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# Simultaneous determination of ethyl glucuronide, cocaine, cocaethylene, and benzoylecgonine in hair by using LC–MS/MS

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**Abstract**

Alcohol and cocaine (COC) are commonly co-used drugs that cause addiction and have harmful effects. Their abuse may threaten the health of the abuser and public safety by causing serious accidents or crimes. The recidivism rate of drug-related crimes closely correlates with alcoholism. Several incidences of alcohol consumption in combination with drug abuse have been reported. Here, liquid chromatography tandem mass spectrometric method was developed to simultaneously analyze ethyl glucuronide (EtG), a metabolite of ethanol; COC; cocaethylene (CE), an alcohol-derived metabolite of COC; and benzoylecgonine (BZE), a major metabolite of COC, to determine the concurrent use of alcohol with COC. For pre-treatment, ultracentrifugation (5 min, 50,000 g) and mixed-mode anion exchange solid-phase extraction were used to increase the recovery of target compounds and minimize the matrix effect of hair. The lower limits of quantification were: 7 pg/mg (EtG), 2 pg/mg (COC), 10 pg/mg (CE), and 1 pg/mg (BZE). The correlation coefficient (r) of the calibration curve within the quantified range of target compounds was ≥ 0.9978. The intra- and inter-day accuracies were − 6.1–9.7% and − 9.3–8.3%, and intra- and inter-day precisions were 0.5–10.3% and 0.6–14.4%, respectively. The recovery, matrix effect, process efficiency, and autosampler stability were 89.2–104.8%, 81.6–105.4%, 81.5–107.1%, and 96.6–109.7%, respectively. The novel analytical method was validated with hair samples from individuals suspected of alcohol and COC use, and the method could distinguish between independent and concurrent use. Based on the findings, the analytical approach developed in this study is anticipated to be valuable in drug and alcohol dependence tests that require the simultaneous detection of alcohol and COC abuse.

**Keywords** Ethyl glucuronide, Cocaine, Cocaethylene, Benzoylecgonine, Hair, Alcohol, LC–MS/MS

## Introduction

Abuse of alcohol and cocaine (COC) disturbs the central nervous system, leading to addiction and disease as well as criminal offenses such as drunk driving, violence, sexual violence, robbery, and murder (Kim et al. 2020). Various countries around the world, including South Korea, are striving to prevent social disruption by controlling the distribution and use of illicit drugs. Despite these efforts, the distribution of illicit drugs in South Korea has increased owing to the ease of access provided by the Internet, and drug-related crimes have become a serious problem in Korean society (An 2020; Chung and Lee 2018; Lee 2019; Park and An 2021).

In South Korea, public awareness of the consequences of crime induced by the concurrent use of alcohol and drugs is low, and related measures are undeveloped. Meanwhile, other nations are constantly examining the medical and pharmacological aspects of

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combined alcohol and drug abuse with efforts to investigate and report on drug-related crimes (Akhgari et al. 2020; Lightowlers and Sumnall 2014; Shimomura et al. 2019). The use of narcotics is characterized by its close association with alcohol dependence. Among the narcotic drug offenders in South Korea, the percentage of those who drink frequently was 98.9%, and the Alcohol Use Disorder Identification Test, Korean version (AUDIT-K) indicated 66.7% of "non-problem drinking," 16.8% of "problem drinking," and 16.5% of "alcohol use disorder" (Ministry of Health and Welfare 2021). Hence, the percentage of drinkers in the group of drug abusers was higher than that of non-abusers.

The effect of narcotics can be maximized by the consumption of alcohol. COC is a stimulant with an enhanced euphoric effect when combined with alcohol; approximately 50–90% of COC users are known to concurrently use alcohol (Kim et al. 2013; Pennings et al. 2002; Schmitz et al. 1997; Singh 2019). Notably, the concurrent use of alcohol and COC can be speculated to be strongly correlated with an increased probability of crimes and accidents. According to studies analyzing the level of aggression in individuals with alcohol and COC dependence, the level of aggression is higher in those concurrently using the two drugs than in those independently using a single drug, and the risk of suicidal ideation is also higher in concurrent users (Czermainski et al. 2020; Salloum et al. 1996). In addition, notable levels of alcohol metabolites and COC have been detected in the biopsy results of drivers with fatal injuries from road accidents (Budd 1989; Marzuk 1990). These data suggest that the combined use of alcohol and COC may result in impulse-control disorders and increase the likelihood of criminal behavior and accidents.

Alcohol consumption results in the production of ethyl glucuronide (EtG) through glucuronidation metabolism, where glycosidic acid mediates the binding of glucuronide and ethanol (Høiseth et al. 2013; Langman et al. 2009). The use of COC results in the production of benzoylecgonine (BZE) as a major metabolite. BZE is a metabolite produced through hydrolysis mediated by the liver carboxylic-ester hydrolase. Its half-life in the body is 4–7 h (Dean et al. 1991). If COC and alcohol are concurrently used, the activity of liver carboxylic-ester hydrolase is suppressed by ethanol, reducing the production of BZE. Simultaneously, cocaethylene (CE) is produced during the transesterification of ethanol and COC (Laizure et al. 2003). The stimulation from dopamine accumulation at the synaptic cleft occurs as COC and CE block the reabsorption of dopamine at the receptors and prolong the excitation while ethanol acts to facilitate the release of dopamine by stimulating the neurons (Baler and Volkow 2006; Jones 2019). An identical inhibitory effect on dopamine reabsorption is exhibited by COC and CE, but the half-life of COC in the body is 0.5–1.5 h, whereas that of CE is longer, at 2.5–6 h (Langman et al. 2009). Hence, with the concurrent use of COC and alcohol, the resulting metabolite CE, whose half-life exceeds that of its parent COC, can have the same effect for a longer time. The difference in half-lives between COC and CE explains why many COC abusers concurrently use alcohol (Jones 2019). Although many COC abusers tend to concurrently use alcohol, an analysis method that can simultaneously determine alcohol metabolites EtG, COC, CE, and BZE is not used in Korea. Identifying the pathway of COC into the body according to the concentration of these metabolites is considered to play a highly important role in responding to related crimes.

