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Simultaneous quantification of methanol and ethanol in alcoholic beverage using a rapid gas chromatographic method coupling with dual internal standards

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Abstract

This study presented a direct GC method using dual internal standards to simultaneously determine ethanol and methanol in alcoholic beverages. The sample should only be spiked with proper amount of internal standards (100 μg 2-pentanol and 50 mg acetonitrile), and then directly injected into a mega-pore capillary column (CP-Wax 58 CB) with FID detector for GC analysis. The accurate content of ethanol and methanol in beverages could be obtained in less than 12 min. The reliability, accuracy and precision of the presented method were examined with the test of standard addition and recovery, comparison with standard methods, and the variation between intra-day and inter-day tests. All results from above tests suggested that the direct GC method presented in this study was as reliable as standard method proposed by AOAC but more rapid.

Keywords: Methanol; Ethanol; Alcoholic beverages; GC

1. Introduction

Content of ethanol is one of crucial elements to mouth feeling of an alcoholic beverage, and it is also the basis for taxing the products (Collins, Miller, Altria, & Waterhouse, 1997). The higher ethanol content in a product means more tax shall be imposed on. Thus, to establish an accurate and rapid method for determination of ethanol content in beverages is a very important task. Contrary to ethanol, methanol is a casual byproduct during wine fermentation, which is derived from degradation of pectic substance in raw materials (Bindler, Voges, & Laugel, 1988). Methanol is also a notorious ingredient used illegally in production of imitated spirits and wine, which killed or blinded people in some developing countries almost every year. Methanol is toxic to human health. Accidental intake of methanol would cause victim headache, vertigo, fatigue, nausea, vomiting, blurred vision, irreversible blindness and even death (Bindler et al., 1988). Accordingly, to develop a simple and rapid but also accurate method capable of determining the content of methanol and ethanol simultaneously is urgently required, especially to the countries popular with fake or imported spirits and wine.

There are plenty of methods for the determination of methanol and ethanol, respectively. For example, rosaniline hydrochloride colorimetric method (Upadhyay & Gupya, 1984), chromotropic acid colorimetric method (AOAC, 1998a), titrimetric method (AOAC, 1998b), enzymatic method (Mizgunova, Zolotova, & Dolmanova, 1996), chemical sensor detection (Sun & Okada, 2000), biosensor detection (Sekine, Suzuki, Takeuchi, Tamiya, & Karube, 1993), high performance liquid chromatography (HPLC) (Chen et al., 1998; Kuo, Wen, Huang, Wu, & Wu, 2002; Tagliaro, Dorizzi, Ghielmi, & Marigo, 1991), near-infrared spectroscopy (Van den Berg, van Osenbruggen, & Smilde, 1997), and gas chromatography (GC) (AOAC, 1998c; Cheung & Lin, 1987; Lee, Acree, & Butts, 1975; Liu, Liu, Zhang, & Zhang, 2001; Pollack & Kawagoa, 1991; Wilson, Ding,

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& Woods, 1991) have been developed to determine methanol content in different samples. As to ethanol, due to its content usually high in sample, measuring specific weight was a common method to quantify ethanol in wine industry. Besides, dichromate oxidation method proposed by AOAC was another popular method routinely used in many respects (AOAC, 1990). All published methods for above alcohol determination have several common defects from the view of rapidity and accuracy. Firstly, they could not simultaneously determine methanol and ethanol in wine and spirit sample, because the content of ethanol are usually thousands folds higher than that of methanol in a sample. The methanol content in wine and spirit are generally below 1 mg/mL, but ethanol is the major component in wine and spirit which could be as high as 40–60% in a sample. Secondly, most accurate methods, e.g., HPLC and GC, required time-consuming pretreatment process for obtaining reliable data (Blanch, Tabera, Sanz, Herraiz, & Reglero, 1992; Cheung & Lin, 1987; Davoli, Cappellini, Airoldi, & Fanelli, 1986; Pereira, Santos, Ferreira, & Andrade, 1999; Pollack & Kawagoa, 1991). Thirdly, some rapid methods could not distinguish methanol from ethanol if they were coexisting in a sample (AOAC, 1998a, 1998c).

