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Development and Validation of a High-Performance Liquid Chromatography Method for the Determination of Diacetyl in Beer Using 4-Nitro-*o*-phenylenediamine as the Derivatization Reagent

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ABSTRACT: Diacetyl is a natural byproduct of fermentation and known to be an important flavor compound in many food products. Because of the potential undesirable effects of diacetyl on health safety and beer flavor, determination of its concentration in beer samples is essential and its analytical methods have attracted close attention recently. The aim of the present work is to develop and validate a novel high-performance liquid chromatography method for the quantification of diacetyl in beer based on the derivatization reaction of diacetyl with 4-nitro-*o*-phenylenediamine (NPDA). After the derivatization with NPDA in pH 3.0 at 45 °C for 20 min, diacetyl was separated on a kromasil C_{18} column at room temperature in the form of the resulting 6-nitro-2,3-dimethylquinoxaline and detected by the ultraviolet detector at 257 nm. The results showed that the correlation coefficient for the method was 0.9992 in the range of 0.0050−10.0 mg L^{-1} and the limit of detection was 0.0008 mg L^{-1} at a signal-to-noise ratio of 3. The applicability of the proposed method was evaluated in the analysis of beer samples with the recovery range of 94.0−99.0% and relative standard deviation range of 1.20−3.10%. The concentration levels of diacetyl detected in beer samples from 12 brands ranged from 0.034 to 0.110 mg L^{-1}. The proposed method showed efficient chromatographic separation, excellent linearity, and good repeatability that can be applied to quantification of diacetyl in beer samples.

KEYWORDS: *Determination, diacetyl, high-performance liquid chromatography (HPLC), precolumn derivatization, 4-nitro-o-phenylenediamine (NPDA)*

INTRODUCTION

Diacetyl (2,3-butanedione) is a natural byproduct of fermentation and known to be an important flavor compound in many food products.[1,2] It can be found in beer, wine, butter, cheese, milk, and yogurt. During the production of beer, diacetyl may impart a negative effect on beer flavor when it is above a certain concentration.[3,4] In lagers, its concentration is generally slightly lower than 0.10 mg L^{-1}, but it is usually higher in stouts and ales.[5] It may also potentially cause diseases, including bronchiolitis obliterans syndrome, obliterative bronchiolitis, or impaired lung function, when the inhaled doses or exposure time increases.[6−9] Because of the potential undesirable effects of diacetyl on beer flavor and health safety, quantification of diacetyl in beer samples is critical for quality control and development of new beer products.

In response to the concern, close attention has been paid to studies on the analytical methods for diacetyl, which mainly involved spectrophotometry,[10−12] fluorescent determination,[13] voltammetric determination,[5,14] gas chromatography with an electron capture detector (GC−ECD),[15,16] gas chromatography with a flame ionization detector (GC−FID),[17,18] gas chromatography coupled with mass spectrometry (GC−MS),[19−24] high-performance liquid chromatography with a fluorescent detector (HPLC−FL),[25] and high-performance liquid chromatography with an ultraviolet detector (HPLC−UV).[26−32] For the sake of enhancing the absorptivity in the detector, the diacetyl was usually derivatized with a labeling reagent. In the earlier time, 4-aminosulfonyl-7-hydrazino-2,1,3-benzoxadiazole,[33] 2,4-dinitrophenyl hydrazine,[34] and dimethylglyoxime[10] were commonly used as the label reagents. Recently, more labeling reagents were developed and validated, such as rhodamine B hydrazide (RBH),[13] 4,5-dichloro-1,2-diaminobenzene (DCDA),[19] 6-hydroxy-2,4,5-triaminopyrimidine (TRI),[25] or *o*-phenylenediamine (OPDA).[3,5,14,26,27,29−32] Among these methods, the spectrophotometry method was simple but inferior in terms of sensitivity and selectivity. GC−MS could achieve a low detection limit but was usually expensive and required complex pretreatment. Recently, the derivatization of diacetyl with OPDA followed by HPLC quantification of the resulting quinoxaline derivative attracted more attention because it is sensitive, selective, and can be accomplished in aqueous solution at a low temperature without further treatment.[3,26,30] Although these methods can meet the analysis need in many fields, new derivatization reagents are still needed to improve the detection properties.

