Analysis of volatile flavor compounds in top fermented wheat beer by headspace sampling-gas chromatography

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Abstract: Headspace sampling-gas chromatography (HS-GC) coupled with an internal standard method (ISM) was developed to analyze the volatile flavor compounds in top fermented wheat beer in our laboratory. Eight compounds, i.e. acetaldehyde, N-propanol, ethyl acetate, isobutyl alcohol, isoamyl alcohol, isoamyl acetate, ethyl hexanoate and ethyl octanoate were separated and quantified. This method was validated to ensure the quality of the results: the precision was satisfactory with relative standard deviation (RSD) in the range of 1.51%-4.22%, recoveries for all the analytes ranged from 95.15% to 99.85%, and the limits of detection were in the range of 0.0002-0.024 mg/L. Results of real wheat beer samples analyzed using this method showed that the volatile compounds' concentrations were in the range of 0.08-99.91 mg/L. Results suggested that this method exhibited good reproducibility, selectivity and high precision, and it can be useful for the analysis of routine beer samples.

Keywords: top fermented wheat beer, volatile flavor compounds, Headspace sampling-Gas chromatography, internal standard method

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1 Introduction

Beer is one of the most popular alcoholic beverages in the world, and it has been consumed in steadily increasing amounts in almost every country[1,2]. Wheat, as one of the traditional materials of brewing industry, is used more and more in beer fermentation in recent decades. The main reason for this is its widely available and high leaching ratio[3], and wheat beer is well received, a crucial factor in consumer-acceptance of this product is its mellower flavour and greater nutrition in comparison with traditional beer. Top fermentation is the most primitive way in beer brewing. Compared to bottom fermented wheat beer, top fermented wheat beer is richer in foam and more unique in mouth-feeling. Moreover, the shorter production cycle and quicker turnaround of equipment meet most of the manufacturers' demands[4,5].

Beer volatile flavor compounds are very important as they make a major contribution to the quality of final product[6]. Furthermore, flavor substances are uncontrollable during fermentation of beer, making it difficult for brewers to assure constant product quality or meet some consumers' expectations. Therefore, in order to monitor beer quality, it is necessary to build fast and reliable methods to investigate organoleptic characteristics such as aroma and flavor[7]. Generally speaking, volatile compounds in beer consist of several aliphatic and aromatic alcohols, esters, acids, carbonyl compounds, terpenic substances and others. Studies on the key aroma compounds are very important for modern brewing technology, which help the selection of raw materials and yeast strains, and the control of routine quality[2].

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Several sampling methods have been employed for the analysis of beer volatiles[8], such as gas sampling methods (including headspace sampling and purge-and-trap technique), solvent extraction/ concentration methods, distillation, headspace solid-phase micro-extraction (HS-SPME)[9,10], gas chromatography coupled to mass spectrometry (GC–MS)[11,12] and others. Headspace gas chromatography (HS-GC) is a technique of gas extraction and can be carried out comparable to a solvent extraction. HS analysis is done by analyzing a portion of the upper gas phase being in equilibrium with the liquid phase in a closed via, it is a well demonstrated analytical tool and has been widely used in the field of volatile flavor compounds in beverages and other related areas[13-15]. Static headspace sampling is a one-step extraction, which separates analytes in the capillary column and is utilized at low oven temperatures, and it is a simple, fast, sensitive technique for detection and identification of aroma components and those time-consuming and costly procedures could be avoided[7,16].

Beer is a very complex matrix and its analysis presents a particular problem due to the presence of CO_2. Furthermore, it is difficult in determining the target compounds in beer as the interference of the foam formation. Many decarbonation methods had been employed to solve this problem, but all of them have drawbacks, not only time consuming but also altering the actual composition of the sample, which can affect the determination of analytes[17,18]. On the other hand, the contents of flavor compounds in beer are greatly different and some of them are too slight to be detected. Therefore, the aim of this study was to develop a rapid and sensitive method for the determination and quantitative of aroma compounds in wheat beer using static headspace sampling with capillary columns gas chromatography[19]. The basic theory of quantitative analysis in HS-GC involves the measurement of peak area. To determine the concentration of a compound, the peak area is plotted versus the concentration of the substance[20]. Three different calibration methods (external standard, internal standard and addition method) can be utilized in quantitative analysis[21,22]. In this work, the application of chemometrics in the handling of HS-GC response data was investigated using the Internal Standard Method (ISM). Experimental results showed that this method could be successfully applied for the analysis of commercial and experimental beers[23].