In our lab, we have developed a GC method coupling with a capillary column to successfully measure the content of methanol in several alcoholic beverages (paper in publishing). Due to highly different in content, methanol and ethanol in a sample could not be determined in one shot. Therefore, the aim of this study was made to test if contents of the two alcohols in an alcoholic beverage could be determined simultaneously when two internal standards were used. Modification of previous method for method determination was also discussed.

2. Material and methods

2.1. Samples and reagents

Alcoholic beverage samples were purchased from supermarkets located at Pingtung or Tainan area. The sample of whisky and red wine was imported from France. Distilled rice wine, medicinal wine and Gaoliang were produced from Taiwan Tobacco and Liquor Corporation. Liquid-chromatographic-grade solvents (purity above 99.5%), such as methanol, ethanol, acetonitrile, 1-propanol, 1-butanol, tert-butanol, 2-pentanol, amyl alcohol and isoamyl alcohol were purchased from TCI (Tokyo, Japan). Standard solutions for methanol and 2-pentanol were made up with 100 mg in 100 mL distilled water (0.1% w/v), respectively. These for ethanol and acetonitrile were made up with 5 g in 100 mL distilled water (5% w/v), respectively.

2.2. GC condition for analysis

The analysis of methanol was conducted in a Trace GC 2000 GC (TermoQuest, Milan, Italy) equipped with a computer containing an integrator software (ChromCard version 1.06 for Trace GC, TermoQuest, Milan, Italy), a 30 m CP-Wax 58 CB mega-pore capillary column (0.53 mm id, film thickness: 1.5 μm; ChromPack, Netherlands) and a flame ionizing detector (H_2: 30 mL/min and air: 300 mL/min). Flow rate of carrier gas nitrogen was set at 3 mL/min. The temperature at injector port and detector was set at 210 and 280 °C, respectively, and splitless injection (about 0.1 μl for each injection) was used. In order to avoid cross contamination between samples, the syringe for injection was completely dried out by heating with a lighter for several seconds after rinsing the syringe with distilled water. Oven temperature was controlled with a temperature elevation program during analysis, which was initially set at 39 °C for 3 min, initially elevated temperature to 65 °C at the rate of 5 °C/min, then continuingly elevated to 250 °C at the rate of 50 °C/min and maintained for 1 min.

2.3. Calculation of Relative Response Factor (RRF)

In this study, 2-pentanol (2-P) was used as internal standard for methanol (MeOH), and acetonitrile (AN) was for ethanol (EtOH). Standard solutions of methanol and 2-pentanol (both in 0.1% w/v) and ethanol and acetontrile (both in 5% w/v) were mixed as following combinations: 20:1,10:1, 5:1, 2:1, 1:1, 1:2,1:5, 1:10, 1:20 (v/v) etc., then subjected to GC analysis. In order to calculate RRF value, peak area ratio of alcohol to internal standard (EtOH/AN or MeOH/2-P) was regressed on their concentration ratio. According to the Eq. (1), the slope of the regression equation was used as RRF value for analysis.

$$(A_1)/(A_2) = \mathrm{RRF}_{(\mathrm{MeOH/2-P\,or\,EtOH/AN})} \times (W_1)/(W_2), \quad (1)$$

where A_1, peak area of methanol or ethanol obtained from GC analysis; A_2, peak area of 2-pentanol or acetonitrile obtained from GC analysis; W_1, weight in μg of methanol or weight in mg of ethanol used in analysis; W_2, weight in μg of 2-pentanol or weight in mg of acetonitrile used in analysis.

The r^2 values of two regression equations for determination of RRF were found to be higher than 0.999.