As a fine chemical, 4-nitro-*o*-phenylenediamine (NPDA) has been widely used in the determination of selenium[35,36] and synthesis of 6-nitroquinoxaline compounds.[37,38] In comparison to OPDA, NPDA has one nitro group on the benzene ring, which gives it stronger absorptivity in both ultraviolet and

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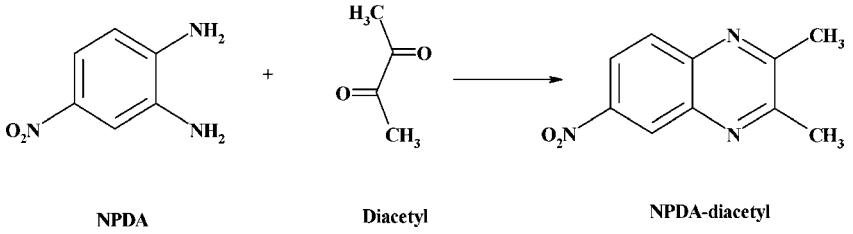

Figure 1. Reaction scheme of diacetyl with NPDA.

visible bands. Because of the stronger UV absorption of the 6-nitroquinoxaline derivatives produced from the reaction of NPDA with α-diketones, NPDA has the potential to be a new efficient derivatization reagent for the determination of α-diketones. The aim of the present work is to develop and validate a novel derivatization method for the quantitation of diacetyl in beer by HPLC. To the best of our knowledge, this is the first application of NPDA as the derivatization reagent for the determination of diacetyl.

MATERIALS AND METHODS

Chemicals and Reagents. Diacetyl, NPDA, and OPDA were analytical-grade reagents and purchased from Sinopharm Chemical Reagent Beijing Co., Ltd. Methanol and acetonitrile were HPLC-grade and purchased from J.T. Baker (Phillipsburg, NJ). The ultrapure water was obtained by a Milli-Q water purification system (Millipore, Billerica, MA). All other chemicals and solvents were analytical-grade and from commercial sources. The stock solutions of 100 mg L^{-1} diacetyl were prepared in redistilled water. The stock solutions of 200 mg L^{-1} OPDA and 200 mg L^{-1} NPDA were prepared in methanol. Aqueous acetate buffer (0.04 M, pH 4.5) was prepared with ammonium acetate, and the pH was adjusted with hydrochloric acid (1.0 M). The stock solutions were stored in the dark at 4 °C when not in use.

Instrumentation. A HPLC system, consisting of two LC-20ATvp pumps and a SPD-20Avp UV detector (Shimadzu, Japan), was applied for the separation and analysis. A reversed-phase kromasil ODS C_{18} column (250 × 4.6 mm inner diameter, with a particle size of 5 μm) and a Chromatograph Solution Light Chemstation for LC system were employed to obtain and process the chromatographic data. A 7890A gas chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a mass spectrometer detector (MSD) 5975C and a 7693 automatic liquid sampler was used to analyze MS data of the derivative.

Derivatization Procedure. First, 1.00 mL of diacetyl standard solution (1.0 mg L^{-1}), 0.20 mL of HCl (0.1 M), 0.60 mL of methanol, and 0.20 mL of NPDA standard solution (200.0 mg L^{-1}) were added to a test vial. The total solution was then homogenized with a vortex mixer and incubated at 45 °C for 20 min. After cooling, the resulting solution was filtered through a 0.22 μm filter membrane and injected into the chromatographic system.

Identification of the Derivative. A mixture of 10.0 mL of diacetyl standard solution (1.0 mg L^{-1}), 2.0 mL of HCl (0.1 M), 6.0 mL of methanol, and 2.0 mL of NPDA standard solution (200.0 mg L^{-1}) was added to a 50 mL flask. The total solution was stirred with a magnetic stirrer at 45 °C for 20 min. Then, the solution was extracted by liquid−liquid extraction in three stages using 10, 5, and 5 mL of CH_2Cl_2. The organic solvent was evaporated under rotary evaporators and a stream of nitrogen at 45 °C until dry. The residue was reconstituted with ethyl acetate and subjected to GC−MS. The MSD was operated in electron ionization mode at 70 eV. The source, quadrupole, and transfer line temperatures were 230, 150, and 290 °C, respectively. Detection was achieved in selected ion monitoring (SIM) mode with a solvent delay of 5 min. Full-scan MS data were acquired over the range of m/z 10−500 to obtain the fragmentation spectra of the transformation products.