Hair samples from COC consumers can be used to detect the combined use of COC and alcohol. Unlike urine or blood samples that only identify recent drug use, hair samples are advantageous and useful indicators in the detection of the cumulative use of alcohol and COC over a relatively long time, from several months to a year, so that even the concurrent use from a distant period can be identified (Kronstrand et al. 2012). The detection of CE in hair indicates that the hair belongs to an individual who concurrently used alcohol and COC, and the EtG concentration in hair simultaneously allows the determination of the level of alcohol drinking (Kintz 2006). The Society of Hair Testing (SoHT) states that the EtG concentration in the hair at 7–30 pg/mg indicates a social drinker, and that above or equal to 30 pg/mg indicates a chronic drinker, so that the level of alcohol can be determined in accordance with the EtG concentration in hair (Society of Hair Testing 2019).

For drug-use determination via hair analysis, analyzing small quantities of hair is preferable because the collection of a large quantity of hair may be an infringement of human rights. However, based on the analytical method, the use of a small amount of hair may not guarantee a positive test result, even on the hair of an actual narcotics user (Park et al. 2008). Consequently, determining the minimum amount of hair that maximizes the detection sensitivity while preventing the potential infringement of human rights is important. The amount of hair used in previous studies was 15–200 mg, and it was 25–50 mg in the most recent study. However, the incubation time was required to obtain maximum extraction efficiency, and the sonication time was more than 2 h (Bastiani et al. 2020; Cabarcos et al. 2013; Kummer et al. 2015; Luginbühl et al. 2018; Mueller et al. 2020; Oppolzer et al. 2017; Palumbo et al. 2018; Pragst et al. 2020; Triolo et al. 2022). The method developed in this study used 30 mg of hair for preventing the potential infringement of human rights and satisfied the lower limit of quantification (LLOQ) of

EtG at a practical level despite a relatively short extraction time. The hair was analyzed following the hair pulverization method recommended by the SoHT (Crunelle et al. 2014; Society of Hair Testing 2019). To perform the simultaneous analysis of EtG, CE, COC, and BZE, liquid chromatography tandem mass spectrometry (LC–MS/MS) with polarity switching ionization was used, and the analysis was performed in the positive (COC, CE, and BZE) and negative (EtG) ionization modes.

In this study, an analytical method based on LC–MS/MS was developed and validated to simultaneously analyze EtG, COC, CE, and BZE in hair. The novel method can be used in forensics to detect EtG and CE in hair to determine independent COC use and combined COC and alcohol use, and to provide specific circumstantial evidence for criminal cases.

## Experimental

### Chemicals and reagents

The standards EtG, COC, CE, and BZE, and the deuterium-labeled internal standards (IS) EtG-$d_5$, COC-$d_3$, CE-$d_3$, and BZE-$d_3$, were purchased from Cerilliant (Round Rock, TX, USA) and Medichem (Barcelona, Spain). Formic acid and acetic acid were purchased from Sigma-Aldrich (St. Luis, MO, USA); dichloromethane was purchased from Honeywell Burdick & Jackson (Muskegon, MI, USA). Cyclohexane was purchased from J.T.Baker/Avantor (Phillipsburg, NJ, USA), and methanol, acetonitrile, and water were purchased from Fisher Scientific (Pittsburgh, PA, USA). All chemicals were of LC–MS grade. The stock standard solutions for the target compounds and their ISs were prepared at 100 μg/mL and 10 μg/mL, respectively, using methanol as the solvent. The working standard solutions for the standards were diluted with methanol to appropriate volumes. The working standard solutions for the IS were diluted with methanol to 200, 10, 10, and 10 ng/mL for EtG-$d_5$, COC-$d_3$, CE-$d_3$, and BZE-$d_3$, respectively. All standard solutions were stored at − 20 °C for subsequent use.

### Hair specimens

The blank hair samples in this study were collected from an individual who did not consume alcohol and COC. To validate the developed analytical method for forensic samples, positive samples were set as the hair from individuals ($n = 26$) suspected of using alcohol and COC. The QC samples were prepared to the following concentrations: 7, 20, 100, and 500 pg/mg for EtG; 2, 5, 25, and 125 pg/mg for COC; 10, 25, 125, and 625 pg/mg for CE; and 1, 2.5, 12.5, and 62.5 pg/mg for BZE. The concentrations used for the calibration curve were as follows: 7, 14, 35, 70, 210, 350, and 700 pg/mg for EtG; 2, 4, 10, 20, 60, 100, and 200 pg/mg for COC; 10, 20, 50, 100, 300, 500, and 1000 pg/mg for CE; and 1, 2, 5, 10, 30, 50, and 100 pg/mg for BZE.

### Sample preparation

For the pre-treatment of hair samples, mixed-mode anion exchange solid-phase extraction (MAX) cartridges (Oasis MAX 3 cc Extraction cartridges, Waters, Milford, MA, USA) and Supelco VISIPREP 24-port (Bellefonte, PA, USA) were used to conduct solid-phase extraction (SPE). Hair samples (30 mg) were washed twice with dichloromethane and water, and then dried. The dry hair and six steel grinding beads (3 mm) were placed in a 2-mL centrifuge tube (Safe-lock tubes, 2.0 mL, Eppendorf, Hamburg, Germany) for 15 min of pulverization at 30 Hz. Next, the pulverized hair sample was mixed with water (950 μL) and IS (50 μL). A sonicator with temperature control (Q500 system sonicator, QSONICA, CT, USA) was used for 1 h ultrasonic extraction from the hair-sample mixture at room temperature, and a high-speed centrifuge (3-30 K refrigerated centrifuge, Sigma, Osterode am Harz, Germany) set for 5 min at 4 °C and 25,000 g. Furthermore, the supernatant (700 μL) of the hair extract was transferred to a 1.5-mL centrifuge tube (Safe-lock tubes, 1.5 mL, Eppendorf), and water (700 μL) was added again to the hair extract residue for mixing by vortex mixer. The mixture was centrifuged for 5 min at 4 °C and 25,000 g, and then, the supernatant (800 μL) was transferred to the 1.5-mL centrifuge tube containing the supernatant of the first extract to perform the centrifugation for 5 min at 4 °C and 50,000 g. Subsequently, the supernatant (1,450 μL) of this final hair extract was passed through methanol (2 mL) and water (2 mL) for loading to the activated MAX cartridge for the adsorption of target compounds. The cartridge was washed with cyclohexane (1 mL) and dried for 10 min in a vacuum condition. The dried cartridge was eluted with methanol (1.5 mL) containing 2% formic acid in a test tube (Glass screw thread culture tube, 16 × 100 mm, 12 mL, Millville, NJ, USA). The eluate was dried in $N_2$ condition and at 40 °C for 25 min. The final sample residue was dissolved in the solvent (80 μL) containing 0.1% of acetic acid and acetonitrile in 95:5 ratio (v/v), and this solution (8 μL) was applied to LC–MS/MS (Fig. 1).