2.4. Measurement of alcohol content in beverages

Determination of the content of methanol and ethanol in beverages was conducted by direct injection GC method coupling with dual internal standards. Practically, 1 mL of sample was mixed with 100 μg 2-pentanol in 0.1 mL and 50 mg acetonitrile in 1 mL, then 0.1 μL

mixture was subjected to GC analysis. The contents of alcohol were calculated according to below equations.

$$\text{Methanol}(\mu g/mL) = (A_{MeOH}/A_{2\text{-P}}) \times (W_{2\text{-P}}/RRF_{MeOH/2\text{-P}}) \times 1/V,$$

$$\text{Ethanol}(mg/mL) = (A_{EtOH}/A_{AN}) \times (W_{AN}/RRF_{EtOH/AN}) \times 1/V,$$

where V, volume of sample in mL.

The GC method developed in this study was compared with three standard methods suggested by AOAC. A spectrophotometric method (AOAC, 1998a) and a GC method (AOAC, 1998c) were used to check methanol quantification, and dichromate oxidation method (AOAC, 1990) was used to check ethanol quantification. The comparison of alcohol quantification was conducted on a same whisky sample. Firstly, the whisky sample was distilled with water vapor. In order to determine methanol content in whisky sample, the distillate was further oxidized with potassium permanganate, reacted with chromotropic acid, and then the changes of color intensity were read at 580 nm. Or, with addition of 3 mL 1% *n*-butanol as internal standard to 50 mL distillate, following with making up sample volume to 100 mL, then 1 μL of sample mixture was injected to GC analysis. For ethanol quantification, the distillate was oxidized with dichromate, followed by titration of excess amount of dichromate with ferrous oxide to measure ethanol content.

2.5. Limit of quantification (LOQ) of direct GC method

Standard methanol with 1.0 mg/mL and ethanol with 100 mg/mL were added separately with distilled water to prepare a serial dilution. Each dilution was mixed with 0.1 mL 0.1% 2-pentanol for methanol quantification and 1 mL 5% acetonitrile for ethanol quantification, and then subjected to GC analysis. Limit of quantification (LOQ) was determined when coefficient of variation of recovery was higher than 15%.

2.6. Recovery test

The standard addition was conducted with supplementing 100 or 500 μg methanol and 50 or 200 mg ethanol into a whisky or Gao-liang sample, followed by adding with 0.1 mL 0.1% 2-pentanol and 1 mL 5% acetonitrile. The recovery of methanol and ethanol from spirits was determined by triplicate tests using direct GC analysis.

2.7. Determination of the validation of direct GC method

A mixture containing standard methanol and ethanol was prepared with the concentration ratio (μg methanol per mL/mg ethanol per mL) of 40/20, 80/50, 200/100, 400/250, and 1000/500, respectively. Each of 1 mL above standard alcohol mixture was added with 0.1 mL 100 μg 2-pentanol and 1 mL 50 mg acetonitrile, mixed well, and then subjected 0.1 μL of mixture into GC analysis. The determination of alcohol content in the same sample was conducted three times (morning, noon, and night) in a day and the following three days. Precision of the proposed method was evaluated by coefficient of variation (CV) of the tests. Accuracy of the method was evaluated by determination of relative error of mean (REM) of the tests. Generally, less than 5% is an acceptable value to determine the precision and accuracy of a method.

