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^{1}H NMR spectroscopic identification of a glue sniffing biomarker

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ABSTRACT

Organic solvent abuse typically involves sniffing organic solvents to experience the mind-altering conditions they induce. In Republic of Korea, organic solvent abuse is a serious social problem, especially among teenagers. Several studies have addressed the effects of organic solvent abuse on mind and body, but there are no simple methods by which such abuse can be positively identified. In this report, we describe a method for analyzing toluene metabolites (toluene is the main ingredient of glue) in glue-sniffers' urine using ^{1}H NMR spectroscopy. Toluene is a commonly used solvent in the rubber, paint, plastics, leather, printing, and chemical industries. Inhaled toluene is metabolized to hippuric acid in the liver and excreted in the urine. Hippuric acid is known as a good biomarker for biological monitoring of toluene exposure. We have scanned hippuric acid and other toluene metabolites by NMR spectroscopy and performed statistical multivariate analysis of the data. Based on this analysis, we sought to determine parameters by which glue-sniffing (toluene inhalation) behavior may be verified. We also demonstrate the use of a pattern recognition method for accurate and efficient analysis of NMR data. In comparison to conventional methods, such as mass spectroscopy coupled with liquid chromatography or gas chromatography, nuclear magnetic resonance spectroscopy has several advantages, including simple sample preparation, non-destructive sampling, accuracy, short acquisition time, and reproducibility in the determination of urinary hippuric acid.

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1. Introduction

Organic solvents are commonly used in Korean industries. These organic solvents are toxic, either through occupational or intentional exposure [1–3]. Guidelines which regulate working environments and the use of personal protective devices can reduce occupational exposure, but organic solvent abuse among teenagers who sniff industrial glue is easily concealed and can occur over long periods of time, leading to excessive exposure.

Organic solvent abuse is a serious social problem, especially among adolescents between the ages of 13 and 18. Glue sniffing is a common form of organic solvent abuse and frequently causes sudden death in adolescents and young adults [4]. The behavior is becoming popularized among adolescents in the Republic of Korea, with some teenagers abusing organic solvents nearly to the point of addiction. Many organic solvents are abused among teenagers have become dependent on them. Glue is inexpensive, easily obtained, legal to purchase, and simple to sniff, all of which contribute to its common use as a hallucinogen. It is thought that organic solvent abuse is related to behavioral and personality disorders. Medical poisoning could be induced by organic solvent abuse in teenagers, and users should be required psychiatric evaluation and appropriate treatment.

A 2003 survey by the Korean National Youth Commission studied nationwide rates of glue sniffing and the results of the survey showed that 96% of teen boys and 66% of teen girls have sniffed glue at least once ($p < 0.01$) (the survey included 100 adolescents in a young offender institution, aged 10–19, 50 males and 50 females). The development of an analytical method for the identification of solvent abuse will aid in the treatment and management of adolescent offenders [5,6].

The glues in use within the Republic of Korea are comprised of approximately 74–88% organic solvents including acetone, methyl ethyl ketone, methyl cyclopentane, cyclohexane, toluene, and cyclohexanone. Toluene is the main solvent constituent of glue, although it can be as low as 10.8% in low-toluene glues [7]. Toluene is a mono-substituted benzene derivative and a lipophilic aromatic hydrocarbon, typical of organic solvents and the main ingredient of lacquer, thinner, and glue. As toluene has very low water solubility, it must be metabolized prior to elimination via the urine and perspiration. Toluene is inhaled and absorbed through the lungs; 18% is excreted in the expired air and the remainder is metabolized

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to benzyl alcohol by the liver cytochrome P-450 system [8]. Benzyl alcohol is dehydrogenated to benzaldehyde by alcohol dehydrogenase and to benzoic acid by alcohol oxygenase [9]. Benzoic acid conjugates with glycine to form hippuric acid, which is water soluble. Thus toluene is mainly excreted in the urine as hippuric acid and benzoic acid [10]. We report the observation of hippuric acid in urine samples using NMR spectroscopy. The concentration of hippuric acid was strongly elevated in the urine of glue sniffers; other identified metabolites included citrate and creatinine. The results provide definitive evidence of toluene inhalation.