2 Materials and methods

2.1 Materials

The volatile flavor compounds studied were: acetaldehyde, ethyl acetate, isobutyl alcohol, N-propanol, isoamyl alcohol, isoamyl acetate, ethyl hexanoate and ethyl octanoate. N-butanol was used as internal standard. All chemicals were of the chromatographic purity and purchased from Chemical Reagent Co., (Tianjin, China).

2.2 Sample preparation of beer

Wheat beer was prepared by simulating the traditional brewing method: Wort was fermented in 100 L fermentation tanks at 20℃ for about 10 days, and then wheat beer was cooled to 0℃ and stored for another 10 days.

About 350 mL wheat beer was sampled directly by flask iodine from the fermentation tank, and then plugged immediately with its ground glass stopper. After filtration, 200 mL of beer was added to a 250 mL volumetric flask and a 25 μL volume of N-butyl alcohol was added as internal standard to the sample and enough amount of beer samples were used to obtain a final volume of 250 mL and shake well.

The procedure of sample detecting followed the steps described below: 10 mL beer sample was injected into a 20 mL vial with a syringe, and then the vial was tightly sealed immediately with crimp caps. Each analysis was undertaken in triplicate with different vials. In order to minimize the loss of volatile compounds, the samples were kept at 4℃ when they were not analyzed.

2.3 Preparation of mixed standard solution

Mixed standard solution was prepared for calibration. Acetaldehyde (25 μL), N-propanol (50 μL), ethyl acetate (16 μL), isobutyl alcohol (25 μL), isoamyl alcohol (100 μL), isoamyl acetate (12 μL), ethyl hexanoate (2 μL),

and ethyl octanoate (2 μL), were injected accurately into 1 000 mL volumetric flask with 4% (v/v) of ethanol, respectively. And the final concentration were 19.70 mg/L acetaldehyde, 40.18 mg/L N-propanol, 14.40 mg/L ethyl acetate, 20.05 mg/L isobutyl alcohol, 81.25 mg/L isoamyl alcohol, 10.51 mg/L isoamyl acetate, ethyl 1.74 mg/L hexanoate, 1.58 mg/L ethyl octanoate, 80.95 mg/L N-butanol, correspondingly. Subsequently, 10 mL mixed standard solution was injected into 20 mL vial and tightly sealed immediately. Five parallel analyses were done.

2.4 Instrumentation

The GC-FID instrument (Agilent 6890N capillary gas chromatograph, Agilent Co., USA) was used to perform this study, connected to a static headspace auto-sampler (Agilent 7694E headspace auto-sampler, Agilent Co., USA). This sampler applies the principle of time-controlled injection. For improving the signal-to-noise ratios and minimizing chromatographic peak widths, the chromatograph was fitted with a 30 m × 0.32 mm i.d. HP-5, 5% phenyl methyl siloxane capillary column from Agilent with a low-film thickness of 0.25 μm. The optimized parameters are listed in Table 1.

Table 1 HS-GC parameters used in this study

Parameter	Optimized settings
GC	
Oven temperature 45℃ for 3 min, increased at 10℃/min to 180℃, held for 3 min	Vaporizer temperature 200℃
Detector temperature	250℃
Carrier gas	Nitrogen with high purity at 3.5 mL.min^{-1}
Split ratio	5:1
Equilibrium time	20 min
Headspace	
Vials equilibrium temperature	85℃
Injection loop temperature	100℃
Transfer line temperature	100℃
Equilibrium time	30 min
Carrier gas pressure	139 kPa
Pressurization time	0.13 min
Injection time	0.4 min
Injection loop filling time	0.13 min
Injection loop equilibration time	0.2 min
FID	
Temperature	250℃

Carrier gas was nitrogen (Evapotranspiration Materials Co., Zibo, China), and its purity was >99.999%. The 20mL headspace vials and the aluminum crimp caps were obtained from Derian Instrument Co., (Shanghai, China). The 1 000 mL micro injectors were obtained from Dragon (Shanghai, China).

2.5 Headspace sampling–gas chromatograph analysis

Peak identification of volatile flavour substances was based on the retention time of the individual reference standards. Head-space chromatographic profiles were compared with known reference flavour compound profiles for identification purposes. Volatile components were evaporated into vial headspace, and their separations were achieved by the capillary column. Nitrogen was used as carrier gas and kept at a constant flow-rate of 3.5 mL/min. Concentrations were determined by measuring peak area of volatile component compared with standard curves. The current signals from detector were stored and integrated by computer software.