Chromatographic Method. The mobile phase consisted of methanol/water (65:35, v/v). All of the mobile-phase solutions were filtered with a 0.22 μm membrane filter before use. The analysis was executed with an injection volume of 20 μL, flow rate of 1.0 mL/min, and UV detection wavelength at 257 nm. Each sample was injected in triplicate, and the analysis was carried out at 25 °C.

In this work, OPDA was employed as the contrast derivatization reagent to validate this novel HPLC method. The derivatization conditions and HPLC separation method were referenced to the report by Barros et al.[26] The beer sample (1.00 mL) with and without spiking standard diacetyl was filtered, derivatized with OPDA (200 mg L^{-1}) at 25 °C for 30 min, and then injected into the HPLC system. The HPLC separation was completed within 12 min, with the mobile phase consisting of acetonitrile/aqueous acetate buffer (80:20, v/v), a flow rate of 1.0 mL/min, and an UV detection wavelength at 315 nm.

Analysis of Beer Samples. A total of 12 beer brands (Tsingtao, Snow, Yanjing, Landai, Carlsberg, Beijing, Laoshan, Haerbin, Lanbei, Tiger, Budweiser, and Heineken) were purchased at local supermarkets in Beijing and stored in glass bottles at 4 °C in a refrigerator. First, 1.00 mL of beer sample solution was filtered through a 0.22 μm membrane filter and transferred to a vial. Then, the vial was added with 0.60 mL of methanol, 0.20 mL of NPDA (200 mg L^{-1}), and 0.20 mL of HCl (0.1 M). The total solution was mixed on a vortex mixer and incubated in a water bath at 45 °C for 20 min. After cooling, the resulting solution was filtered through a 0.22 μm membrane filter and injected into the chromatographic system.

RESULTS AND DISCUSSION

Identification of the Derivative. Diacetyl has the molecular formula of $C_4H_6O_2$, and its relative molecular masses before and after derivatization were 86 and 203, respectively. The molecular formula of NPDA is $C_6H_7N_3O_2$ with the relative molecular mass of 153. The product ions in the GC−MS spectra for the NPDA derivative of diacetyl were recorded in the positive-ion mode. The precursor ion of NPDA−diacetyl was at m/z 203 (M^+, 100), and three abundant fragments were found at m/z 162 (35), 116 (60), and 75 (20). The results of this study were consistent with the previous research.[37,38] According to the GC−MS data of the NPDA−diacetyl derivative, this product should be 6-nitro-2,3-dimethylquinoxaline, with the molecular formula of $C_{10}H_9N_3O_2$.

Optimization of Derivatization Conditions. The synthesis of quinoxaline rings generally requires a high reaction temperature, strong acidic media, and usually long reaction times.[39,40] However, the reaction of NPDA with α-diketones can easily and quickly form the single product 6-nitroquinoxaline derivative in the presence of catalysts.[37,38] The derivatization reaction of diacetyl with NPDA is shown in Figure 1. Because most of these reactions are reversible, the concentration ratio of NPDA/diacetyl, reaction temperature, and pH may greatly influence the reaction velocity and reaction yield. To determine the optimum derivatization conditions, the effects of the three factors on the required derivatization time (velocity) and the resulting peak areas of NPDA−diacetyl (yield) were investigated and the results are shown in Figure 2.