2. Method and experimental design

2.1. Sample collection

A total of 9 urinary samples were available for this study. Four samples were collected from people who self-identified as glue-sniffers (toluene concentration was over 0.1 μg/mL by gas chromatography using urine; data not shown). One sample was collected within 2 weeks of last use; however, no physical evidence was available to verify this assertion (“2W glue sniffer”). The remaining samples were from 4 healthy volunteers with no history of glue sniffing. Collected samples were stored at 4 °C prior to analysis.

2.2. Preparation of urinary samples for NMR analysis

Urine samples were prepared for ^{1}H NMR spectroscopy by adding 50 μL of D_2O containing 20 mM TSP (sodium trimethylsilyl [2,2,3,3-$^2H_4$]propionate) to 450 μL urine to a final TSP concentration of 2 mM. TSP was used as a chemical shift reference with a δ 0.0 singlet peak and the deuterium oxide (D_2O) was used as the lock signal. All samples were placed in 5-mm NMR tubes for analysis and were stored at 4 °C except during analysis.

2.3. NMR spectroscopy

^{1}H NMR spectral data were acquired on a 500 MHz Varian Unity-Inova (Varian Inc., Palo Alto, CA) and 298 K operating at a proton NMR frequency of 499.789 MHz. Water signals were suppressed by using a presaturation pulse sequence. All measurements were processed with an ID/PFG 5 mm probe. 512 transitions were accumulated and the ^{1}H NMR spectral acquisition time was about 30 min per sample. All data were calibrated to TSP at δ 0.00 ppm and an exponential function using a line broadening of 0.2 Hz was applied to each free induction decay prior to Fourier transformation.

2.4. Statistical analysis of NMR spectra

All NMR spectra were manually phased and baseline-corrected by MestreNova Suite 5.3.1 (Mestrelab Research, USA). Full-resolution NMR data were imported into Chenomx NMR Suite 6.01 software (Chenomx Inc., Canada) and regions corresponding to water/HDO and urea (δ 4.7–6.0) and TSP (δ −0.25–0.25) were excluded from statistical analyses. All remaining regions of the spectra were reduced to ppm spectral buckets and were normalized to the region of 0.5–9.5 ppm by MestreNova Suite 5.3.1.

A spectral assignment and estimated concentration of each metabolite was generated by Chenomx NMR Suite 6.01 software. Principal component analysis (PCA) was applied to all samples using Simca-P+.

3. Results

3.1. ^{1}H NMR spectral analysis of urine

A single spectrum was selected from each test group as examples for detailed signal assignment. Assignments were made using the Chenomx 500 MHz library database and a literature compilation [11–13]. Fig. 1 displays the chemical shift information for several metabolites. All spectra were normalized to the TSP peak; however, the water and urea peaks (from δ 4.7 ppm to δ 6.0 ppm) were excluded from whole spectra. The major identified compounds were hippuric acid, creatinine, dimethylamine, alanine, 3-hydroxybutyrate, citrate, and TMAO (Trimethylamine N-oxide). In the middle frequency region, δ 2.7–6.3 ppm, citrate, hippuric acid, TMAO, and creatinine were observed. In the high frequency region, δ 6.3–8.5 ppm, hippuric acid and other aromatic compounds were observed.

Fig. 1 shows the assigned urine spectrum of a control, a glue sniffer and the 2W glue sniffer with TSP and water resonances

