Analytical method validation and monitoring of diacetyl in liquors from Korean market

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Abstract Diacetyl is a natural fermentation by-product and is an important flavor component of certain liquors. This paper aims to validate the high performance liquid chromatography (HPLC) method based on derivatization with 1,2-diaminobenzene for diacetyl quantification in liquor samples. A limit of quantitation of 0.039 mg/L was obtained. Coefficient regression (R^2) of calibration curve for the HPLC–UV method exceeded 0.999, showing adequate linearity on the standard curve. Relative standard deviation values obtained from intraday and interday analysis for precision were 2.5 and 4.1%, respectively. Using the validated method, the contents of diacetyl in liquors (12 types, 389 samples) distributed throughout Korea were monitored. The average diacetyl content of all analyzed liquor samples ranged from trace amounts to 3.655 mg/L (microbrewery beer). The highest average diacetyl content was found in fruit wines (0.432 mg/L), followed by red wine (0.320 mg/L) and general distilled spirits (0.249 mg/L). In *takju* and *yakju*, no distinctive effect of sterilization on diacetyl content was found.

Keywords Diacetyl · Method validation · Liquor · Liquid chromatography

Introduction

The 2014 average per capita liquor consumption in Korea was 9.0 L, similar to the average value of 9.0 L for the 25 member countries of the Organization for Economic Cooperation and Development (OECD) [1]. According to the National Tax Service of Korea (2014), four types of liquors including beer, diluted *soju*, *takju*, and *cheongju* account for 91% of the total liquor consumption [2]. Recently, the Korean consumer market expanded to include liquors with added flavors and tastes, together with low-alcohol content liquors.

Diacetyl (2,3-butanedione) is present in some food products (milk, beer, coffee, etc.) and has a butter-like flavor. It is known to contribute to the favorable flavor of foods but it gives the food products a bad odor if the diacetyl concentration exceeds a certain level [3]. In particular, it is one of the vicinal diketones found in beer, which exhibits a negative effect by generating bad smell when it is present at levels exceeding 1 mg/L [4, 5]. The cause of diacetyl production is variable. This compound is a known fermentation by-product produced when pyruvate cannot be properly metabolized into ethanol [6–8].

Diacetyl is a generally recognized as safe (GRAS) substance according to the United States Food and Drug administration (USFDA). Thus, the standards for diacetyl content in foods are not yet specified [9]. However, since the respiratory toxicity of diacetyl was reported in the 2000s [10], the National Institute for Occupational Safety and Health (NIOSH) limited the 8 h time-weighted average diacetyl level to lower than 0.005 mg/L. In this way, an environmental specification for diacetyl was established by limiting its permissible concentration in the workplace, where it is easily inhaled through the respiratory system [11–13].

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The American Society of Brewing Chemists (ASBC) suggests spectrophotometric and gas chromatographic methods for the analysis of diacetyl in beers. Since beers contain carbon dioxide and floating matter, pretreatment by pouring, ultrasonication, or filtration was proposed for accurate quantitative analysis [14]. Furthermore, the International Organization of Vine and Wine (OIV) proposed an analytical method featuring diacetyl derivatization with 1,2-diaminobenzene (OPDA) followed by analysis by high performance liquid chromatography (HPLC) or gas chromatography (GC) [15, 16]. In Korea, a spectrophotometric method is used by the National Tax Service [17]. In addition, there have been reports about diacetyl analysis using gas chromatography (GC) [18]. However, studies about other instrumental analysis and for liquors other than *yakju* have been very limited.

Generally, HPLC-ultraviolet (HPLC-UV) detection can be applied to the analysis of diacetyl after its derivatization with OPDA, and this method was adopted for the present study. Furthermore, for liquors forming air bubbles, such as beer, *takju*, and sparkling wine, a degassing method proposed by the ASBC was used after slight modification. Additionally, a pretreatment method for *takju*, which has a large quantity of floating matter, was also studied. A calibration curve was prepared using diacetyl and 2,3-hexanedione as an internal standard (IS), and the linearity, precision, accuracy, limit of detection (LOD), and limit of quantitation (LOQ) were also determined. Subsequently, the validated method was used for the investigation of 12 types of liquors (i.e., *yakju*, *takju*, wine, whiskey, distilled *soju*, general distilled spirit, diluted *soju*, liqueur, fruit wine, beer, *cheongju*, and other liquors) consumed in Korea.