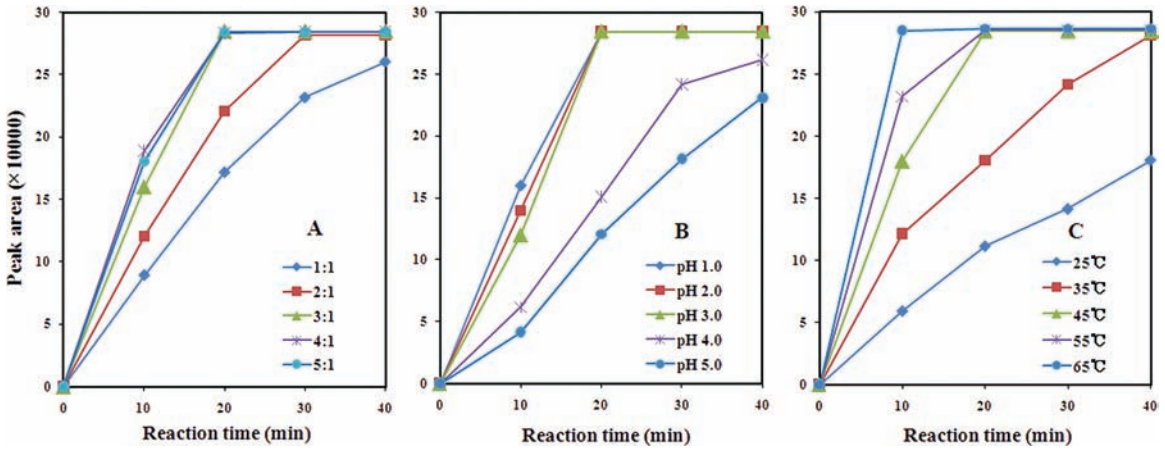

Figure 2. Effects of the (A) concentration ratio of NPDA/diacetyl, (B) derivatization pH value, and (C) derivatization reaction temperature on the peak areas of NPDA−diacetyl and the required derivatization time. Conditions: (A) 10 μM diacetyl, pH 3.0, and 45 °C, (B) 10 μM diacetyl, 30 μM NPDA, and 45 °C, and (C) 10 μM diacetyl, 30 μM NPDA, and pH 3.0.

The optimum concentration ratio of NPDA/diacetyl was of primary importance. Diacetyl solutions of 10 μM (0.86 mg L^{-1}) were reacted with NPDA concentrations of 10, 20, 30, 40, and 50 μM (ratio of 1:1, 2:1, 3:1, 4:1, and 5:1). The results indicated that, when the concentration ratio was ≥3:1, the peak area could reach the largest value within 20 min (Figure 2A). Thus, the minimum ratio of NPDA/diacetyl was 3:1 in the derivatization reaction. In beer, wine, water, butter, or milk samples, the detection threshold concentrations of diacetyl have been reported to be in the range of 0.06−4.37 mg L^{-1}.[29,41] To keep the concentration ratio of NPDA/diacetyl > 3:1, the addition of NPDA of 200 mg L^{-1} in the derivatization solutions was sufficient.

The reaction of NPDA with diacetyl was found to be pH-dependent. The previous study suggested that a strong acid media is needed for these reactions. However, too low of pH is caustic and may also have potential adverse effects on the C_{18} column. Thus, it is essential to optimize the reaction pH. The effects of pH (values of 1.0, 2.0, 3.0, 4.0, and 5.0) on the derivatization time and the resulting peak areas were studied using hydrochloric acid (HCl). Figure 2B shows that the peak areas increase to the maximum value within 20 min when the pH range is 1.0−3.0. When the pH value increases to 4.0 or 5.0, the required derivatization time is more than 40 min. Considering the reaction efficiency and safety aspects, pH 3.0 was selected for the derivatization procedure.

The temperature usually greatly influences the derivatization time and yield. A temperature range of 25−65 °C was used to study the optimum derivatization temperature and the corresponding derivatization time. The results showed that, with the temperature increase, less derivatization time was required. The peak area could reach the largest value within 20 min when the temperature was ≥45 °C (Figure 2C). Because too high of a temperature may have undesirable effects on the components in beer samples, 45 °C was selected as the derivatization temperature, and the corresponding derivatization time was 20 min.

Because of the poor solubility of NPDA in water, methanol was added to the derivatization medium to avoid precipitation of the reagent. It was found that the proportion of methanol should be at least 30% in the derivatization solution.

Optimization of Separation Conditions. NPDA and NPDA−diacetyl were scanned using a SPD-20Avp UV detector. Figure 3 shows that the maximum absorption wavelength of NPDA and NPDA−diacetyl are both less than

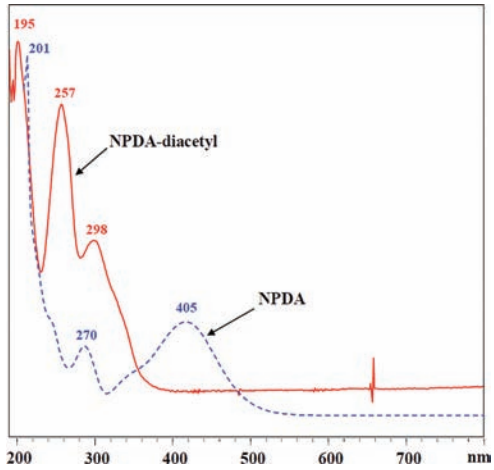

Figure 3. Scanned UV absorption spectra of NPDA and NPDA−diacetyl.