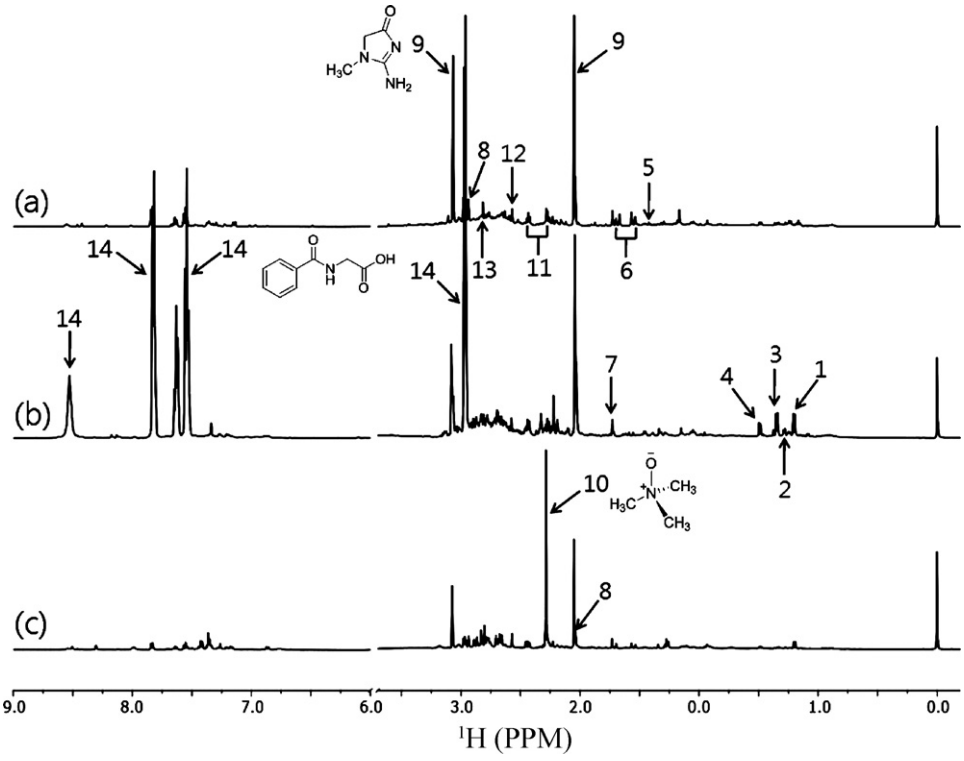

Fig. 1. Representative ^{1}H NMR assigned spectra of controls, glue sniffers and the 2W glue sniffer with TSP (δ 0.00 ppm) and water resonances removed. All spectra were normalized to the TSP peak (from δ −0.026 ppm to δ 0.023 ppm). The following metabolites were identified with Chenomx. (a) Control, (b) glue sniffer, (c) 2W glue sniffer. Assigned list: (1) 3-hydroxybutyrate, (2) 3-hydroxyisovalerate, (3) lactate and threonine, (4) alanine, (5) succinate, (6) citrate, (7) dimethylamine, (8) creatine, (9) creatinine, (10) TMAO (Trimethylamine N-oxide), (11) taurine, (12) glycine, (13) guanidoacetate, (14) hippuric acid.

Table 1
Metabolite concentrations in each group. Control, glue-sniffing, and 2W glue sniffer. Three metabolites were shown in this table which changed considerably. The concentration of metabolites were calculated with integration of peak areas using chenomx.

	Control1 (mM)	Control2 (mM)	Control3 (mM)	Control4 (mM)	Control5 (mM)
Creatinine	19.23	16.81	30.86	16.24	4.89
Hippuric acid	1.34	2.52	2.06	5.05	0.33
Citrate	2.82	2.78	4.72	2.33	0.84
	Glue sniffer1 (mM)	Glue sniffer2 (mM)	Glue sniffer3 (mM)	Glue sniffer4 (mM)	2W-glue sniffer (mM)
Creatinine	1.86	0.87	12.07	17.04	6.11
Hippuric acid	6.88	10.06	127.83	63.44	1.63
Citrate	0.07	0.08	1.6	0.04	1.15

removed. Hippuric acid resonances appeared at δ 4.0 ppm (doublet), δ 7.5 ppm (triplet), δ 7.6 ppm (triplet), δ 7.8 ppm (doublet), and δ 8.5 ppm (singlet). After glue sniffing, the hippuric acid concentration is extremely elevated as toluene is metabolized. Hippuric acid concentration can be affected by various factors, including diet, medical treatment, or alcohol consumption; however, measured hippuric acid peaks were substantially different in glue sniffers, despite these variable factors. 18% of toluene is excreted in the expired (through the expired) air and the remaining 95% is oxidized to become benzyl alcohol by cytochrome P-450 [8]. This benzyl alcohol is dehydrogenated to benzaldehyde by alcohol dehydrogenase and the benzaldehyde is dehydrogenated to benzoic acid [9,10]. The benzoic acid conjugates with glycine to hippuric acid [10]. Toluene is almost excreted in urine as hippuric acid and benzoic acid with extremely small amount of toluene, so hippuric acid peaks were clearly observed but a toluene peak was not appear in the NMR spectrum. Although GC–MS analysis indicated the presence of toluene in 4 samples, the NMR spectra did not show any detectable praks for the toluene. The reason is that the concentration of the toluene is below the detection limit of NMR. Creatinine, one of the major components of urine, seemed to be reduced in glue sniffer samples in comparison to controls. In Table 1, the metabolite concentrations observed in each group are shown. Creatinine and citrate concentrations were reduced and the hippuric acid was extremely high in the glue sniffers.