2.6 Qualitative analysis

Eight top fermented wheat beer samples (10 mL, added with 0.1 μL internal standard each) were injected in a 20 mL headspace vial carefully and then added acetaldehyde (1 μL), N-propanol (1 μL), ethyl acetate (0.5 μL), isobutyl alcohol (0.5 μL), isoamyl alcohol (1 μL), isoamyl acetate (0.5 μL), ethyl hexanoate (0.1 μL), ethyl octanoate (0.1 μL), respectively, after which the vials were immediately sealed.

2.7 Statistical analysis

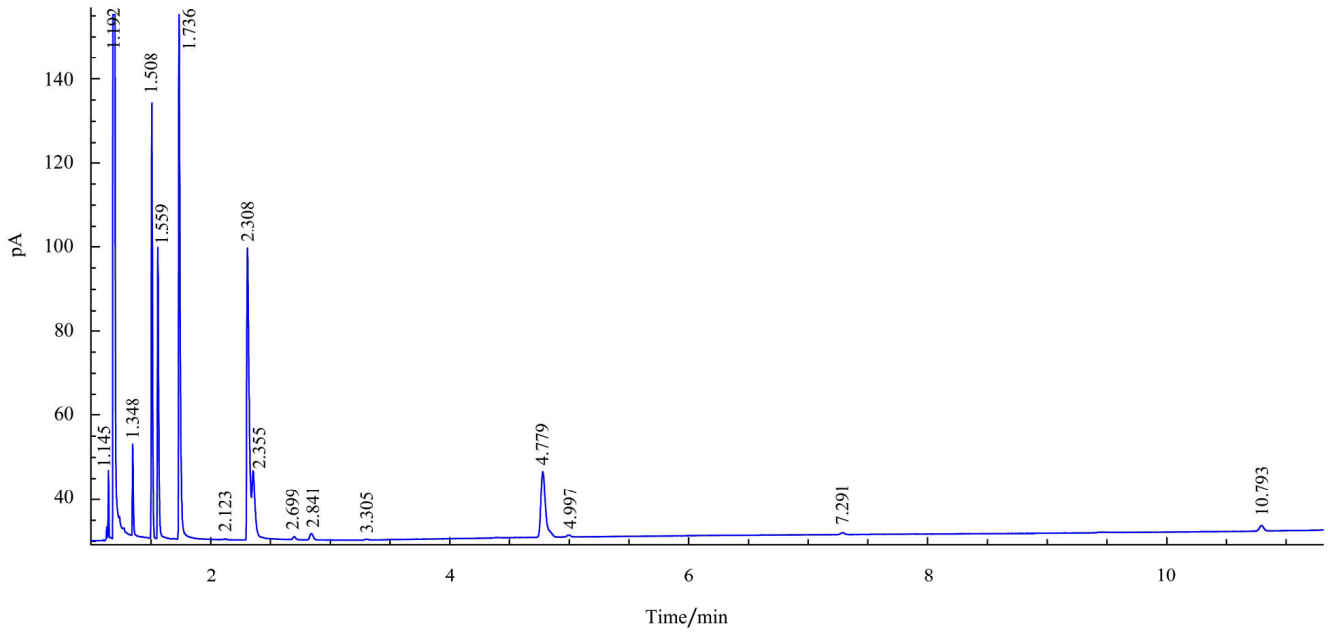
All the data used for method validation were analyzed according to SPSS14.0, and the data used for qualitative analysis were analyzed by Origin8 SR3. The statistical significance level was set at $P = 0.05$.

