

Rapid Monitoring Method of Active and Passive Smoker with Saliva Cotinine by Gas Chromatography-Mass Spectrometry

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Environmental tobacco smoke (ETS) is the material released into the ambient atmosphere by smoking tobacco products, which consist of a heterogeneous mixture of gases, uncondensed vapors, tar and particle. ETS is a group A human carcinogen as defined by the US Environmental Protection Agency. Exposure to ETS is a cause of heart disease and respiratory tract illness. Nicotine is the principal alkaloid in tobacco and is present as a major component of tobacco smoke. Nicotine is absorbed by both active and passive smokers exposed to ETS, however, its short half-life ($t_{1/2} = 1-2$ h) precludes its use as an accurate marker for ETS. Cotinine, a primary metabolite of nicotine, has a much longer half-life ($t_{1/2} = 18-20$ h) than nicotine, resulting in higher and more stable plasma concentrations.¹⁻³ Therefore, the determination of cotinine in biological fluids has aroused particular interest.^{4,5} While cotinine is not carcinogen, the presence of it indicates ETS exposure. And cotinine has been used as a useful screening tool of ETS exposure. The biochemical marker has been used to estimate active smoking behavior, to validate smoking cessation, and to evaluate the levels and significance of ETS exposure.⁶⁻¹³

In order to estimate of ETS exposure, a technique for the rapid and simple determination of cotinine in biological samples is required. Many methods have been proposed for the determination of nicotine and cotinine in human urine, plasma, hair and saliva. Measurements of cotinine level in biological samples of ETS exposed have been analysed by radio immunoassay,¹⁴ high-performance liquid chromatography,¹⁵⁻¹⁹ and gas chromatography using electron-capture,²⁰ or nitrogen-phosphorus²¹⁻²⁴ detectors. For the determination of ng/L concentrations of nicotine and cotinine in biological samples, the most frequently used method is gas chromatography-mass spectrometry (GC-MS).²⁵⁻³¹ We reported in previous work that salivary cotinine was found to be significantly correlated to the concentration of cotinine in plasma and it can be used as an indicator for the ETS exposure.³¹ The extraction method in the work needed a concentration step, and then semi-volatile nicotine or cotinine was affected by evaporation losses. Also, the long extraction procedure could not be used easily for the monitoring of ETS.

This paper describes an extremely rapid and simple extraction, and a sensitive and rapid measurement procedure as a monitoring method of ETS utilizing gas chromatography-

mass spectrometry and d3-cotinine as internal standard.

Experimental Section

Materials. Cotinine and d3-cotinine (internal standard) were obtained from Sigma (St. Louis, MO, USA). Sodium sulfate (Junsei, Japan), methylene chloride, methanol (J. T. Baker, USA), purified water (Millipore Corp., Milford, MA) were used as reagents and solvents.

Extraction of cotinine from saliva. For the analysis of cotinine in saliva, 0.5 mL of saliva was placed in a 3 mL micro extraction vessel. 25 μ L of d3-cotinine (2.0 μ g/mL in methanol/water, 1 : 50) as an internal standard were added to the solution, and the sample was mechanically vibrated with 100 μ L of methylene chloride for 2 min, centrifuged at 5000 rpm for 3 min. The organic phase was transferred into a V-shape in auto vial and a 3 μ L of that was automatically injected into the GC system.

Gas chromatography-mass spectrometry. All mass spectra were obtained with a Agilent 6890/5973 N instrument. The ion source was operated in the electron ionization mode (EI: 70 eV, 230 °C). Full-scan mass spectra (m/z 40-800) were recorded for analyte identification. Separation was achieved with an HP fused-silica capillary column with 5% diphenyl poly(dimethylsiloxane (HP 5), ~30 m length, 0.2 mm i.d., 0.33 μ m film thickness. Samples were injected in the splitless mode. The flow rate of the helium was 0.9 mL/min. The operating parameters were as follows: injector temperature, 280 °C; transfer line temperature, 280 °C; oven temperature, programmed from 140 °C at 25 °C/min to 270 °C (held for 0 min). The ions selected in this study were m/z 98 and 176 for cotinine, and m/z 101 and 179 for d3-cotinine.

Calibration and quantitation. Calibration curve of cotinine was established by extraction after adding amounts in range of 2.5-250 ng of standards and 50 ng of internal standard in 0.5 mL of saliva. The ratio of the peak area of standard to that of internal standard was used in the quantitation of the analyte.