Fig. 2 shows a representative ^{1}H NMR assigned spectrum for a control, a glue sniffer, and the 2W glue sniffer in the expanded areas from δ 2.485 ppm to δ 2.79 ppm. Citrate resonances appeared at δ 2.5 ppm (doublet) and δ 2.7 ppm (doublet). In the control samples, citrate and dimethylamine peaks appeared in this region; however, citrate peaks disappeared in glue sniffer samples and were recovered after 2 weeks.

In Fig. 3, we compare the means of creatinine, citrate and hippuric acid in control and glue sniffer samples to identify a biomarker of glue sniffing. Control samples had higher creatinine content when compared to the glue sniffing samples. Creatinine could serve as a glue sniffing biomarker, but it varies between individuals and is therefore not ideal. Citrate and hippuric acid were significantly different in the control and glue sniffer samples. A combination of strong signals for hippuric acid and reduced citrate was found to be a reliable discriminator between glue sniffers and controls. The standard error of the mean, or the standard deviation of sample means, can be reduced in a larger study, which may verify the confident relationship between glue sniffing and metabolites change.

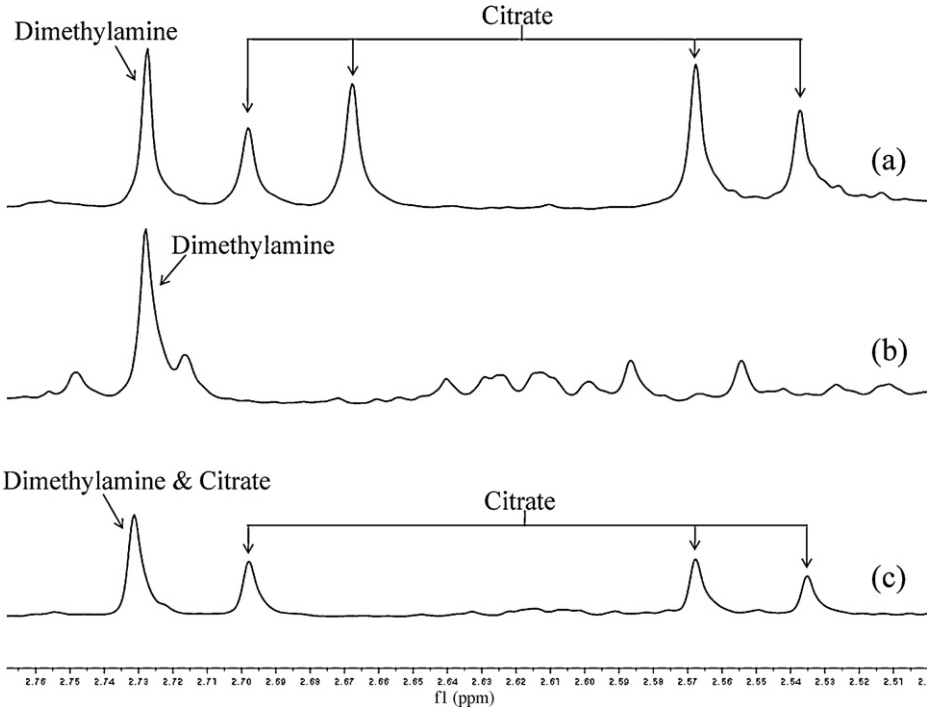

Fig. 2. Representative ^{1}H NMR expanded spectra of controls, glue sniffers, and 2W glue sniffer, from δ 2.48 ppm to δ 2.77 ppm, with TSP (δ 0.00 ppm) and water resonances removed. All spectra were normalized to the TSP peak (from δ −0.026 ppm to δ 0.023 ppm). (a) Control, (b) glue sniffer, (c) 2W glue sniffer. We verified that citrate peaks were extremely reduced in the glue sniffing samples.