3 Results and discussion

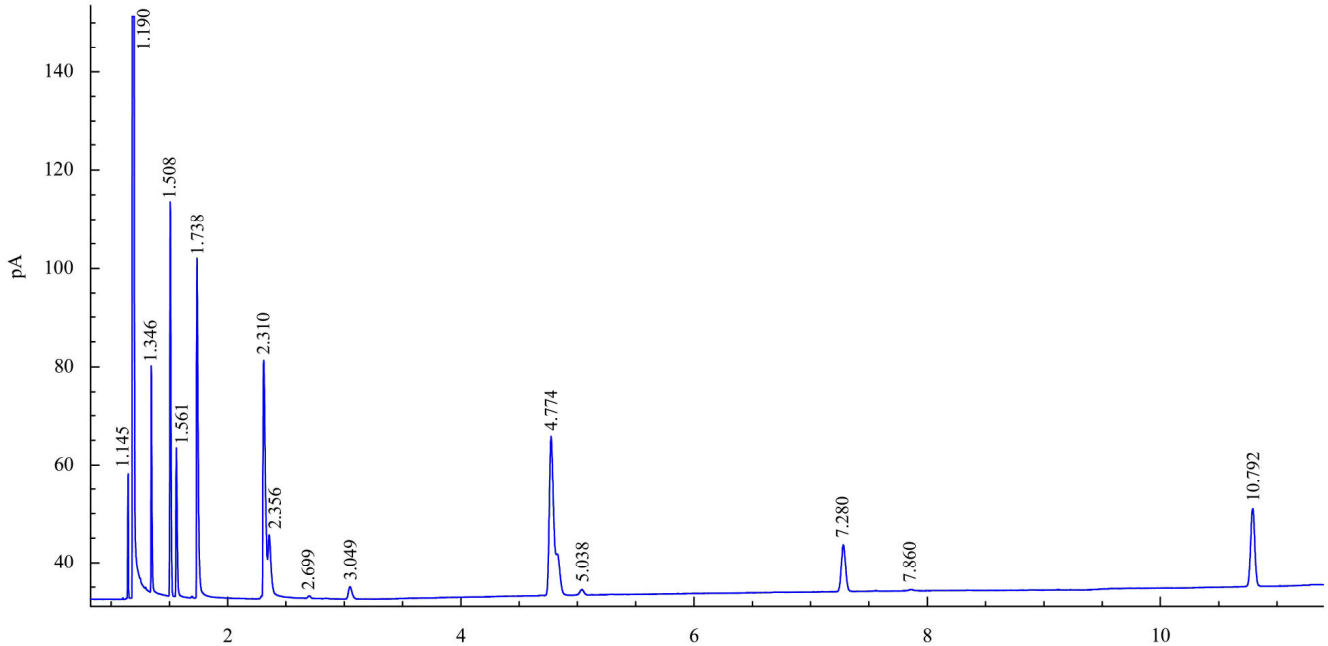
3.1 Chromatographic separation of volatile flavor compounds

Beer is a complex matrix in which the contents of flavor compounds are differed greatly, so the demands on a method for the analysis of aroma compounds in beer are very high. A beer sample and a mixed standard solution were analyzed by HS-GC and the optimized parameters

(Table 1) in this study, respectively. Compared to the GC profiles of the beer sample and the mixed standard solution in Figure 1, all the peaks of interest were sufficiently separated in beer sample. Eight compounds in wheat beer were analyzed besides N-butanol (the internal standard) and ethanol. The observed separation was found sufficiently to continue qualitative and quantitative analysis though some of the peaks were not baseline separated.