3. Results and discussion

In order to develop a method simultaneously with rapidity and accuracy for wine industry and inspection sector in government to determine ethanol and toxic methanol, a direct injection GC method with dual internal standards was tested. Following with previous study (Wang, Choong, Lee, & Su, 2003), a polar column, CP-Wax 58 CB, for GC analysis was also used to determine alcohol in beverages by a direct injection method. The GC profile indicated that current method could separate at least 8 different alcoholic compounds clearly (data not shown). The retention time for methanol and ethanol revealed by this method was at 2.87 and 3.41 min, respectively. When 3 typical alcoholic beverages with distinct nature were subjected to above GC analytical system, as shown in Fig. 1, only acetonitrile and 2-pentanol was absent in the chromatogram comparing with standard profile. This result suggested that acetonitrile and 2-pentanol could be used as internal standard for this GC analysis. Due to the content of ethanol in alcoholic beverages always higher than that of methanol in several magnitudes, only one internal standard used is obviously not able to cover the determination of two alcohols in one GC analysis. In this study, we applied acetonitrile and 2-pentanol together as internal standards for simultaneous determination of ethanol and methanol in beverages by direct GC analysis. Because water solubility of acetonitrile is higher than 2-pentanol, we used acetonitrile as internal standard for ethanol and 2-pentanol for methanol. In order to reduce the time for GC analysis, oven temperature was elevated rapidly at 8 min of analysis to 250 °C, when most low molecular alcohols was eluted. With this treatment, an analysis of alcohols in a beverage sample could be finished within 12 min.

Relative response factor of ethanol and methanol to their internal standard was also determined. Results indicated RRF for ethanol to acetonitrile was 0.89 and that for methanol to 2-pentanol was 0.43. As shown in Fig. 2, the linear range ($r^2 > 0.999$) for measurement was from 5