Materials and methods

Reagents, chemicals, and samples

HPLC-UV analysis utilized OPDA for diacetyl derivatization, and 2,3-hexanedione was used as an internal standard. All reagents were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). HPLC grade solvents such as ethanol, water, and methanol were used for the analysis. The alcoholic beverages used for monitoring, i.e., *yakju* (25 sterilized, 14 nonsterilized samples), *takju* (23 sterilized, 49 nonsterilized samples), wines (8 red wines, 5 white wines, 7 sparkling wines, and 4 rose wines), 12 whiskies, 9 distilled *soju*, 38 general distilled liquors, 15 diluted *soju*, 25 liqueurs, 31 fruit wines, 33 other liquors, beers (10 domestic and 30 foreign beers), and 15 *cheongju* were purchased from markets (Seoul, Korea), while 36

microbrewery beers were purchased from small-scale beer shops in Seoul, Korea.

Sample pretreatment

For liquors such as beers, *takju*, and sparkling wines, CO₂ was removed by repeated pipetting until no more air bubbles were generated, whereas turbid liquors (e.g., *takju*) were centrifuged (Hanil, HA-1000-3, Hanil Science Co., Daejeon, Korea) at 1821×*g* for 10 min, and the clear supernatant was used. Subsequently, 10 mL of the supernatant was transferred into a 25 mL vial, and the pH was adjusted to 8 using 0.5 M NaOH solution (tested with pH testing paper, Advantec® test paper, Toyo Roshi Kaisha Ltd., Tokyo, Japan). OPDA (5 mg) and 50 µL of 0.4 µg/mL solution of 2,3-hexanedione in 50% ethanol were added. The vial was sealed using Teflon tape, and derivatization was performed in a shaking water bath at 60 °C for 3 h, followed by cooling and stirring using a vortex mixer for 1 min.

Calibration curve, linearity, LOD, and LOQ

The analytical method proposed in OIV-MA-AS315-20 [15] was slightly modified for diacetyl analysis by HPLC-UV equipped with a Waters spherisorb® ODS2 (5 µm particle 4.6 × 250 mm, Waters Technology Ireland, Ltd., Wexford, Ireland) column. The mobile phase comprised solvent A (water/acetic acid, 100/0.05, v/v) and solvent B (100% methanol) with gradient condition [15]. Qualitative results were confirmed by the relative retention time (RRT), which is a ratio between the peak retention times of the diacetyl standard and the added internal standard (2,3-hexanedione).

Relative retention time (RRT)

$$= \text{Diacetyl peak retention time} \div \text{IS peak retention time}$$

Quantitative analysis was performed using the values obtained by dividing the areas of the diacetyl standard peak by the internal standard peak as Y-values, and the concentration of the diacetyl standard as X-values. For obtaining the calibration curve, the prepared diacetyl standard solution (0.05, 0.1, 0.5, 1, and 5 mg/L prepared by 10% ethanol) and the 2,3-hexanedione solution (2 µg/mL) were mixed. The ratio of each peak area in the calibration curve was calculated using the average values obtained for five independently repeated experiments.

The LOD and LOQ for the analytical method used for diacetyl quantification were determined based on the International Conference on Harmonization guidelines [19]. Five blank samples were analyzed to estimate the signal to noise ratio of LOD and LOQ. Blank peaks were

integrated at the retention time of diacetyl, and then the average of peak area in blank samples was multiplied by 3.3 and 10 for obtaining each minimum concentration of LOD and LOQ. The minimum concentrations were calculated from the standard curve equation by substituting the calculated areas. The two minimum concentrations calculated for LOD and LOQ were analyzed five times, respectively, and then standard deviations (SD) from the measured areas of their concentrations were obtained. In addition, each external standard including minimum concentration was prepared to establish the corresponding curve, and each slope (S) was determined. Eventually, the LOD and LOQ were calculated from the slope and standard deviation using the equations below.

$$\text{LOD} = 3.3 \times \text{SD} \div S$$

$$\text{LOQ} = 10 \times \text{SD} \div S$$

where S is the slope of the standard curve and SD is the standard deviation of the response areas obtained by repeatedly analyzing the minimum concentration.