a. Beer sample



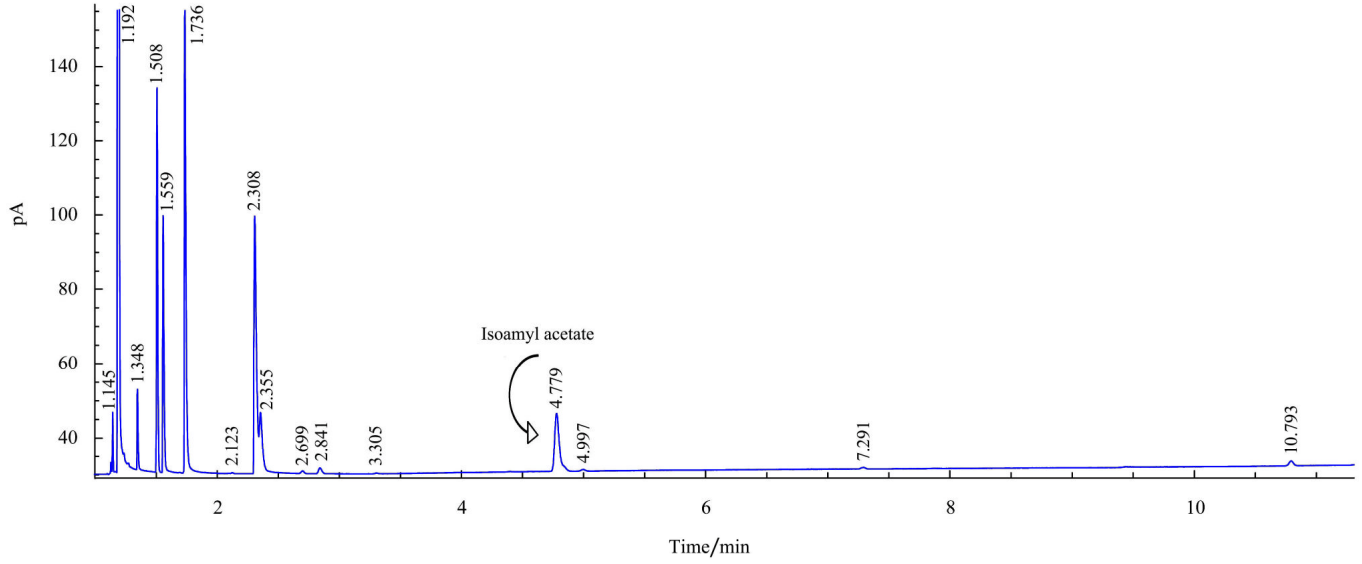
b. Mixed standard solution

Figure 1 GC chromatograms of beer sample added with N-butanol and the mixed standard solution: acetaldehyde (retention time 1.145 min); ethyl alcohol (1.192 min); N-propanol (1.348 min); ethyl acetate (1.508 min); isobutyl alcohol (1.559 min); N-butanol (1.736 min); isoamyl alcohol (2.308 min); isoamyl acetate (4.779 min); ethyl hexanoate (7.291 min); ethyl octanoate (10.793 min)

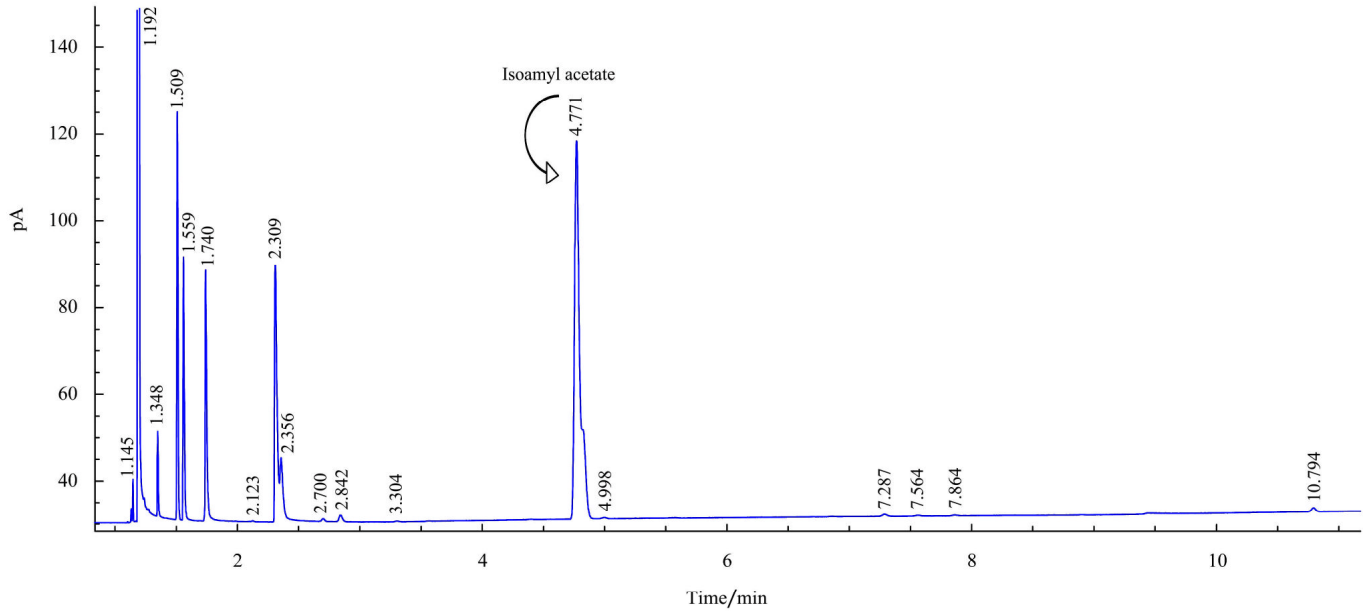
3.2 Qualitative analysis

This part was based on the peak area increase (combined with retention time method) of the individual reference standards. As an example, the chromatogram

of the beer sample added with isoamyl acetate shown in Figure 2b was analyzed by the method (described in section 2.4) and the results of the qualitative analysis of other samples are shown in Figure 3. Figure 2 shows a comparison of the GC profiles obtained for the beer sample added with isoamyl acetate (b), compared with the profile of the sample added with internal standard (a). Although some trace peaks had a slight change in retention time in corresponding to standard peaks, they do not affect the qualitative analysis as small changes occurred in beer composition after injecting standard volatile component. Compared with Figure 2a, the peak height and area (Figure 2b the peak of retention time at 4.771 min) increased significantly while other peaks remain unchanged, the peak corresponding to the volatile substances can be determined to isoamyl acetate. Similar results are shown in Figure 3, moreover, the peak area of samples added with individual standards were significantly higher than others.