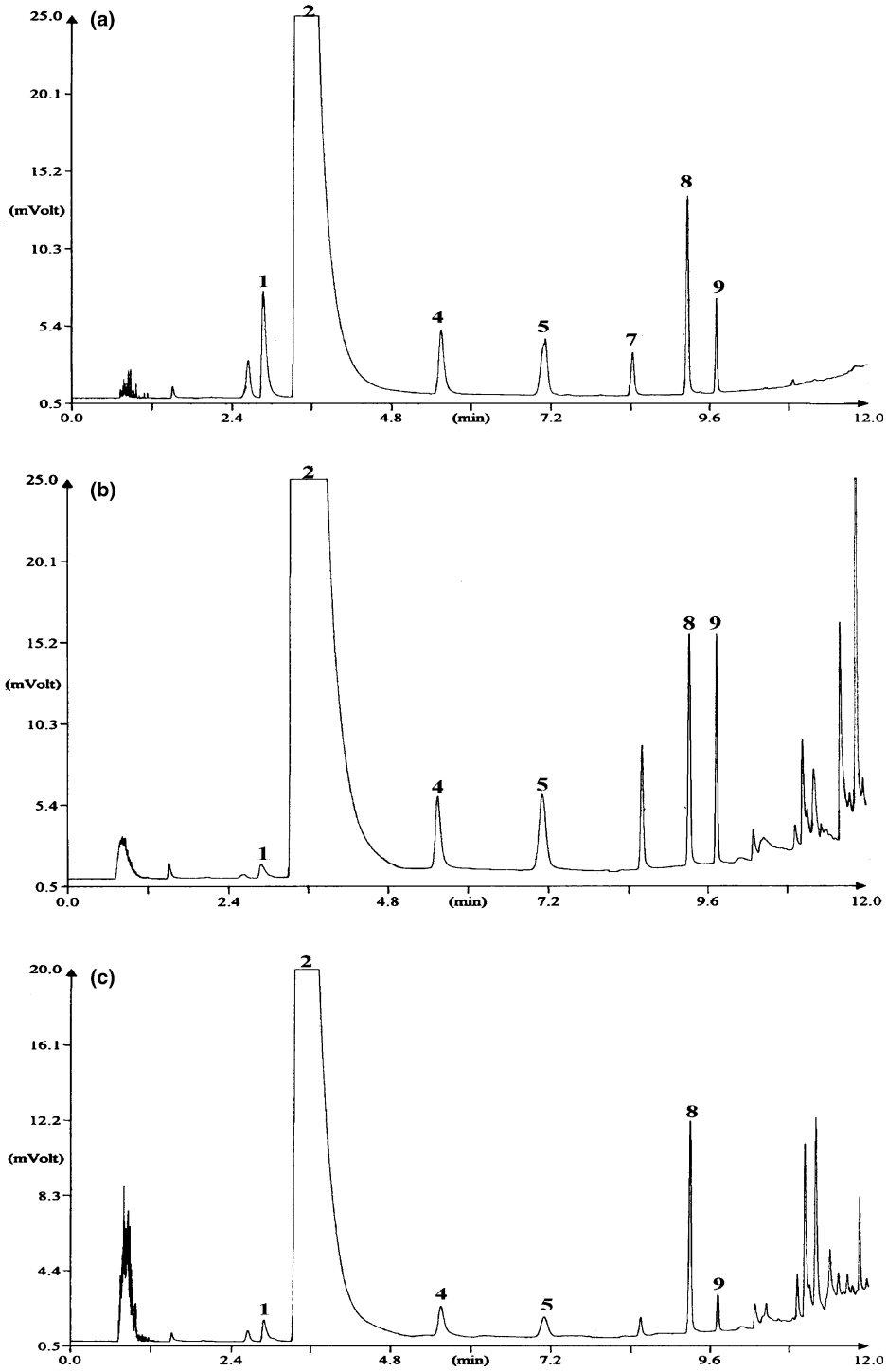

Fig. 1. Gas chromatograms of alcohols in three common commercial products. (a) whisky; (b) medicinal wine; (c) red wine. Peak identification: 1, methanol; 2, ethanol; 3, acetonitrile; 4, 1-propanol; 5, isobutanol; 6, 2-pentanol; 7, isoamyl alcohol; 8, amyl alcohol; 9, tert-butanol.

to 2000 μg/mL for methanol and 10–500 mg/mL for ethanol. The LOQ of this method was 5 μg/mL for methanol and 5 mg/mL for ethanol, when FID was used accompanying with the settings of range and attenuation at 1 and coefficient of variation was set at 15%.

The effect of addition of dual internal standards on the direct determination of methanol and ethanol was evaluated by comparing with single standard. In this study, the amount of internal standards was supplemented with 100 μg 2-pentanol in 0.1 mL and 50 mg acetonitrile in 1 mL. As shown in Table 1, neither the content of methanol nor that of ethanol showed significant difference between dual standard supplements and single standard supplement ($p < 0.05$), when whisky and

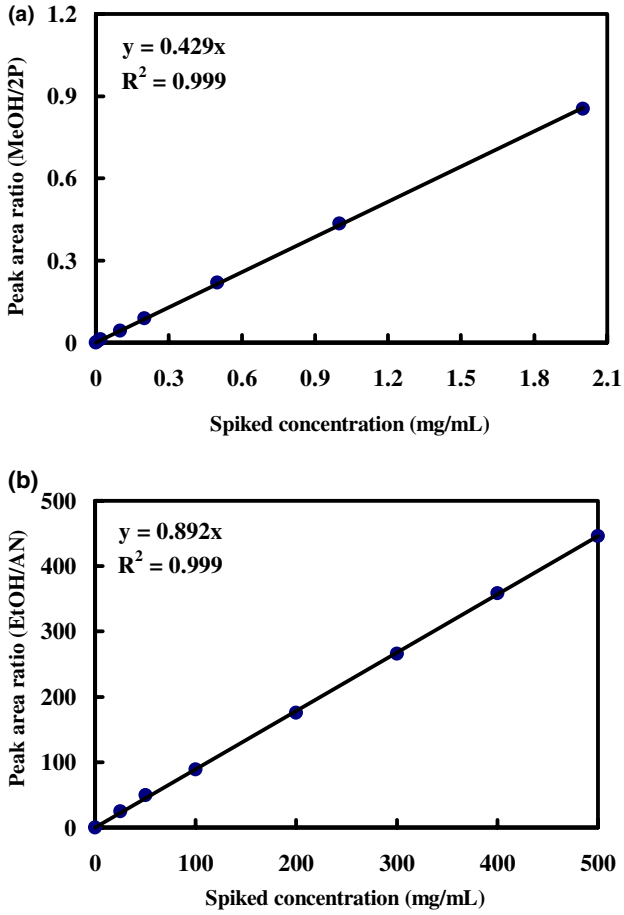

Fig. 2. Calibration curves for methanol and ethanol using direct GC method coupling with dual internal standards: (a) for methanol; (b) for ethanol.