### LC–MS/MS conditions

The LC–MS/MS analysis was conducted using Nanospace NASCA HPLC (Shiseido Co., Tokyo, Japan) with Sciex QTrap 6500 triple-quadrupole mass spectrometer (AB SCIEX, Foster city, CA, USA). Xselect HSS T3 XP C18 column (2.5 μm, 2.1 × 150 mm, Waters, USA) was used as the column to isolate the target compounds. 0.1% acetic acid (water with 0.1% (v/v) acetic acid) and acetonitrile were used as mobile phase A and mobile phase

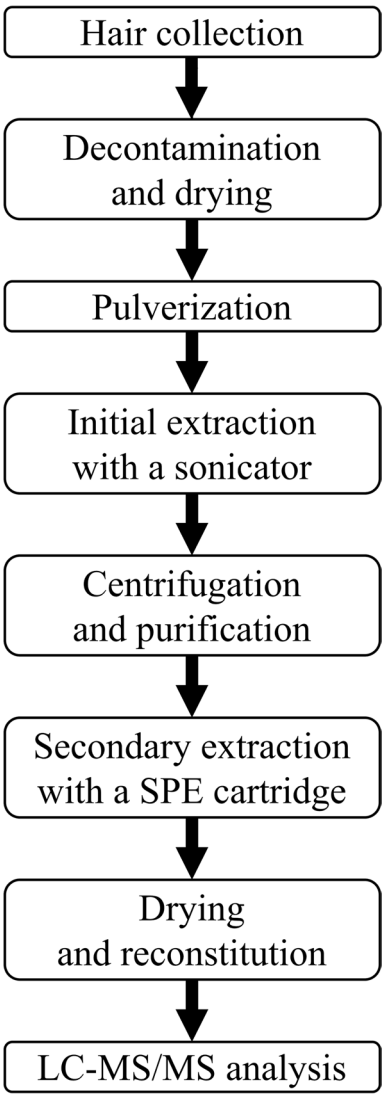


**Fig. 1** Schematic workflow of hair-sample preparation

B, respectively. The column temperature in the LC system and the sample storage temperature in the autosampler were maintained at 40 °C and 4 °C, respectively. The gradient elution condition was applied with the flow rate of the mobile phase maintained at 220 μL/min. For mobile phase A, the initial level was 95%, which was changed to 5% for 9 min. The changed level was maintained for 3 min and then returned to the initial level of 95% for 0.5 min, followed by 3.5 min stabilization.

Using rapid positive–negative ion polarity switching time of 5 ms, a method that simultaneously applies the negative mode for analyzing EtG and the positive mode for analyzing COC, CE, and BZE, electrospray ionization was performed. To quantify the target compounds in hair, the method of multiple-reaction monitoring (MRM) was applied. In the negative mode, curtain gas was set at 45, collision gas at medium, ionspray voltage at −4,500 V, turbo-gas temperature at 550 °C, ion source gas 1 at 50, and ion source gas 2 at 55. In the positive mode, curtain gas was set at 30, collision gas at medium, ionspray voltage at 5,500 V, turbo-gas temperature at 600 °C, ion source gas 1 at 50, and ion source gas 2 at 50. The MRM ion pairs for the target compounds were set as one quantifying ion pair and one qualifying ion pair. The MRM ion pair for the IS was set as one qualifying ion pair. Table 1 presents the retention time (RT), precursor ion, product ions, declustering potential (DP), entrance potential (EP), collision energy (CE), and collision-cell exit potential (CXP) for the standards and IS.

### Method validation

The validation followed the Food and Drug Administration Bioanalytical Method Validation Guidance for Industry, whereby the following were tested for effectiveness: selectivity, limit of detection (LOD), LLOQ, linearity, accuracy, precision, recovery (RE), matrix effect (ME),

**Table 1** The retention time and multiple-reaction monitoring (MRM) parameters of target compounds and deuterium-labeled internal standards

| Compound | RT (min) | Ions monitored (m/z) | | MRM parameters of quantifier ion | | | |
|---|---|---|---|---|---|---|---|
| | | Quantifier | Qualifier | DP | EP | CE | CXP |
| EtG | 2.5 | 85.0 | 75.0 | −45.0 | −10.0 | −20.0 | −11.0 |
| COC | 5.6 | 182.1 | 77.0 | 16.0 | 10.0 | 23.0 | 24.0 |
| CE | 5.9 | 82.0 | 196.1 | 1.0 | 10.0 | 47.0 | 8.0 |
| BZE | 5.5 | 168.1 | 77.0 | 61.0 | 10.0 | 27.0 | 6.0 |
| EtG-$d_5$ | 2.5 | 85.0 | – | −20.0 | −10.0 | −20.0 | −7.0 |
| COC-$d_3$ | 5.6 | 185.1 | – | 71.0 | 10.0 | 27.0 | 16.0 |
| CE-$d_3$ | 5.9 | 199.1 | – | 81.0 | 10.0 | 27.0 | 14.0 |
| BZE-$d_3$ | 5.5 | 171.1 | – | 131.0 | 10.0 | 25.0 | 14.0 |

process efficiency (PE), and stability (The Food and Drug Administration 2018).

To test the selectivity, the blank sample contained hair from an individual who did not consume alcohol or use COC. This is to find any interfering compounds that could affect the analysis of the target compounds and IS in the RT. To test the LOD and LLOQ, the signal-to-noise ratio (S/N) was calculated. The criteria were as follows: S/N > 3 for LOD and S/N > 10 with ± 20% accuracy, and 20% precision for LLOQ. The calibration curve was expressed as the ratio of peak areas between the target compounds and IS, and the linearity was verified by calculating the correlation coefficient (r) of the calibration curve. In the tests of intra-day and inter-day accuracy and precision, LLOQ and QC samples of three concentrations (low, intermediate, and high) were used for measurements, and using six replicates of each concentration, four repeated inter-day experiments were performed. In the tests of RE, ME, and PE, six replicates of low, intermediate, and high concentrations of QC samples were used for measurements. The tested samples were as follows: Sample (A) of the solution with the standards free of pre-treatment; Sample (B) of the blank sample to which standards were added, followed by pre-treatment; Sample (C) of the blank sample for which pre-treatment was given, followed by the addition of standards. The applied formula was ME (%) = B/A × 100, RE (%) = C/B × 100, and PE (%) = C/A × 100 = (ME × RE)/100. In the test of stability, six replicates of low and high concentrations of QC samples were used for measurements. The analyzed QC samples were stored in an autosampler set at 4 °C for 24 h, then reanalyzed to test the autosampler stability.