210 nm, a wavelength range indicating that many solvents and matrix interferences also have strong UV absorption. To obtain better absorption of NPDA−diacetyl and minimize the interferences, a wavelength of 257 nm was selected in the HPLC−UV analysis.

In the present study, chromatographic separation was optimized under a reversed-phase condition on a kromasil C_{18} column. The mobile phase composition was optimized to achieve fast and optimum separation of NPDA, NPDA−diacetyl, and matrix interferences in the HPLC system. Methanol (eluent A) and water (eluent B) were used as the mobile phase. The influence of the mobile phase ratio on chromatographic separation was also investigated. Results indicated that, when the proportion of eluent B increased, the retention time of NPDA−diacetyl increased from 4.8 to 14.5 min. To achieve better separation and avoid interferences, the mobile phase ratio was selected to be 65:35 (methanol/water). The typical chromatograms of the derivatization of diacetyl with NPDA obtained in optimum separation conditions are shown in Figure 4. The retention time of

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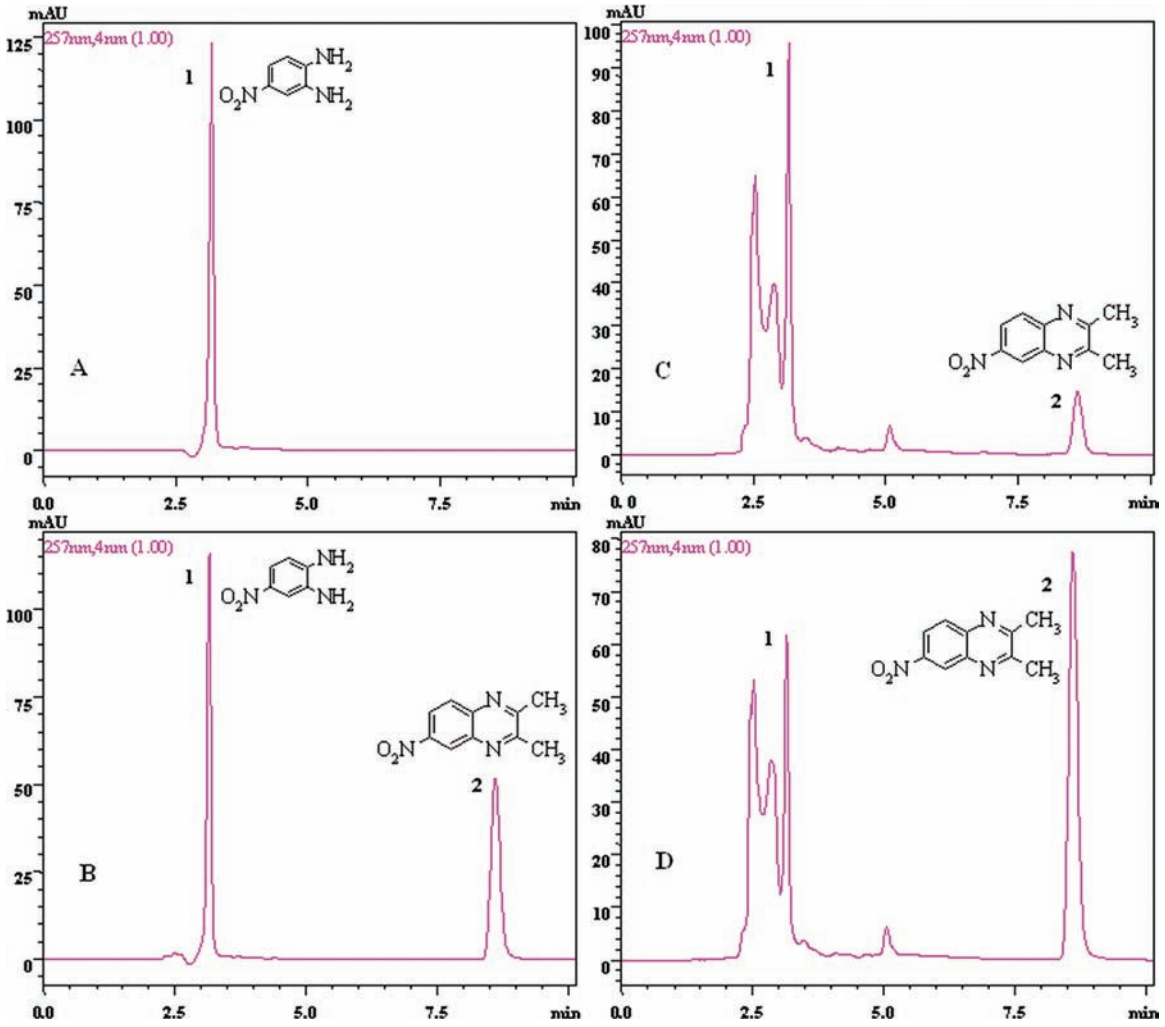