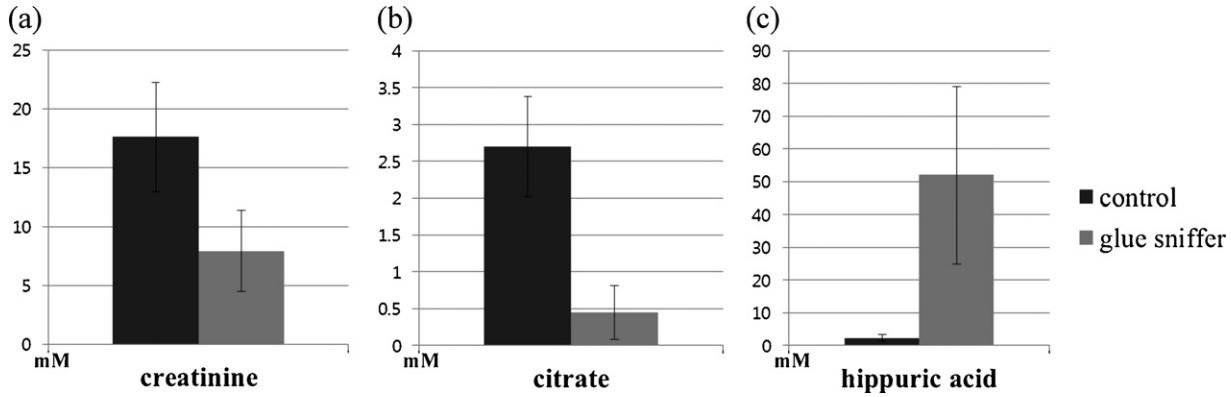

Fig. 3. Mean metabolite concentration values for controls and glue sniffers with standard error bar. Concentrations of creatinine (a) and citrate (b) were lower in the glue sniffers. Glue sniffers had higher concentrations of hippuric acid (c) than the controls (compare to the control).