## Results and discussion

### Evaluation of the extraction

To achieve the LLOQ of EtG with relatively low sensitivity in a simultaneous multi-compound analysis, solid-phase extraction was evaluated using dilution-and-shoot, Clean screen EtG (Clean screen FAST EtG, 200 mg, 3 mL, Bristol, PA, USA), and mixed-mode anion exchange solid-phase extraction (MAX), and the peak height and signal-to-noise ratio (S/N) of target compounds were compared. All three pre-treatment methods used 30 mg of pulverized hair for 1 h of ultrasonic extraction by water at ambient temperature, followed by 5 min of centrifugation (25,000 g, 4 °C). In dilution-and-shoot, the supernatant (450 μL) from centrifuged hair extract (600 μL) in a 2-mL centrifuge tube was transferred to a 1.5-mL centrifuge tube to be diluted with acetonitrile (900 μL) and mixed by vortex mixer. The hair-sample mixture after dilution with acetonitrile was centrifuged (50,000 g, 4 °C) for 5 min, and the supernatant (1300 μL) was transferred to a test tube to be dried (60 °C, 25 min) in $N_2$ condition. The final sample residue was re-dissolved in the solvent (80 μL) containing 0.1% acetic acid and acetonitrile in a 95:5 ratio (v/v), of which 8 μL was injected into the LC–MS/MS. In Clean screen EtG, supernatant (700 μL) from centrifuged hair extract (1,000 μL) in a 2-mL centrifuge tube was transferred to a 1.5-mL centrifuge tube and water (700 μL) was added for mixing by a vortex mixer. The mixture was centrifuged for 5 min at 4 °C and 25,000 g, and supernatant (800 μL) was transferred to the 1.5-mL centrifuge tube containing the supernatant of the first extract for another 5 min centrifugation at 4 °C and 25,000 g. The final hair extract was applied as follows: supernatant (1,450 μL) was adsorbed onto a Clean screen EtG cartridge activated with methanol (2 mL) containing 1% formic acid (2 mL), which was followed by washing with water (2 mL) and drying in vacuum condition for 10 min. The dried cartridge was used in the elution with the test tube of methanol (1.5 mL) containing 1% formic acid, and the eluate was dried in $N_2$ conditions and at 40 °C for 25 min. The final sample residue was re-dissolved in the solvent (80 μL) containing 0.1% acetic acid and acetonitrile in a 95:5 ratio (v/v), of which 8 μL was injected into the LC–MS/MS. The results indicated that only EtG but not COC, CE, or BZE was detected when the hair sample was pre-treated with Clean screen EtG, and with MAX, compared to dilution-and-shoot, the relative S/N of EtG, CE, and BZE was improved to 159%, 22%, and 66%, respectively (Fig. 2).

Additionally, the maximum volume of the eluent (methanol containing 2% formic acid) for MAX was evaluated to increase the EtG RE. The maximum eluent volume was found to satisfy the conditions for both maximum RE and the shortest drying time. The eluate was eluted five times in sequence by 0.5 mL each time, and the peak area of the target compound from each fraction was compared. For EtG, 1.0 mL and 1.5 mL of eluent volume led to 95% and 98% elution, respectively. For COC, CE, and BZE, 1.5 mL of eluent volume led to 84–96% elution (Fig. 3). In the case of COC, compared to the other compounds, the rate of elution was low (84%), but eluent volumes above 1.5 mL were considered inappropriate as the drying time exceeded 30 min. In addition, the RE at the eluent volume of 1.5 mL was 89.2% or higher for all target compounds, indicating that the level was acceptable, and the drying time of residual eluate was 25 min or lower, while the elution rate of EtG was 98%. Considering the elution time, elution rate, and RE, the eluent volume was set to 1.5 mL.

### Optimization of LC–MS/MS conditions

To ensure adequate levels of target compound isolation and sensitivity, two columns were compared. For EtG, in particular, higher sensitivity than other

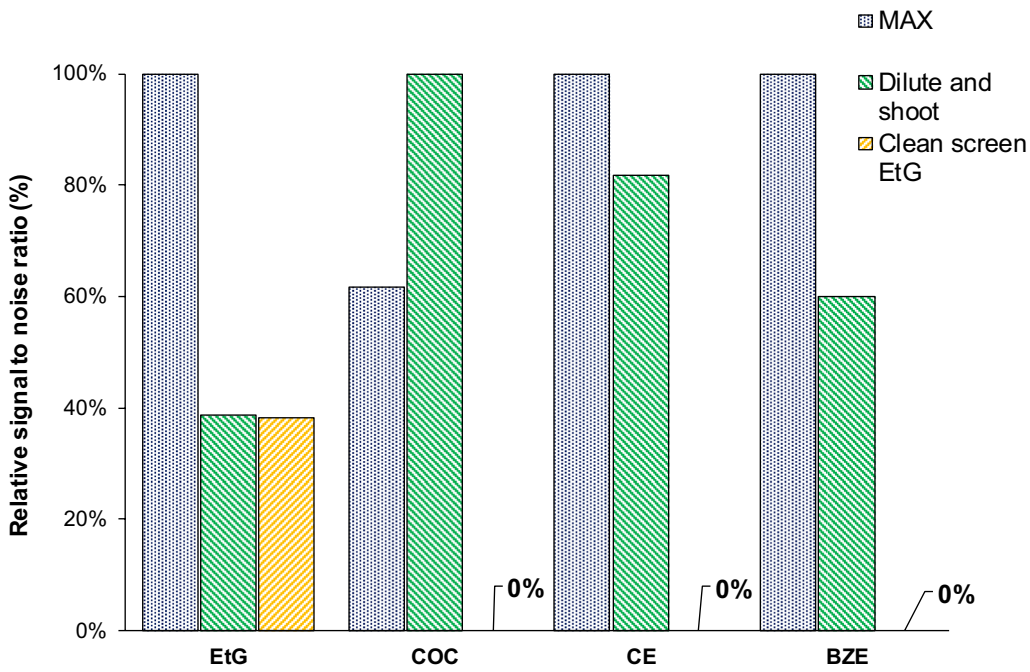


**Fig. 2** Comparison of sample preparation methods of mixed-mode anion exchange solid-phase extraction (MAX), dilution-and-shoot, and Clean screen EtG for EtG, COC, CE, and BZE