Precision and accuracy

In general, the acceptable precision of an analytical method for diacetyl was evaluated as the relative standard deviation (RSD, %) obtained from values of repeatedly analyzed samples. The intraday precision was obtained by performing the experiment five times daily under the same conditions ($n = 5$), whereas the interday precision was determined by repeating the experiment for four days under the same conditions ($n = 4$). RSD for the evaluation of precision was determined for both the intraday and interday precision as the following equation:

$$\text{RSD (\%)} = \frac{\text{Standard deviation of the values}}{\div \text{mean value}} \times 100$$

The accuracy of the analytical method for diacetyl was evaluated as the recovery percentage (%) determined from red wine spiked with a known content (10 mg/L) of diacetyl. The recovery rate (%) was calculated as follows:

$$\text{Recovery rate} = \frac{\text{Recovery value}}{\div \text{true value}} \times 100 \quad (20)$$

Results and discussion

Linearity, LOD, and LOQ

The coefficient regression (R^2) of the calibration curve was determined accordingly (Table 1). As suggested in the OIV method [15], R^2 for the HPLC–UV method exceeded 0.999, showing adequate linearity on the standard curve.

Table 1 Precision (RSD, %), accuracy (recovery rate, %), limit of detection (LOD, mg/L), limit of quantitation (LOQ, mg/L) and calibration curves for analysis of diacetyl in liquor

Method validation on HPLC–UV	
Precision	
Interday (RSD, %)	4.1
Intraday (RSD, %)	2.5
Accuracy	
Recovery rate (%) from red wine	93.6
LOD (mg/L)	0.004
LOQ (mg/L)	0.039
Calibration curve	$y = 0.699x + 0.024$
Coefficient regression (R^2)	0.999

The retention time of diacetyl was about 28 min, and it was clearly separated from that of the 2,3-hexanedione (about 36 min) [Fig. 1(A), (B)]. RRT was found in the range of 0.779 ± 0.097 min.

The LOD and LOQ of the HPLC–UV method are presented in Table 1. The average area after injection of the blank solution was around 1.9, and the LOD and LOQ of each component were 0.004 and 0.039 mg/L, respectively.

Precision and accuracy

To determine the intra- and inter-day precision of diacetyl analysis, relative standard deviations (RSD, %) were calculated from the averages and standard deviations of diacetyl levels (Table 1). Precision was evaluated at a concentration level of 0.5 mg/L. The intraday RSD was 2.5%, and the inter-day value was 4.1%. According to the Association of Official Analytical Chemists (AOAC), an RSD of 15% is acceptable if the analyte concentration is 10 ppb, with a value of 8% being acceptable for a concentration of 1 mg/L [20]. Good precision for the analytical method of diacetyl was observed, showing below 5% of RSD (Table 1).

Furthermore, the recovery rate for evaluating the accuracy was performed by spiking the red wine with a known amount of diacetyl (10 mg/L). The recovery rate of diacetyl from red wine was 93.6% in agreement with the guideline suggesting 80–115% for 10 mg/L of analytes [20].