a. Sample added with N-butanol



b. Sample added with ethy1 hexanoate

Figure 2 Comparison of the GC profiles

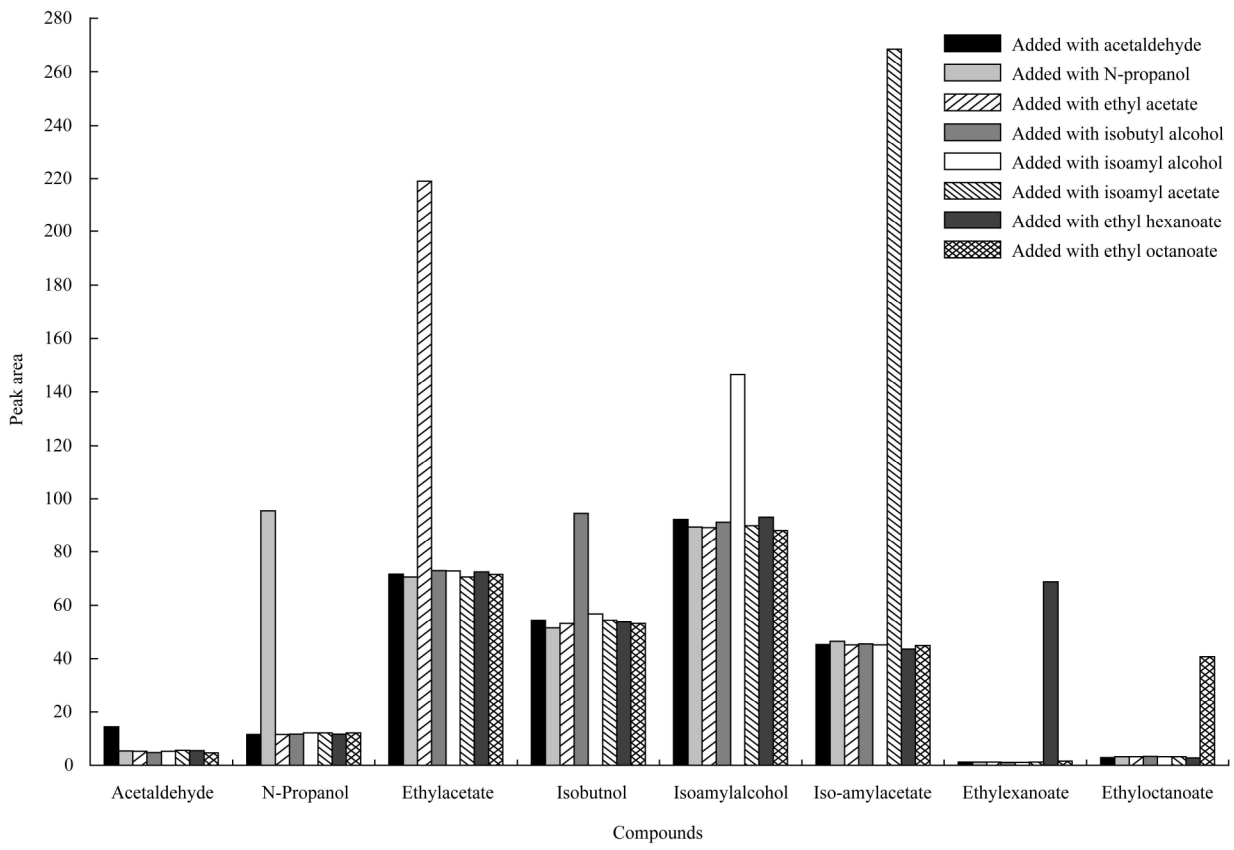
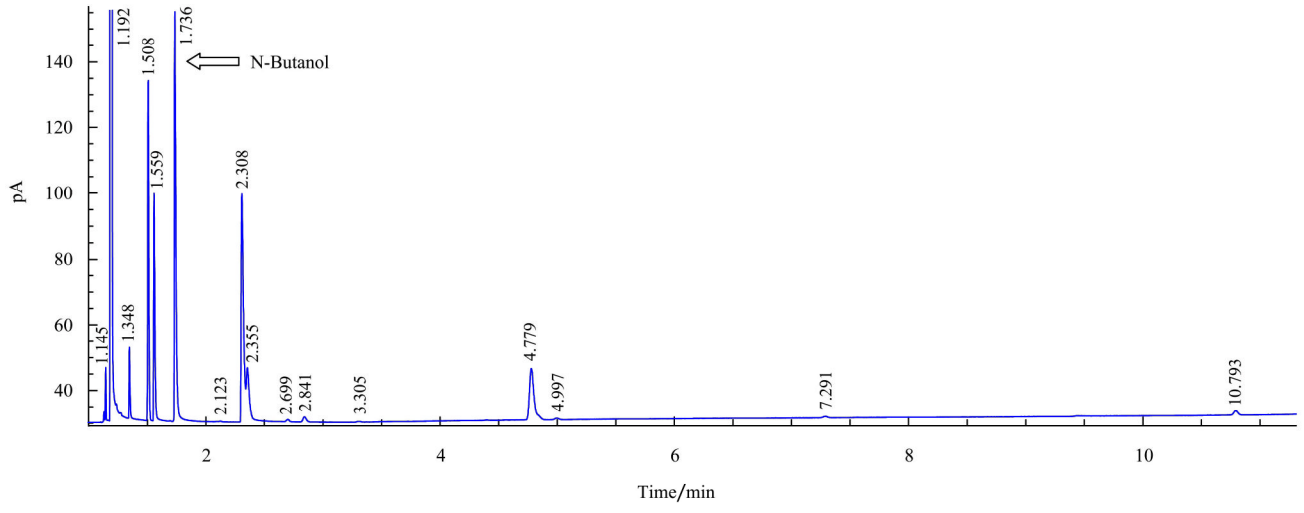