Results and Discussion

Chromatogram. For the GC separation of cotinine, the use of the nonpolar stationary phase was found to be

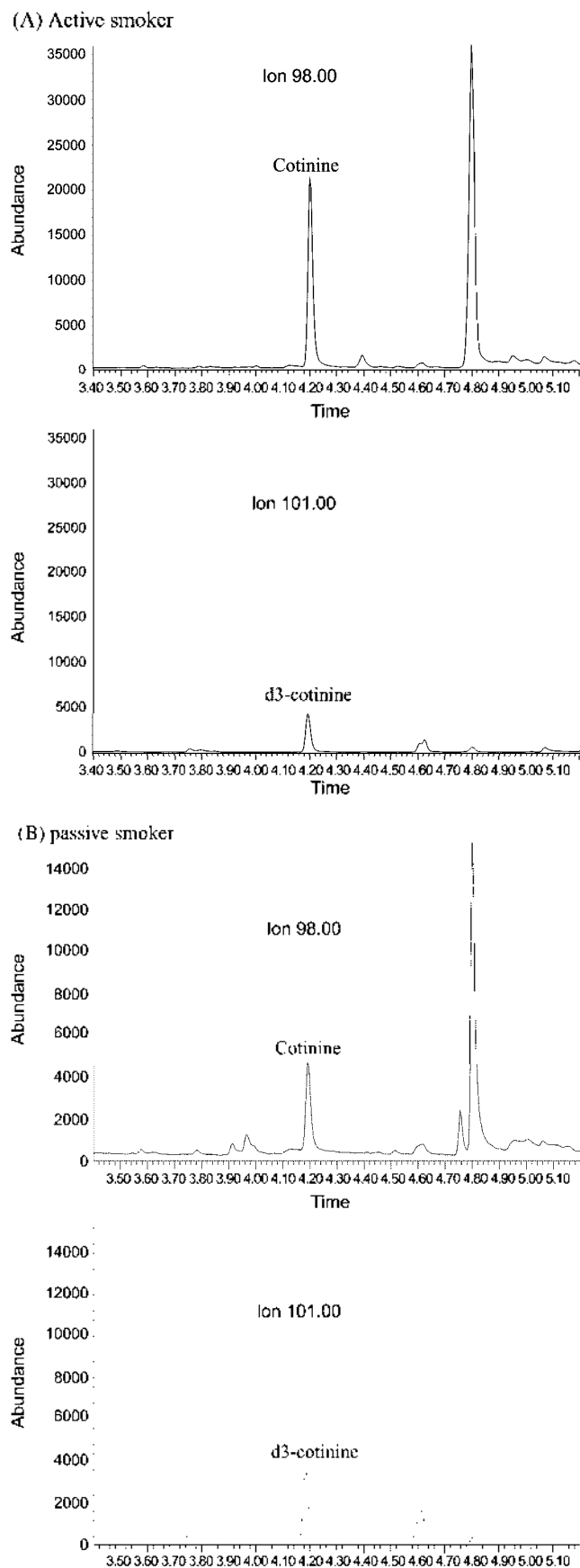


Figure 1. Chromatograms of the saliva extracts.

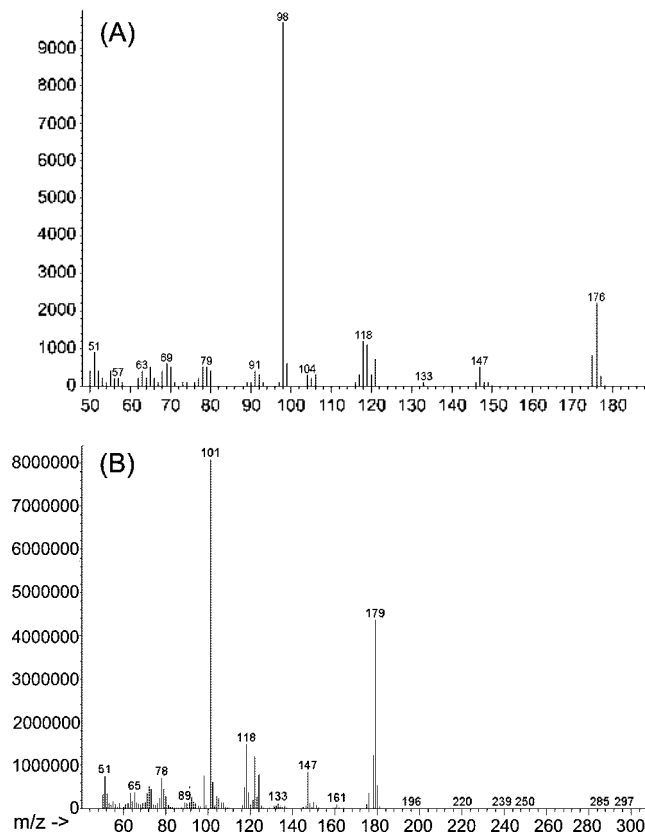


Figure 2. Mass spectra of cotinine (A) and d3-cotinine (B).

efficient. Chromatograms are shown in Figure 1. As can be seen from the figure, the peaks of cotinine and internal standard are symmetrical and separation of the analyte from the background compounds in biological samples was very good. The retention times of cotinine and d3-cotinine were 4.21 and 4.20 min. There were no susceptible peaks observed in a chromatogram of blank saliva in the time range of between 3.4 and 5.2 min.