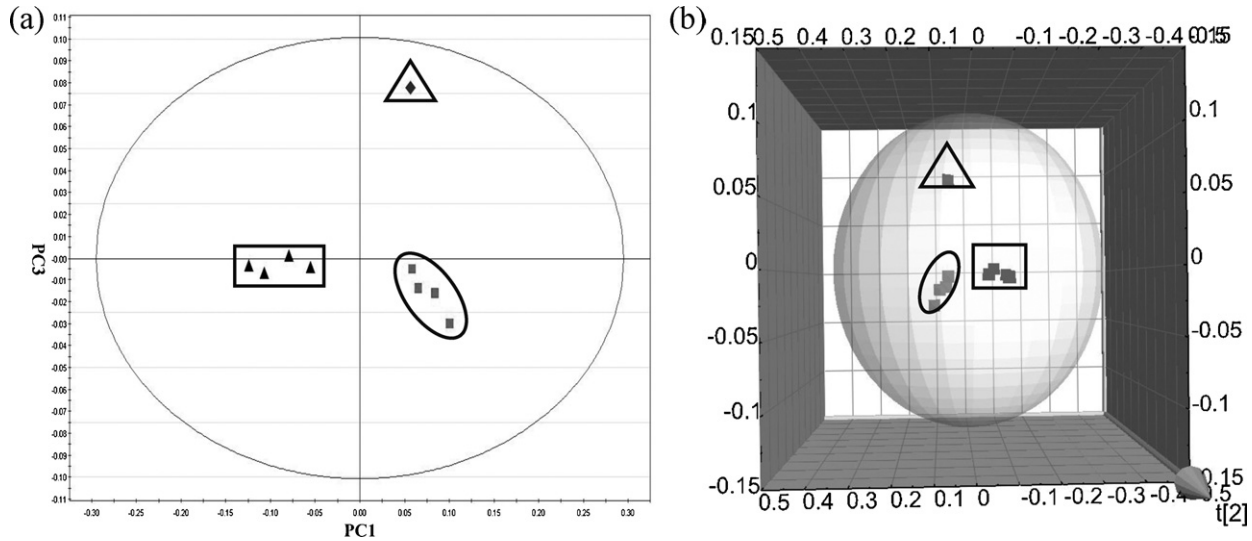

Fig. 4. Principal component analysis of glue sniffers and the 2W glue sniffer in comparison to controls. (a) PCA 2D scores plot of PC1 vs. PC3, (b) PCA 3D scores plot of PC1 vs. PC2 vs. PC3. We observed segregation of the controls (spots in ellipse) from the glue sniffers (spots in quadrilateral) and 2W glue sniffer (spot in triangle). In addition, the glue sniffers were further sub-divided into the glue sniffing and 2W glue sniffer groups.

3.2. Principal component analysis of ^{1}H NMR data

We performed principal component analysis (PCA) of ^{1}H NMR spectra of urine samples from glue sniffers, the 2W glue sniffer, and the control samples to distinguish a glue sniffer from a non glue sniffer and to analyze metabolite changes. PCA results and clustering of samples in the principal component 1 (PC1) and principal component 3 (PC3) score plots are shown in Fig. 4(a) and (b). It was clear that each group were clearly separated in Hotelling's T2 region with a 95% confidence interval for the modeled variation. We verified that the control and 2W glue sniffer were divided from the glue sniffers along PC1 ($p < 0.05$) on the PCA scores plot. In addition, the controls and 2W glue sniffer were further divided along PC3 ($p < 0.05$), thus demonstrating our ability to distinguish each group, even with respect to the time of use.

To identify the metabolite profile change in a glue sniffing PCA, loading plots were generated and the results are shown in Fig. 5. The areas of the loading plot of PC1 and PC3 represent those metabolites which contribute to the division of samples into the sample groups. In Fig. 5(a), there were two components, hippuric acid and creatinine, which distinguish glue sniffers from the controls and 2W glue sniffer. Hippuric acid was increased and creatinine was decreased in glue sniffer's urine (Fig. 1). Creatinine was reduced in the glue sniffer group; however, the amount of creatinine depends on the inter-individual variation. We consider creatinine to be a component of separation as it is a major component of the loading plot. We confirmed that the 2W glue sniffer segregated from the controls with TMAO, creatinine, and hippuric acid (Fig. 5(b)). However, we measured only 1 sample of a suspected glue sniffer who claimed his last exposure was 2 weeks prior to sampling; more samples are required to establish confidence in this result. There is no evidence of other factors which may have caused the 2W glue sniffer sample to appear different in a multivariate statistical analysis.

4. Discussion

Those who deliberately abuse organic solvents expose themselves to 100–1000 times the established occupational exposure limits for the safe use of solvents in industry [6,14]. Exposure to large amounts of toluene can cause injury, even sudden death [15–17]. Therefore, analysis of metabolites in organic solvents abusers will be crucial in the forensic field because organic solvents are rapidly metabolized. Here, we track these rapid changes in metabolites and demonstrate a method to discriminate those who are exposed to high concentrations of toluene. Discrimination