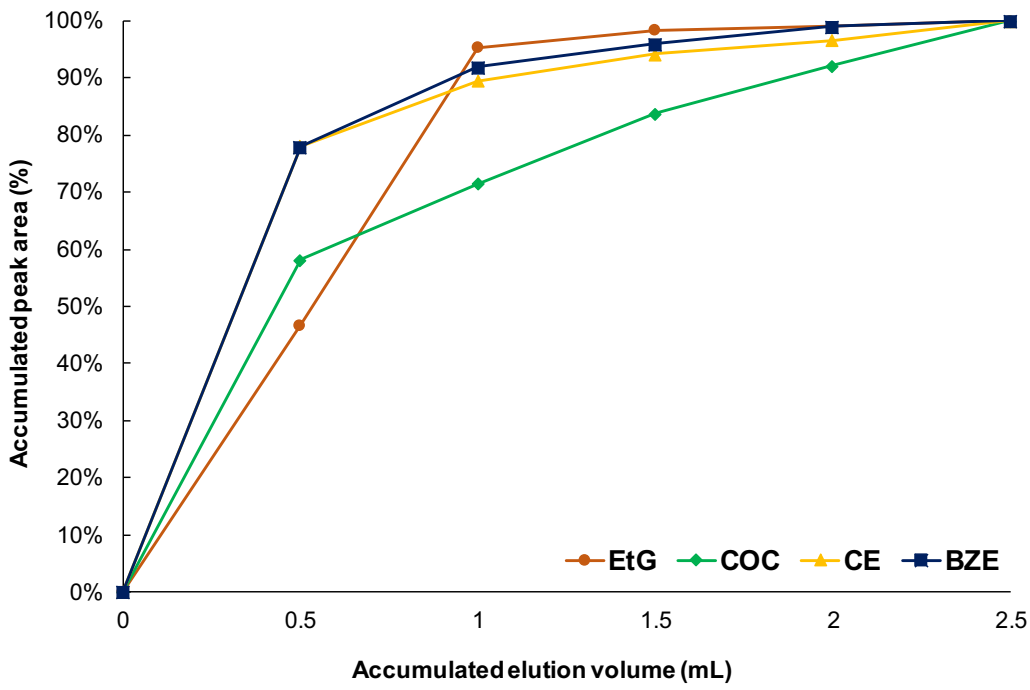


**Fig. 3** Relationship of MAX eluent volume and accumulate peak area for EtG, COC, CE, and BZE

compounds is required, and hence, the focus was on testing the S/N for EtG in comparing and selecting the column. The compared columns were Xselect HSS T3 XP C18 column (2.5 µm, 2.1 × 150 mm, Waters, USA) and Atlantis PREMIER BEH C18 AX column (2.5 µm, 2.1 × 100 mm, Waters, USA). The selected column that

led to an adequate level of isolation with EtG peak symmetry was Xselect HSS T3 XP C18, whereby two-fold higher S/N than Atlantis PREMIER BEH C18 AX was obtained (Fig. 4).

In another approach to achieve the LLOQ of EtG, the chromatography efficiency was compared for the target compounds in varying mobile phase conditions. In mobile phase A, the concentrations of formic acid and acetic acid were adjusted to 0.01–0.5%. In mobile phase B, acetonitrile and methanol were selected to compare various mobile phase acid compositions. For the combinations of formic acid and acetonitrile, the peak symmetry and S/N were the best for the combination of 0.1% formic acid (mobile phase A) and acetonitrile (mobile phase B). For the combinations of acetic acid and acetonitrile, the best result was obtained from the combination of 0.1% acetic acid (mobile phase A) and acetonitrile (mobile phase B). For the combinations of formic acid and methanol, the best result was obtained from the combination of 0.5% formic acid (mobile phase A) and methanol (mobile phase B). For the combinations of acetic acid and methanol, the composition with the most outstanding result was 0.1% acetic acid (mobile phase A) and methanol (mobile phase B). Among the four compositions, that of 0.1% acetic acid and acetonitrile led to the best peak symmetry and S/N (Fig. 5). The optimal RT for EtG was 2.5 min.

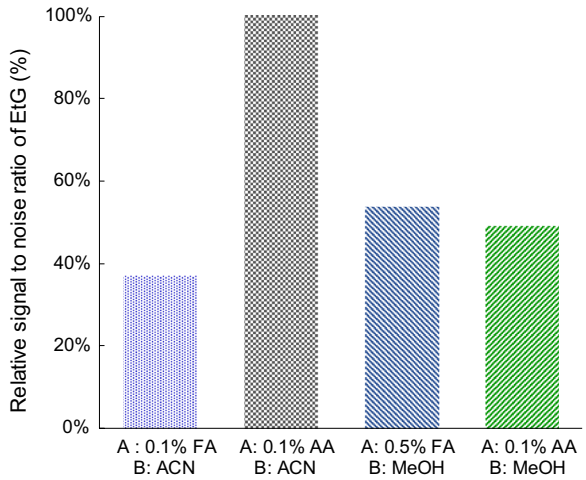


**Fig. 5** Relative S/N ratio of EtG for four types of mobile phases. Mobile phase (**A**) was 0.1% acetic acid (0.1% AA), 0.1% fomic acid (0.1% FA), and 0.5% formic acid (0.5% FA) in $H_2O$. Mobile phase (**B**) was acetonitrile (ACN) and methanol (MeOH)

## Method validation

Figure 6 presents the result of sample analysis for the blank hair sample with IS (Fig. 6a) and target compounds at low quality control (QC) (Fig. 6b) concentrations. No interfering compound was present in the blank hair sample that affected the analyses of COC, CE, and BZE. In the case of EtG, a trace amount may be detected even without any alcohol consumption owing to the intake of foods containing a small amount of ethanol, such