Mass spectrum. The mass spectra of cotinine and d3-cotinine are shown in Figure 2. Cotinine shows molecular ion at m/z 178 and the base peak at m/z 98, and the characteristic ions at m/z 118, 119 and 147. d3-Cotinine illustrates molecular ion at m/z 181 and the base peak at m/z 101, and the characteristic ions at m/z 121, 122 and 150, which are both due to the loss of the pyridyl group from the molecular ion.

Linearity. Examination of typical standard curve by computing a regression line of peak area ratios of cotinine to internal standard on concentration using a least-squares fit demonstrated a linear relationship with correlation coefficient being greater than 0.998.

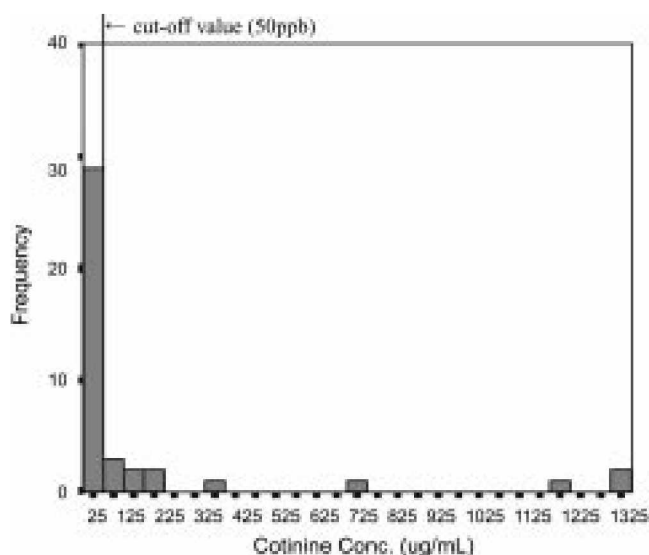
Recovery. Several saliva samples of varying composition were prepared and the relative recovery was calculated by percentage of cotinine recovered. The mean recoveries were 40.6 and 43.5% at the concentration of cotinine 50 and 100 ng/mL, respectively.

Precision and accuracy. The reproducibility of the assay was very good, as shown in Table 1. For five independent determinations at each concentration, the coefficient of vari-

Table 1. Precision and accuracy of cotinine in saliva (n=5)

Spiked Conc. (ng mL)	Mean + SD (RSD ^o %)
10	10.1 ± 0.2 (2.2 ^o %)
50	50.4 ± 0.7 (1.3 ^o %)
100	100.5 ± 1.3 (1.3 ^o %)

SD – standard deviation; RSD – relative standard deviation

**Figure 3.** Distribution of salivary cotinine concentrations.

ation was less than 2.3%.

Sensitivity. The combination of the low background and the high sensitivity of the analyte by the detector permit their determination in saliva at low concentrations. A detection limit of cotinine was 1.0 ng/mL in saliva. Limit was defined by a minimum signal-to-noise ratio of 3 and coefficients of variation for replicate determinations (n=5) of 15% or less.

Turnaround time. By the described method, a laboratory technician can extract and prepare 20 samples in approximately 25 min. These samples are then loaded into an Agilent 7673 automatic sampler. The GC cycle time is 8.0 min per sample. Fifth sample run was used in the longer retention time to remove interference peaks. So, in theory, 150 samples could be analyzed each day.

Monitoring of active and passive smoking volunteers. Saliva samples of 31 non-smoking and 10 smoking volunteers from high school in Korea were quantified by the described methods. As a results, the concentration of cotinine in saliva ranged from 0-1349 ng/mL (Figure 3).

Conclusions

The extraction method described in this study needs not any additional concentration step, and then is not affected by evaporation losses of the analyte. Turnaround time for up to 150 samples was one day. The peaks have good chromatographic properties and offer very sensitive response for the EI-MS (SIM). Analytical procedure of cotinine in saliva with the limit of detection of 1.0 ng/L was established. The

detection limits from this study was lower than or similar to those obtained by a more costly procedure involving conventional liquid-liquid extraction and SPME.¹⁶⁻³¹ The developed method is simple and rapid, and can be used easily for the monitoring of active and passive smoking. Through the accurate determination of cotinine in saliva the risk of lung cancer and heart disease can be predicted.

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