Figure 3 The qualitative analysis of eight sample results: 1. beer sample added with acetaldehyde (1 μL); 2. N-propanol (1 μL); 3. ethyl acetate (0.5 μL); 4. added isobutyl alcohol (0.5 μL); 5. isoamyl alcohol (1 μL); 6. isoamyl acetate (0.5 μL); 7. ethyl hexanoate (0.1 μL); 8. ethyl octanoate (0.1 μL)

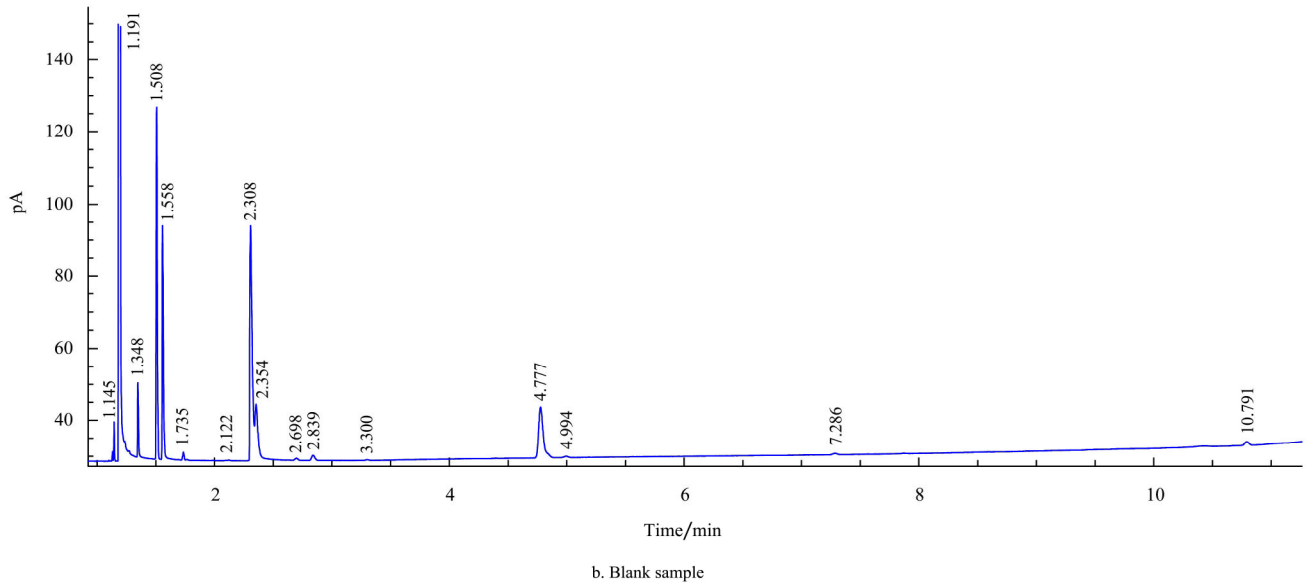
3.3 Quantitative analysis of flavor compounds in wheat beer

3.3.1 Choice of internal standard

N-butanol was chosen as the internal standard in this work since it was rarely detected in beer sample, besides the location of its peak appears just at the gap between the peaks of the studied compounds. An addition of 1 μL/mL was chosen since it gave a moderate response. Figure 4 shows a comparison of the GC profiles obtained for the beer sample (b) and the sample added with N-butanol (a). A new peak was appeared at the retention time of 1.736 min in Figure 4A, while other peaks remain unchanged, the peak was determined as N-butanol.



a. Sample added with N-butanol



b. Blank sample

Figure 4 GC chromatograms of sample added with N-butanol and blank sample

3.3.2 Determination of quantitative calibration factor

To obtain quantitative results from GC, it is necessary to use calibration factors. The relative calibration factor is described as below[24]:

$$f_i = \frac{A_s \times W_i}{A_i \times W_s}$$

where, A_i, A_s are the peak areas of compound i and reference compounds (N-butanol here); W_i, W_s are the contents of compound i and reference compounds.