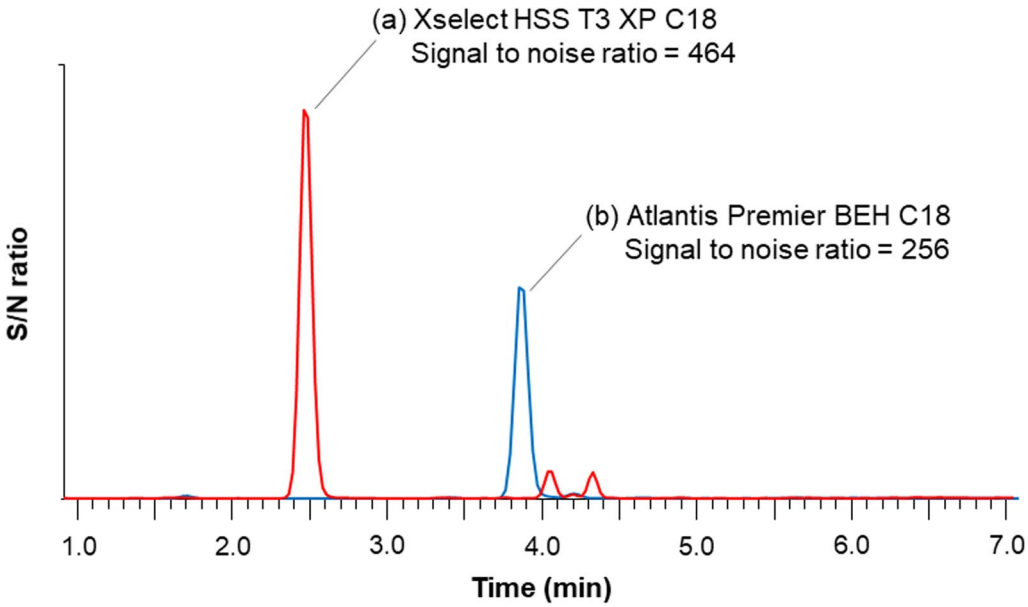


**Fig. 4** Comparison of two different types of C18 columns for EtG: **a** Xselect HSS T3 XP C18 column (2.5 µm, 2.1 × 150 mm particle size) and **b** Atlantis PREMIER BEH C18 AX column (2.5 µm, 2.1 × 100 mm particle size)

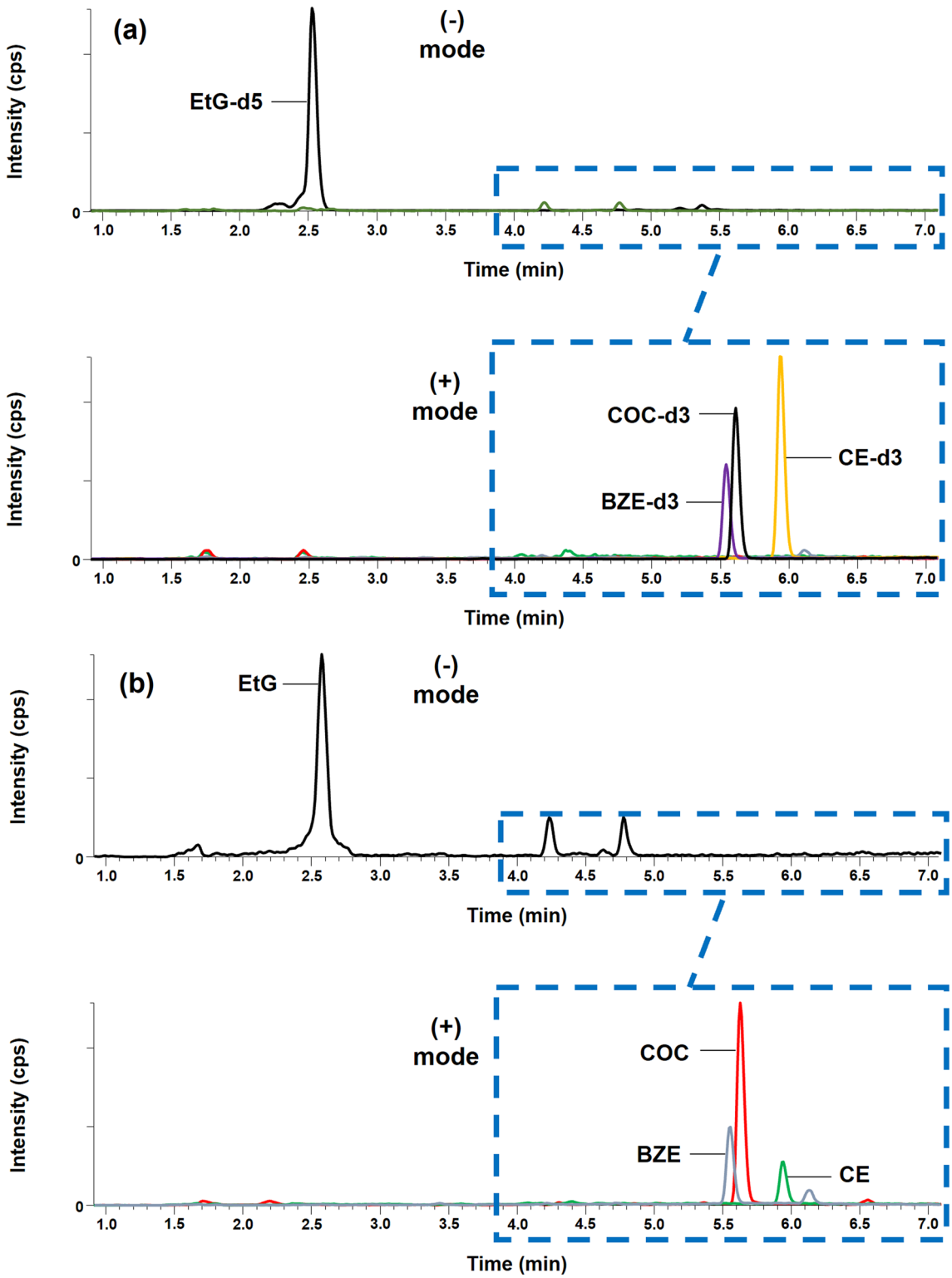


**Fig. 6** LC–MS/MS chromatograms of (**a**) blank hair sample with IS, (**b**) low QC hair sample containing 20 pg/mg for EtG, 5 pg/mg for COC, 25 pg/mg for CE, and 2.5 pg/mg for BZE, respectively, (**c**) positive hair sample for all compounds (forensic sample-1), and (**d**) EtG-positive sample (forensic sample-2). Positive ionization mode was performed for COC and its metabolites, whereas EtG was operated in negative ionization mode