Monitoring of diacetyl content in liquors from Korean market

The diacetyl content of commercially available *yakju* (25 sterilized and 14 nonsterilized samples), *takju* (23 sterilized and 49 nonsterilized samples), wines (8 red, 5 white, 7

Fig. 1 HPLC chromatograms of the diacetyl. (A) standard (0.5 mg/L), (B) standard (5 mg/L), (C) general distilled spirits, (D) fruit wine, (E) microbrewery beer (diacetyl 3.655 mg/L), and (F) microbrewery beer (diacetyl 0.024 mg/L). Peak 1, diacetyl; Peak 2, 2,3-hexanedione (IS)

sparkling, and 4 rose wines), whiskies (12 samples), distilled *soju* (9 samples), general distilled spirits (38 samples), diluted *soju* (15 samples), liqueurs (25 samples), fruit wines (31 samples), other liquors (33 samples), beers (10 domestic, 30 imported, and 36 microbrewery beers), and *cheongju* (15 samples) is presented in Tables 2 and 3. Additionally, the selected sample chromatograms of different liquor types are presented in Fig. 1. Since the difference between the minimum and maximum diacetyl levels in the same kind of liquor was large, the standard

deviation was also large. The diacetyl content for all 389 liquor samples ranged from trace levels to 3.655 mg/L, with no diacetyl detected in 35 liquor samples. The highest diacetyl content was found for one sample of microbrewery beers (3.655 mg/L, Table 3).

For the 12 whiskey samples, 0.093 mg/L of diacetyl was detected on an average, with a minimum of 0.006 mg/L and a maximum of 0.413 mg/L. Previously, Lee et al. [21] reported that the average diacetyl content of 10 types of whiskey was 0.04 mg/L. However, the 9 distilled *soju*

Table 2 Concentration ranges of diacetyl in several types of liquor

Samples	Number	Average (mg/L)	Minimum (mg/L)	Maximum (mg/L)
Whiskey	12	0.093 ± 0.144	Trace ^a	0.413
Distilled <i>Soju</i>	9	0.070 ± 0.110	ND ^b	0.329
General distilled spirits	38	0.249 ± 0.672	ND	3.443
Diluted <i>Soju</i>	15	0.067 ± 0.155	ND	0.571
Liqueur	25	0.034 ± 0.048	ND	0.129
Fruit wine	31	0.432 ± 0.767	Trace	3.305
Others	33	0.042 ± 0.066	ND	0.320
<i>Cheongju</i>	15	0.076 ± 0.135	Trace	0.545
Total	178	0.163 ± 0.471	ND	3.443

All data are shown as mean ± standard deviation (number = n)

^a Trace, LOD ≤ to ≤ LOQ

^b ND not detected

Table 3 Diacetyl contents from different types of beer, wine, *yakju* and *takju*

Type	Number	Average (mg/L)	Minimum (mg/L)	Maximum (mg/L)
Beer				
Domestic				
Lager	7	0.032 ± 0.024	Trace ^a	0.080
Ale	3	0.043 ± 0.004	Trace	0.047
Total	10	0.035 ± 0.020	Trace	0.080
Foreign				
Lager	20	0.020 ± 0.017	ND ^b	0.066
Ale	10	0.066 ± 0.110	Trace	0.374
Total	30	0.035 ± 0.066	ND	0.374
Microbrewery	36	0.200 ± 0.598	ND	3.655
Total	76	0.113 ± 0.419	ND	3.655
Wine				
Red wine	8	0.320 ± 0.214	Trace	0.616
White wine	5	0.044 ± 0.035	Trace	0.086
Sparkling wine	7	0.147 ± 0.246	ND	0.698
Rose wine	4	0.082 ± 0.045	Trace	0.121
Total	24	0.172 ± 0.207	ND	0.698
Yakju				
Nonsterilized	14	0.114 ± 0.111	Trace	0.465
Sterilized	25	0.096 ± 0.066	Trace	0.241
Total	39	0.102 ± 0.084	Trace	0.465
Takju				
Nonsterilized	49	0.152 ± 0.175	ND	0.823
Sterilized	23	0.116 ± 0.169	ND	0.837
Total	72	0.141 ± 0.173	ND	0.837

All data are shown as mean ± standard deviation (number = n)

^a Trace, LOD ≤ to ≤ LOQ

^b ND Not detected

showed an average of 0.070 mg/L, displaying no significant difference in the average diacetyl content between the analyzed whiskeys and distilled *soju* (Table 2). Meanwhile, the 38 general distilled spirits showed an average diacetyl content of 0.249 mg/L, providing higher levels than whiskey and *soju*. High diacetyl content was found from the general distilled spirits especially made of mulberries (odee) and *bokbunja* from the same brewery at 2.545 mg/L (data not shown) and 3.443 mg/L. The chromatogram of the general distilled spirits made of *bokbunja* is presented in Fig. 1(C).