Standard solution samples of five successive concentrations were determined by the optimized parameters described in the section 2.2, and the average results of the quantitative calibration factors of eight samples are showed in Table 2, with a mean value from 0.0320 to 1.5325.

3.3.3 Determination of real beer samples

The chromatogram results of a beer sample added with internal standard N-butanol are shown in Figure 1. Combined peak areas of each compound with the f_i (Table 2), the content of each flavor compounds in wheat beer can be obtained by the formula described below:

$$C_i = \frac{A_i \times C_s \times f_i}{A_s}$$

where, C_i, C_s are the concentrations of compound i and internal standard. The concentrations of eight compounds were calculated and the results were described in Table 2. Results indicated that ethyl hexanoate in top fermented wheat beer was tiny trace and isoamyl alcohol had the highest content among the eight compounds, with an average concentration of 0.08 mg/L and 99.91 mg/L, respectively.

3.4 Method validation

A validation study was carried out by assessing precision; recovery and detection limit (LOD).

3.4.1 Precision

Precision of the optimized method was expressed as the relative standard deviation (RSD) obtained from the determination of the five same beer samples. It was verified that the reproducibility depended on the compounds, with a mean value from 1.51% to 4.22% and it is sufficient for the studied compounds' quantitative analyzes. Similar values have been reported in wine[25], beer[8], water[26], blood and urine[27], using Headspace solid-phase micro-column extraction (HS-SPMCE) and gas chromatography-mass spectrometry analysis.

3.4.2 Accuracy

The recovery was defined as the percentage ratio between concentration of analyte found and concentration of analyte added. In order to check the accuracy of the proposed method confirmatory HS-GC method, a recovery test was carried on blank samples of beer at three concentration levels, each test performed five times, as described in section 2.2. As showed in Table 2, averages of recoveries are ranging from 95.15% to 99.85%. Results provided evidence that this method

guarantees that the volatile components in wheat beer can be properly quantified.

Table 2 Quantitative, Calibration, factor (f_i), precision (RSD %), recovery, detection limit (LOD) and concentration of investigated analytes

Compounds	f_i	RSD/%	Recovery	LOD/mg · L^{-1}	Concentrations/mg · L^{-1}
Acetaldehyde	1.5325	3.41	98.15	0.036	14.17
N-Propanol	1.2529	2.35	99.04	0.042	18.21
Ethyl acetate	0.2138	1.91	96.65	0.089	17.66
Isobutyl alcohol	0.7042	3.72	96.04	0.071	42.87
Isoamyl alcohol	0.8987	3.98	95.15	0.124	99.91
Isoamyl acetate	0.0830	2.29	96.75	0.065	4.46
Ethyl hexanoate	0.0567	4.22	99.85	0.0002	0.08
Ethyl octanoate	0.0320	1.51	95.25	0.0005	0.12

3.4.3 Limit of detection

The detection limit of an analytical procedure is the lowest concentration of analyte in a sample that can be detected. In general, the LOD of the method was determined by successive analyses of chromatographic sample extracts with decreasing amounts of the compounds until a 3:1 signal-to-noise ratio was reached[14,28]. The results were also presented in Table 2.

4 Conclusions

A headspace sampling-gas chromatography coupled with an internal standard method was utilized for the determination and quantification of eight volatile components in top fermented wheat beer samples in our laboratory. The technique was validated and proved to be sensitive, precise and accurate. The results obtained showed that HS-GC can be successfully used in the analysis of flavor compounds in beer, and indicate that it had the potential interest in real analytical applications. As also showed by many authors, the HS-GC is a reliable and reproducible technique[1,21]. In addition, the system error was reduced to the lowest by using internal standard method and had a more accurate quantification. Therefore, this method seems to be very promising in the analysis of volatile components of beer, although it is necessary to improve the studies, such as it is not easy to find a suitable internal standard and an additional step of adding quantitative internal standard in the process of sample preparation was required, before it becomes a widely usable technique. Data in this paper were also helpful to monitor the process of fermentation and determine the concentration of favours in beer.

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