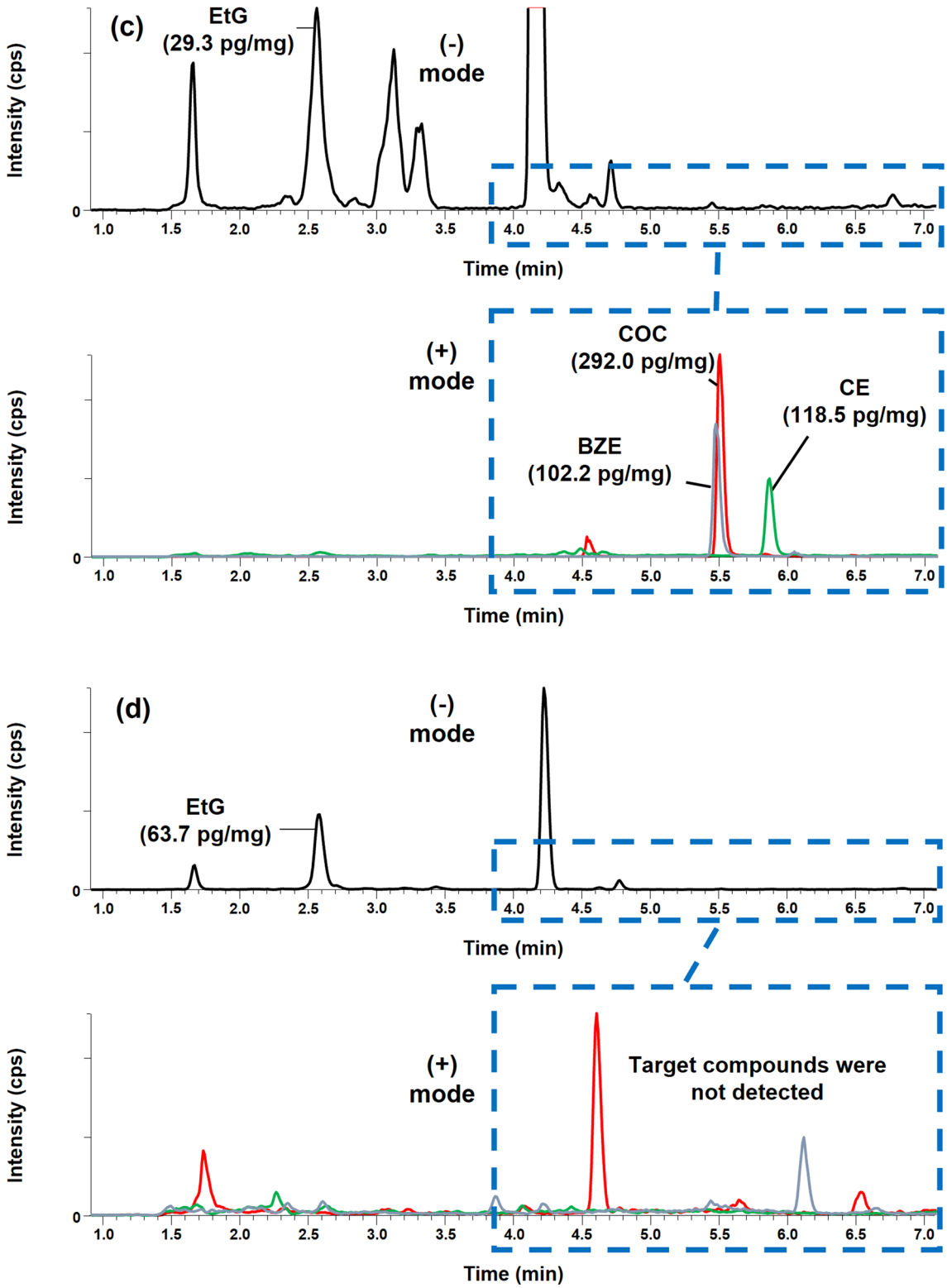


**Fig. 6** continued

as non-alcoholic beer, fermented foods, and ripened bananas left at room temperature for a long time (Musshoff et al. 2018; Ostrovsky 1986; Rosano and Lin 2008; Stephanson et al. 2002). In fact, a peak of EtG around 20% or less of LLOQ peak area was detected in the hair of a nondrinker.

The correlation coefficient ($r$) of the calibration curve within the quantified range of target compounds was ≥ 0.9978, verifying the linearity. The determined LOD and LLOQ for each compound were: 2 pg/mg and 7 pg/mg for EtG; 0.5 pg/mg and 2 pg/mg for COC; 2 pg/mg and 10 pg/mg for CE, and 0.3 pg/mg and 1 pg/mg for BZE, respectively (Table 2).

As can be seen in Table 3, the intra-day accuracy and inter-day accuracy for EtG were − 5.4–4.5% and − 9.3–0.7%, respectively. For COC and its metabolites, the intra-day and inter-day accuracies were − 6.1–4.0% and − 4.1–3.5% for COC; − 4.1–9.7% and − 1.5–3.6% for CE; − 3.4–8.5% and − 4.4–8.3% for BZE. The intra-day precision and inter-day precision for EtG were 3.7–10.3% and 1.0–14.4%, respectively. For COC and its metabolites, the intra-day and inter-day precisions were 0.5–5.9% and 0.6–3.9% for COC; 0.9–10.0% and 2.1–4.3% for CE; 1.9–8.4% and 3.2–7.7% for BZE. The observed accuracy and precision levels were approximately ± 15.0%, verifying the reliability of the analyses.

The RE, ME, and PE for target compounds were determined using low, intermediate, and high concentrations of QC samples, and the measured values were 89.2–104.8%, 81.6–105.4%, and 81.5–107.1%, respectively. To verify the stability of the target compounds in the hair samples after pre-treatment, the analyses were repeated using the samples stored in an autosampler at 4 °C for 24 h. The change in the measured levels in the samples after 24 h in the autosampler was 96.6–109.7%. Based on this result, the stability of the target compounds in

**Table 2** Limit of detection (LOD), lower limit of quantification (LLOQ), linearity, and calibration curve

| Compound | LOD (pg/mg) | LLOQ (pg/mg) | Calibration curve ($n = 3$) | | | |
|---|---|---|---|---|---|---|
| | | | Calibration range (pg/mg) | $r$ | Slope | y-Intercept |
| EtG | 2 | 7 | 7–700 | 0.9978 | 0.0028 ± 0.0001 | 0.0077 ± 0.0025 |
| COC | 0.5 | 2 | 2–200 | 0.9989 | 0.0883 ± 0.0020 | 0.0419 ± 0.0051 |
| CE | 2 | 10 | 10–1000 | 0.9987 | 0.0023 ± 0.0000 | 0.0046 ± 0.0009 |
| BZE | 0.3 | 1 | 1–100 | 0.9989 | 0.1089 ± 0.0030 | 0.0246 ± 0.0125 |