distilled rice wine were tested. The low coefficient of variation (< 4.7%) shown in Table 1 also indicated that the quantification of alcohol by dual standard supplement was feasible and reliable.

The validation of presented method was further evaluated with three criteria, such as recovery, precision and accuracy, and comparison with AOAC standard method. Table 2 shows that the recovery of methanol addition ranged from 93.8% to 103.2% with coefficients of variation ranging from 2.8% to 4.9%; and that of ethanol addition ranged from 95.3% to 96.8% with coefficients of variation ranging from 2.8% to 4.8%, when either whisky or Gao-liang was tested with two levels of alcohol supplements. Precision of the method was determined by examining the coefficient variation of data obtained from intra-day and inter-day quantification on the same sample. As shown in Table 3, no matter the supplement level was as low as 40 μg in methanol determination and 20 mg in ethanol determination or as high as 1000 μg in methanol determination and 500 mg in ethanol determination, the coefficients of variation obtained from intra-day and inter-day quantification were all less than 5%. This result suggested that the precision of this method was as high as any published method. The accuracy of presented method was revealed by absolute value of relative error of mean obtained from the data shown in Table 3. The results indicated that the absolute values of relative error of mean were all less than 5.6%, suggesting that the accuracy of this method was generally comparable to any published method. The method presented in this study was also compared with three AOAC methods for determination of methanol and ethanol. As shown in Table 4, there were no significant difference found in methanol determination between AOAC spectrophotometric method (AOAC, 1998a), AOAC GC method (AOAC, 1998c), and proposed direct GC method ($p < 0.05$). And, the same result was also found between ethanol determination by AOAC titration method (AOAC, 1990) and proposed direct GC method ($p < 0.05$). Furthermore, the lowest coefficient of variation (2.9% and 3.5%) derived from proposed direct GC method, compared with that (6.4–8.7%) from AOAC method, also suggested that the direct GC method performed higher precision than AOAC methods.

Conclusively, the direct GC method presented in this study performed several advantages when comparing with traditional AOAC methods. Firstly, if determination of both methanol and ethanol was required, you needed to set up two protocols to deal with methanol and ethanol separately when AOAC methods were applied. Furthermore, when analyzing non-distilled

Table 1
Comparison of the way of addition of internal standard during quantification of methanol and ethanol in whisky and distilled rice wine by direct GC method

Standards[a]	Methanol (μg/mL)[b]		Ethanol (mg/mL)[b]	
	Whisky	Disted rice wine	Whisky	Disted rice wine
2-Pentanol	214 ± 10 (4.7)[c]	24 ± 1 (2.1)	ND	ND
2-Pentanol + acetonitrile	218 ± 9 (4.1)	24 ± 0 (1.7)	323 ± 14 (4.4)	156 ± 6 (3.9)
Acetonitrile	ND[d]	ND	332 ± 12 (3.7)	155 ± 5 (3.4)

[a] The amount of internal standard was added with 100 μg of 2-pentanol, 50 mg of acetonitrile, or both.
[b] The data were obtained from triplicate analyses and displayed in mean ± SD.
[c] The values in parentheses are coefficient of variation (CV %).
[d] ND: not detected.