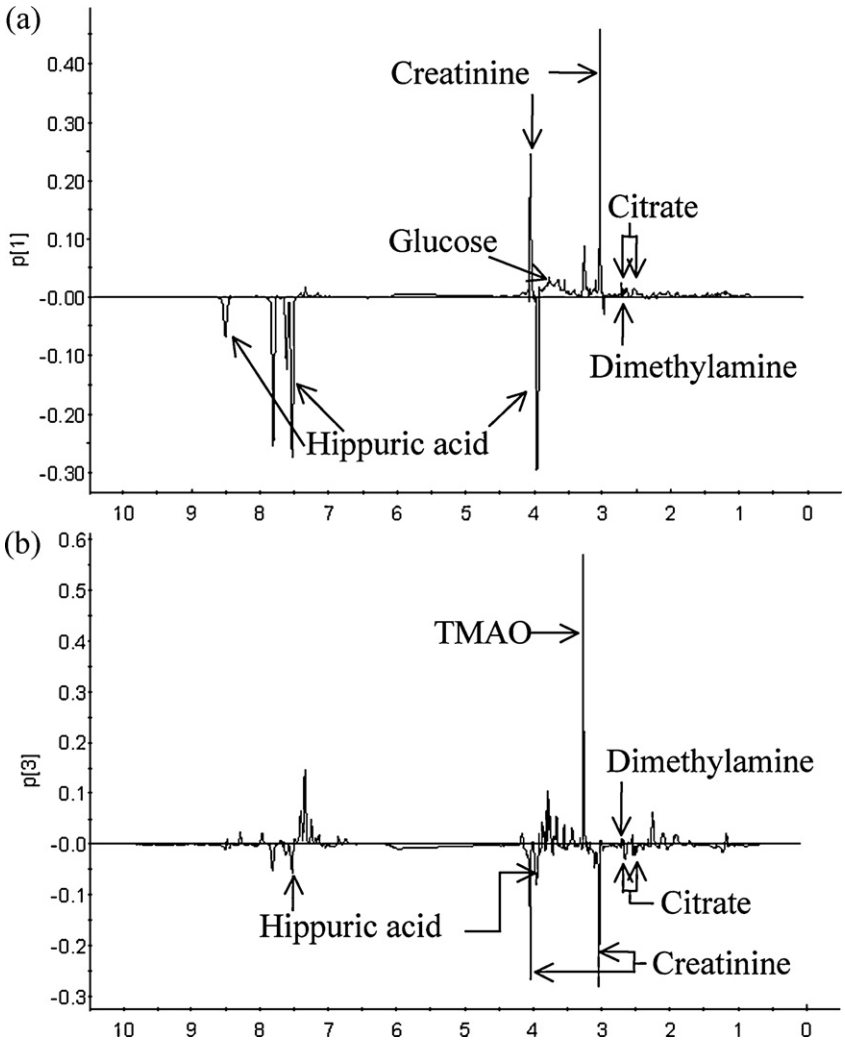

Fig. 5. Principal component analysis loading plots based on ^{1}H NMR spectra of principal component 1 and 3. (a) PCA loading plot of PC1, (b) PCA loading plot of PC3. There were two major components that discriminated between glue sniffers and controls: hippuric acid and creatinine. Hippuric acid was increased in glue sniffer's urine but creatinine was extremely reduced in loading plot (a), indicating that creatinine has a tendency to decrease in glue sniffers. In loading plot (b), we confirmed that TMAO, creatinine, and hippuric acid are the major contributors to segregation from other samples.

was based on the presence of urinary hippuric acid and other compounds as assessed by NMR spectrometry.

We carried out NMR experiments to monitor the metabolite changes in glue sniffers and a 2W glue sniffer in comparison to a control group. Based on these results, we conclude that the concentration of hippuric acid, creatinine and citrate were critical to the discrimination of glue sniffers. In this group, the hippuric acid concentration was extremely high and the citrate concentration was decreased after glue sniffing. Previous reports have mentioned the relationship between toluene and the TCA cycle [18]; our results suggest that toluene influences citrate through the TCA cycle. The amount of creatinine tends to be reduced in glue sniffers; however, creatinine concentration is dependent on inter-individual variability. Further study with a larger sample size will help clarify the relationship between creatinine and hippuric acid. We have demonstrated our ability to identify glue sniffers by the presence of increased hippuric acid with a concurrent decrease in citrate, although toluene peaks were not apparent in the NMR spectrum.

In summary, we verified the identification of glue sniffers based on clear changes in the concentrations of 2 metabolites: hippuric acid and citrate. Creatinine may also serve to identify glue sniffers. Hippuric acid, a toluene metabolite, was detected by NMR spectroscopy and statistical analysis revealed that controls and glue sniffer samples were easily distinguished, allowing us to verify abusers of glue. Because of the difficulties in sample supply, in total of nine samples obtained from the glue sniffers were assessed in the present study. However, it is noted that there were easily detectable differences in NMR spectrum patterns obtained from abusers' samples compared with the non-abuser'. The method described in this paper should be a useful tool to determine glue sniffers in forensic investigation with further verification experiments. Although NMR has a higher detection limit than mass spectroscopy with chromatographic methods, hippuric acid detection in urine by NMR is rapid, provides for easy sample collection, is non-invasive, non-destructive, and highly accurate and reproducible. On the basis of these advantages we suggest that NMR-based hippuric acid detection has great potential utility in the monitoring of glue sniffing behavior.

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