experiments and analyzed the data, and all authors participated in writing the manuscript.

Additional information

Competing financial interests: The authors declare no competing financial interests.

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