Figure 4. Chromatograms obtained from the (A) NPDA blank, (B) reaction of NPDA with diacetyl standard (0.50 mg L^{-1}), (C) beer sample blank, and (D) beer sample spiked with diacetyl standard (0.50 mg L^{-1}). Chromatographic conditions: column, reversed-phase kromasil ODS C_{18} column (250 × 4.6 mm inner diameter, with a particle size of 5 μm); UV detection, λ = 257 nm; mobile phase, methanol/water = 65:35; flow rate, 1.0 mL/min; and temperature, 25 °C. Peaks: 1, NPDA; 2, NPDA−diacetyl.

NPDA−diacetyl is 8.6 min, and the separation can be completed within 10.0 min.

Stability of the Derivative. The stability of the diacetyl derivative in methanol/water (4:6, v/v) at 4 °C was investigated over 7 days without light irradiation. Results showed no significant change in peak areas of the derivative. The average degradation rates of NPDA−diacetyl at room temperature were 3.50% after 7 days. When the sample was illuminated by ordinary light of a 100 W bulb for 24 h, there was a decrease of 8.10% of peak areas. It appeared that the stability of NPDA−diacetyl was light-dependent. Therefore, the analysis samples should be stored in darkness at 4 °C when not in use.

Validation of the Method and Application to Real Analysis. To investigate the linear calibration range, diacetyl samples in the concentration range of 0.0050−10.0 mg L^{-1} were prepared and analyzed using the optimized derivatization procedure and separation conditions. Sensitivity of the method was determined by the detection limit (LOD) at a signal-to-noise ratio of 3 and the quantification limit (LOQ) at a signal-to-noise ratio of 10.[17,42] The slope and intercept of the calibration graph were obtained by linear regression of the peak area versus the concentration: $y = ax + b$, where a is the slope, b is the intercept, x is the concentration, and y is the peak area. The usually numerical value used is the relative standard deviation (RSD) for reproducibility. The reproducibility of this analytical method was evaluated in both intra- and interday with a diacetyl concentration of 0.50 mg L^{-1}. As the mean value of six determinations, RSD reached 1.76% in intraday and 2.50% in interday. The linear calibration range, regression equation, R^2, detection limit, quantification limit, and RSD of diacetyl with this new method were calculated, and the results are listed in Table 1.

The applicability of the developed method was evaluated in Tsingtao beer, Snow beer, and Yanjing beer. The accuracy of an analytical method is the agreement between the true value of the analyte in the sample and the value obtained by analysis. It was assessed by the recovery data calculated using the standard addition method. Diacetyl was measured by adding the standard of three different concentrations (0.050, 0.500, and 2.000 mg L^{-1}) to the beer samples. Three replicates were handled at each concentration, and each sample was injected in six replicates. Because beer samples contain diacetyl, the recovery should be calculated by the formula

$$\text{recovery} = (C - C_d)/C_s \times 100\%$$

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Table 1. Linear Calibration Ranges, Regression Equations, and Detection Limits of NPDA−Diacetyl and OPDA−Diacetyl

parameters	NPDA−diacetyl	OPDA−diacetyl
calibration range (mg L^{-1})	0.0050−10.0	0.050−10.0
regression equation, y^a	330507x + 336.8	167550x + 631
coefficient regression, R^2	0.9992	0.9989
RSD (%), n = 6, within day	1.76	2.12
RSD (%), n = 6, between day	2.50	2.74
detection limit (mg L^{-1})^b	0.0008	0.0092
quantification limit (mg L^{-1})^c	0.0027	0.0310