Table 2
Recovery of methanol and ethanol from spiked commercial alcoholic beverage by direct injection GC method with dual internal standards

Samples	Spiked alcohols	Original content (A)[a]	Added amount (B)	Detected amount (C)[a]	Recovery (%)[b]	CV (%)[c]
		μg				
Whisky	Methanol	259 ± 3	100	353 ± 10	93.8	2.8
		259 ± 3	500	772 ± 29	102.6	3.7
		mg				
	Ethanol	318 ± 12	50	366 ± 10	96.8	2.8
		318 ± 12	200	509 ± 24	95.3	4.8
		μg				
Gao-liang	Methanol	185 ± 7	100	288 ± 11	103.2	3.6
		185 ± 7	500	673 ± 33	97.8	4.9
		mg				
	Ethanol	328 ± 12	50	376 ± 18	96.6	4.8
		328 ± 12	200	520 ± 21	96.4	4.0

[a] The data in original content and detected amount of alcohol were obtained from triplicate analyses and displayed in mean ± SD.
[b] Recovery (%) = (C−A)/B × 100%.
[c] CV: coefficient of variation.

Table 3
Evaluation of precision and accuracy for direct injection GC method to determine methanol and ethanol

Analyses[a]	Tested alcohols	Concentration applied	Concentration detected[b]	Precision CV (%)[c]	Accuracy REM (%)[d]
		(μg/mL)			
Intra-day test	Methanol	40	41 ± 1	2.7	2.8
($n = 3$)		80	80 ± 1	0.8	−0.5
		200	204 ± 4	1.8	1.8
		400	414 ± 19	4.6	3.6
		1000	1056 ± 48	4.5	5.6
		(mg/mL)			
	Ethanol	20	20 ± 0	1.5	−2.5
		50	52 ± 2	4.3	3.0
		100	98 ± 3	2.8	−2.4
		250	249 ± 12	4.7	−0.6
		500	513 ± 16	3.1	2.6
		(μg/mL)			
Inter-day test	Methanol	40	38 ± 1	1.8	5.3
($n = 9$)		80	81 ± 2	1.9	0.8
		200	205 ± 5	2.5	2.4
		400	387 ± 20	5.1	−3.3
		1000	1046 ± 53	5.0	4.6
		(mg/mL)			
	Ethanol	20	19 ± 1	4.1	−3.5
		50	50 ± 2	3.8	−1.0
		100	102 ± 4	3.4	2.4
		250	253 ± 10	3.8	1.6
		500	521 ± 12	2.3	4.1

[a] Intra-day test was conducted with three independent GC analysis within a day, and inter-day test was conducted with three independent GC analysis at each day following with the same analysis for the successive three days.
[b] The data of concentration detected was shown in mean ± SD.
[c] CV: coefficient of variation.
[d] REM: relative error of mean.

alcoholic beverage, all AOAC method would request distillation prior to determination of alcohols. Distillation is generally considered to be the primary step for making error along the process of determination. But, with the method presented in this study, one shall only inject the sample spiked with dual internal standards and then the data will come out in less than 12 min. No pretreatment is required for direct GC method. More-

Table 4
Method comparison between AOAC developed methods and proposed GC method with dual internal standards for determination of methanol and/or ethanol in whisky

Method	Methanol		Ethanol	
	μg/mL[a]	CV (%)[b]	mg/mL[a]	CV (%)[b]
AOAC Photometric method	139 ± 12	8.5	ND	ND
AOAC GC method	133 ± 9	6.4	ND	ND
Proposed method	136 ± 4	2.9	386 ± 14	3.5
AOAC titration method	ND[c]	ND	388 ± 34	8.7

[a] All data were obtained from triplicate analyses and displayed in mean ± SD.
[b] CV: coefficient of variation.
[c] ND: not determined.

over, resolution power and column stability of mega-pore capillary column had been proved to be higher than packed column proposed by AOAC method in other published papers (e.g., Wang et al., 2003). We, therefore, strongly suggest to using this method for routine determination of methanol and ethanol in beverages.

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