The highest average diacetyl content among the 12 analyzed liquor types was found in fruit wines (0.432 mg/L). One of the chromatograms from fruit wine is shown in Fig. 1(D). Among the 31 samples of analyzed fruit wines, the highest diacetyl levels were found in liquors made of *bokbunja* and blueberries (produced in a different brewery), equal to 2.441 (data not shown) and 3.305 mg/L, respectively (Table 2). Similar to general distilled spirits, fruit wines made of berries contained a large amount of diacetyl.

Table 3 shows the differences in diacetyl content due to the fermentation and sterilization of the same liquor type. The studied 76 beers contained 0.113 mg/L of diacetyl on an average. Diacetyl is known to be bad-smelling at levels above 1 mg/L [3]. One beer sample exhibiting a diacetyl content of more than 1 mg/L (i.e., 3.655 mg/L) was obtained from a microbrewery (Fig. 1(E)). Beer was classified into microbrewery beer, domestic beer, and imported beer according to its origin. Domestic and imported beers were again grouped into lager and ale beer. The microbrewery beers (36 samples) showed an average diacetyl content of 0.200 mg/L, whereas foreign beers (30 samples) exhibited levels of 0.035 mg/L and domestic beers (10 samples) showed an average of 0.035 mg/L. Therefore, relatively high diacetyl content was observed from microbrewery beers, and diacetyl content of foreign ale beer showed 3.3 times higher diacetyl content than that of foreign larger beer. The chromatogram exhibiting a diacetyl content of 0.024 mg/L is shown in Fig. 1(F).

According to the research of Swiegers et al. [22], the diacetyl content of wines is affected by their origin, degree of aging, and production method. Therefore, 8 red wines, 5 white wines, 7 sparkling wines, and 4 rose wines were grouped according to the production method and grape-type for comparison. The average diacetyl content of all wines (24 samples) was 0.172 mg/L, and the highest contents of diacetyl were found in one of the red wine samples (0.616 mg/L) and sparkling wine samples (0.698 mg/L). The red wine diacetyl content on an average was 0.320 mg/L, showing a higher diacetyl content as compared with sparkling wines (0.147 mg/L), rose wines (0.082 mg/L), and white wines (0.044 mg/L). Rodrigues et al. [23] estimated the diacetyl content of wine at 0.17 ± 0.03 mg/L, which is similar to the range determined in this study.

Analysis of 39 *yakju* types showed an average diacetyl content of 0.102 mg/L, with a minimum of 0.014 mg/L and a maximum of 0.465 mg/L. Notably, *yakju* containing 0.465 mg/L of diacetyl was produced by the *oyangju* technique that is not a general preparation method. The *Yakju* was divided into 25 sterilized and 14 nonsterilized samples (Table 3). The average diacetyl content in sterilized *yakju* was 0.096 mg/L, while diacetyl levels in nonsterilized *yakju* were at 0.114 mg/L, showing no distinctive difference. The 72 samples of *takju* contained 0.141 mg/L of diacetyl on an average. *Takju* was further categorized into 23 sterilized and 49 nonsterilized samples (Table 3). Nonsterilized *takju* contained 0.152 mg/L of diacetyl on an average, whereas the corresponding value for sterilized *takju* was 0.116 mg/L. However, sterilization resulted in no distinctive difference in the diacetyl content. Min et al. [24] stored *takju* at 4 °C to study the changes in the yeast and organic acid content. After two days, the amount of yeast was drastically reduced partly because of the produced organic acids. Therefore, little diacetyl production during the distribution of nonsterilized *takju* was assumed, since the difference in diacetyl content compared to sterilized *takju* was not detected.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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