^{a}x, concentration of diacetyl (mg L^{-1}); y, peak area of NPDA−diacetyl or OPDA−diacetyl. ^{b}S/N = 3, per 20 μL injection volume. ^{c}S/N = 10, per 20 μL injection volume.

where C represents the total concentration found after spiking, C_d represents the initial measured concentration before spiking, and C_s represents the spiked concentration. The typical chromatograms of the derivatization of spiked diacetyl in the beer sample are shown in Figure 4. The recoveries of diacetyl in the real analysis with this new method were investigated and shown in Table 2. Results showed that the recoveries of diacetyl were from 94.0 to 99.0% and RSDs were from 1.20 to 3.10%, depending upon the sample investigated. Therefore, this new method is well-adapted to quantification of diacetyl in beer samples.

The validation parameters of the method using OPDA as the contrast derivatization reagent were also calculated and listed in Tables 1 and 2. The results of the comparison indicated that the detection limit of the method with NPDA as the derivatization reagent was more than 10 times lower than that with OPDA. Therefore, in comparison to the method using OPDA as the derivatization reagent, this new method had more sensitivity. The comparison also illustrated that there was no significant difference in the method recoveries between the application of NPDA and OPDA.

As mentioned above, there were several labeling reagents that had been used in the derivatization of diacetyl. The comparison of NPDA to other used labeling reagents recently reported for diacetyl is given in Table 3. As shown in Table 3, NPDA was superior to TRI and RBH but inferior to DCDA in the derivatization time and temperature requirements. When the HPLC method was used, the application of NPDA as the derivatization reagent had a lower detection limit than that of OPDA and TRI. However, this new method was less sensitive than GC−MS with DCDA as the derivatization reagent. Considering these properties, this new method showed superiority in the aspects including simplicity, derivatization efficiency, and sensitivity.

Monitoring of Diacetyl in Beer. The developed method was applied to monitor the concentrations of diacetyl in beer samples from 12 beer manufacturers in China. The levels of diacetyl detected in beer samples are given in Figure 5. The concentrations of diacetyl were in the range of 0.034−0.110 mg L^{-1}, with the highest value in Laoshan beer and the lowest value in Budweiser beer. The concentrations of diacetyl in Yanjing beer and Laoshan beer slightly exceeded 0.1 mg L^{-1}, which was generally accepted as the sensory threshold in beer.[23]

In conclusion, as a fine chemical, NPDA reacts with diacetyl to produce the stable 6-nitroquinoxaline derivative, which has strong UV absorption. On the basis of the reaction of NPDA with diacetyl, a novel derivatization method for the determination of diacetyl in beer by HPLC was developed. In comparison to several reported derivatization reagents, application of NPDA as the labeling reagent could increase the method sensitivity and reduce the derivatization time. The developed method was applied to monitor the concentrations of diacetyl in beer samples from 12 beer manufacturers in China. The concentrations of diacetyl in different beer brands were in the range of 0.034−0.110 mg L^{-1}. The proposed method showed efficient chromatographic separation, excellent linearity, and good repeatability that can be applied to the quantification of diacetyl in beer samples.

Table 2. Recoveries of Diacetyl from Beer Samples by the Methods Using NPDA and OPDA as the Derivatization Reagent

derivatization reagent	beer	initial^a (mg L^{-1})	spiked (mg L^{-1})	total^b (mg L^{-1})	found^c (mg L^{-1})	recovery (%)	RSD^d (%)
NPDA	Tsingtao	0.065	0.050	0.112	0.047	94.0	2.45
			0.500	0.560	0.495	99.0	1.52
			2.000	2.045	1.980	99.0	1.20
	Snow	0.082	0.050	0.129	0.047	94.0	2.60
			0.500	0.570	0.488	97.6	1.88
			2.000	2.052	1.970	98.5	1.25
	Yanjing	0.105	0.050	0.151	0.046	92.0	3.10
			0.500	0.597	0.492	98.4	2.40
			2.000	2.075	1.970	98.5	1.34
OPDA	Tsingtao	0.065	0.050	0.111	0.046	92.0	3.12
			0.500	0.543	0.478	95.5	1.85
			2.000	1.975	1.910	95.5	1.52
	Snow	0.082	0.050	0.128	0.046	92.0	2.80
			0.500	0.564	0.482	96.4	1.82
			2.000	2.036	1.954	97.7	1.45
	Yanjing	0.105	0.050	0.151	0.046	92.0	3.28
			0.500	0.588	0.483	96.5	2.95
			2.000	2.045	1.940	97.0	2.07

^{a}Mean value of six initial measured concentrations in beer before spiking. ^{b}Mean value of six initial measured concentrations in beer after spiking. ^{c}The total measured concentration minus the initial measured concentration. ^{d}Mean value of six determinations.