**Table 3** Inter- and Intra-day accuracy and precision, recovery (RE), matrix effect (ME), process efficiency (PE), and autosampler stability (AS) of target compounds in hair

| Analyte | QC sample (pg/mg) | Inter-day ($n = 24$) | | Intra-day ($n = 6$) | | RE ($n = 6$) (%) | ME ($n = 6$) (%) | PE ($n = 6$) (%) | AS stability ($n = 6$) (%) |
|---|---|---|---|---|---|---|---|---|---|
| | | % bias | % CV | % bias | % CV | | | | |
| EtG | 7 | − 9.3 | 14.4 | 2.3 | 10.3 | – | – | – | – |
| | 20 | 0.7 | 4.0 | 4.5 | 4.8 | 89.2 | 91.4 | 81.5 | 109.7 |
| | 100 | − 1.8 | 1.4 | − 2.7 | 3.7 | 98.5 | 88.0 | 86.7 | – |
| | 500 | − 3.9 | 1.0 | − 5.4 | 5.9 | 104.5 | 96.6 | 100.9 | 101.2 |
| COC | 2 | − 3.1 | 3.9 | 1.9 | 5.9 | – | – | – | – |
| | 5 | 3.0 | 0.6 | 4.0 | 1.4 | 100.5 | 81.7 | 82.0 | 96.6 |
| | 25 | 3.5 | 1.4 | 2.0 | 0.5 | 100.0 | 81.6 | 81.6 | – |
| | 125 | − 4.1 | 1.5 | − 6.1 | 1.6 | 99.6 | 83.1 | 82.8 | 100.8 |
| CE | 10 | 3.4 | 4.3 | 9.7 | 10.0 | – | – | – | – |
| | 25 | 3.6 | 2.1 | 5.7 | 7.2 | 104.8 | 96.2 | 100.8 | 99.9 |
| | 125 | 3.6 | 2.2 | 2.7 | 1.8 | 97.2 | 89.9 | 87.3 | – |
| | 625 | − 1.5 | 2.6 | − 4.1 | 0.9 | 100.1 | 82.9 | 83.0 | 100.3 |
| BZE | 1 | − 4.4 | 7.7 | 6.4 | 8.4 | – | – | – | – |
| | 2.5 | 3.7 | 3.2 | 8.5 | 1.9 | 97.5 | 105.1 | 102.5 | 99.6 |
| | 12.5 | 8.3 | 4.8 | 5.1 | 3.9 | 96.6 | 101.9 | 98.4 | – |
| | 62.5 | − 0.1 | 3.3 | − 3.4 | 2.8 | 101.7 | 105.4 | 107.1 | 99.9 |

samples stored in such conditions was verified, implying a lack of sample quality degradation in the reanalysis after 24 h.

### Forensic applications

The concurrent use of alcohol and COC was determined by testing the EtG and CE in hair samples from individuals ($n = 26$) suspected of using alcohol and COC. Figure 6 presents the chromatogram and concentration of each target compound in positive hair samples. The concentration of EtG in forensic sample-1 (Fig. 6c), which tested positive, was 29.3 pg/mg, indicating a social drinker level based on the SoHT criteria. This hair also showed COC (292.0 pg/mg) and its metabolites CE (118.5 pg/mg) and BZE (102.2 pg/mg), indicating that the hair was from an individual who concurrently used alcohol and COC. In forensic sample-2 (Fig. 6d), which was tested positive, the concentration of EtG was 63.7 pg/mg, but no COC, CE, or BZE was detected. This indicated that the hair was from a social drinker who did not use COC. By contrast, the detection of COC and BZE without EtG or CE would have indicated that the hair was from a COC user who did not consume alcohol.

## Conclusions

In this study, an LC–MS/MS-based method was developed to simultaneously analyze COC, CE, BZE, and EtG. First, to increase the extraction efficiency of the target compounds from hair, the hair pulverization method was used according to the recommendations of the SoHT. Notably, a sonicator at constant ambient temperature was used for short-time (1 h) extraction to minimize the hair matrix effect, while an ultracentrifuge (50,000 g) at low temperature (4 °C) was used for effective pellet precipitation of the pulverized hair to effectively remove the background noise from the substrate matrix. In addition, MAX cartridge was used to remove the matrix effect and maximize the process efficiency. To achieve high sensitivity for EtG, 0.1% acetic acid and acetonitrile were selected as the optimal mobile phase combination, and at the same time, an optimal pre-treatment method was developed to ensure optimal conditions for the setting of simultaneous analyses with more varied limitations than independent analyses. As a result, the method developed in this study was shown to enable rapid and simultaneous analyses with no interfering compounds when applied to the hair sample from an individual who concurrently used COC and alcohol. Further studies must collect a diverse set of data based on hair from people who use alcohol and COC concurrently to investigate additional metabolite patterns and a larger number of positive samples to expand the usefulness of the test using the developed analytical approach. The findings of this study will be a valuable resource in narcotics investigations where independent and concurrent use of alcohol and COC should be determined, as well as in alcohol dependence tests.

### Abbreviations

| | |
|---|---|
| BZE | Benzoylecgonine |
| CE | Cocaethylene |
| CE | Collision energy |
| COC | Cocaine |
| CXP | Collision-cell exit potential |
| DP | Declustering potential |
| ME | Matrix effect |
| MRM | Multiple-reaction monitoring |
| QC | Quality control |
| RE | Recovery |
| EP | Entrance potential |
| EtG | Ethyl glucuronide |
| IS | Internal standard |
| LC–MS/MS | Liquid chromatography–tandem mass spectrometry |
| LLOQ | Lower limit of quantification |
| RT | Retention time |
| S/N | Signal-to-noise ratio |
| SoHT | Society of hair testing |

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### Author contributions

JY contributed to the design of the study. DW performed the experiments and analyzed the data. SY and SI gave their valuable suggestions in this study. DW and JY wrote the manuscript. All authors read and approved the final manuscript.

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### Availability of data and materials

Not applicable.

## Declarations

### Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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## Publisher's Note