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Table 3. Comparison of the Derivatization Conditions and Detection Limit of the Labeling Reagents Recently Reported for the Determination of Diacetyl in Different Matrixes[a]

analytical method	derivatization reagent	derivatization conditions	matrix	extraction method	detection limit	reference
fluorescence	RBH	37 °C, 3 h	cancerous cells	enrichment and centrifugation	ND[b]	13
voltammetric	OPDA	RT, 3 min	wine	dilution	1×10^{-8} M	14
GC−MS	DCDA	30 °C, 5 min	beer	filtering and congelation	0.2 μg/L	19
HPLC−FL	TRI	60 °C, 45 min	human urea	ND[b]	99 pmol[c]	25
HPLC−UV	OPDA	RT, 30 min	beer and wine	SPE	ND[b]	26
HPLC−UV	OPDA	60 °C, 3 h	coffee	SPE	0.02 μM	30
HPLC−UV	OPDA	90 °C, 10 min	beer	MELM	3 μg/L	31
HPLC−UV	NPDA	45 °C, 20 min	beer	filtering	0.8 μg/L	this work

[a]RBH, rhodamine B hydrazide; OPDA, *o*-phenylenediamine; TRI, 6-hydroxy-2,4,5-triaminopyrimidine; DCDA, 4,5-dichloro-1,2-diaminobenzene; SPE, solid-phase extraction; RT, room temperature; MELM, membraneless extraction module. [b]ND = not described. [c]The full unit: 99 pmol per injection (20 μL).

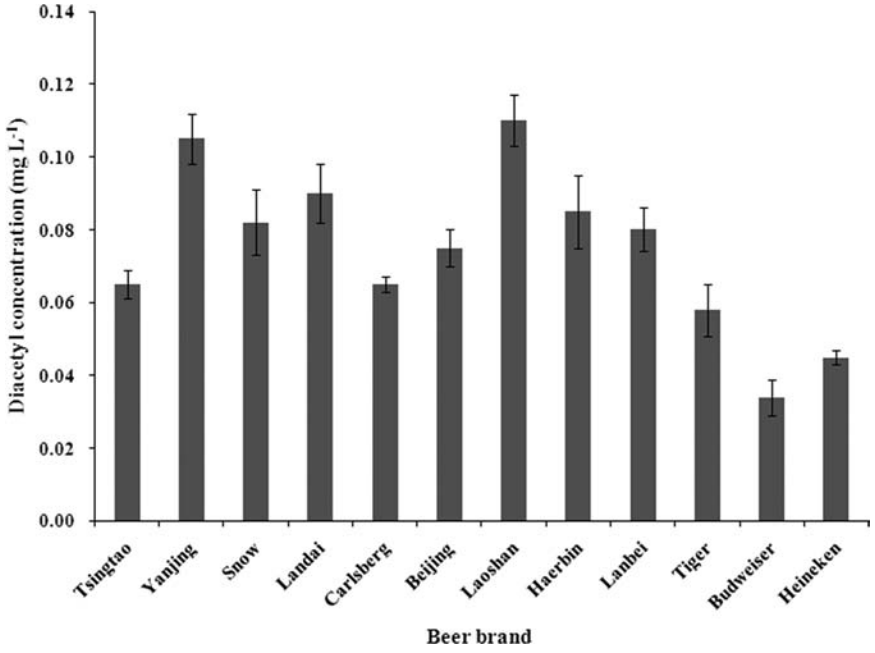

Figure 5. Detected concentrations of diacetyl in beer samples from 12 beer brands. Three samples from each beer brand were determined, and each sample was injected in triplicate. The bars in the figure represent the mean ± standard deviation with 95% confidence intervals.

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Notes

The authors declare no competing financial interest.

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