RAPID AND SIMULTANEOUS DETERMINATION OF ACETONE, BUTANOL AND ETHANOL IN BUTANOL FERMENTATION BROTH BY FULL EVAPORATION HEADSPACE GAS CHROMATOGRAPHY

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Received December 16, 2013

A gas chromatography (GC) method including a full evaporation headspace (FE-HS) sampling technique was developed for rapidly and simultaneously measuring volatile products for acetone-butanol-ethanol (ABE) fermentation. In this method, a small volume (<50 μL) of fermentation broth was directly injected into a sealed headspace sample vial (22 mL) and the mass transfer of acetone-butanol-ethanol from liquid phase to vapor phase achieved equilibration at 105 °C for 5 min, the vaporous solvents were then determined by GC with a flame ionization detector. The results showed that the measurement precision and accuracy were excellent with present method, in which the relative standard deviations (RSD) of ethanol, acetone and butanol were of 1.7%, 1.2% and 0.76%, and their recovery was of 99.7±2.68%, 100±1.78% and 99.6±1.74%, respectively. The present method is simple, practical, automated, and suitable for application in the fermentation in related researches without any sample pretreatment.

Keywords: acetone-butanol-ethanol fermentation, full evaporation headspace, gas chromatography, *Clostridium acetobutylicum*

INTRODUCTION

Butanol fermentation is historically referred to as the bioprocess that produces acetone, n-butanol and ethanol from starch through bacterial fermentation.[1] Butanol fermentation was once the second largest biotechnological industry in the world for production of organic solvents and synthetic rubber precursors in the first half of the 20th century, but it came to a halt in the 1960s because of a failure in competition with the petrochemical industry.[1-4] Recently, with the promoting public environmental awareness and the increasing demand for renewable fuels and chemicals, this process has resurfaced and been redesigned using lignocellulosic biomass as feedstock.[5-8] Usually, the acetone-butanol-ethanol fermentation (ABE) process is accomplished by solvent-producing bacteria of the genus Clostridium under anaerobic conditions,[2] in which either hexoses or pentoses sugars can be fermented to produce acetone, butanol and ethanol.[9] Recent development in high-throughput biotechnology opens up the possibility of obtaining superior biobutanol-producing strains with an excellent adaptability in the saccharification liquor of lignocellulose.[10-12] Clearly, an efficient analytical method is significant for the determination of ABE fermentation products. The method also plays an important role in the process study to clarify how the process parameters, such as substrate concentration, temperature, aeration, pH etc., impact the production of the products from butanol fermentation.

There are three methods (mass spectroscopy, near- or mid-infrared spectroscopy and gas chromatography) available for simultaneous determination of the major products, i.e., acetone, n-butanol and ethanol, from butanol fermentation.[13-15] However, they are associated with problems in sampling and sample preparation that affect the precision and accuracy in quantification. For example, the mass

spectroscopy (MS) method is susceptible to membrane inlet problems, which leads to slow response times, nonlinear calibration, and membrane memory effects.[13] The near- or mid-infrared spectroscopic method[14,16] also relies on a membrane extraction of the products and can hardly provide results of high precision. Since all these products from ABE process are volatile, gas chromatography (GC) is a suitable technique that can give high quality results. However, it is mandatory to pretreat the sample, typically by solvent extraction, due to the non-volatile sample matrix. Such pretreatment is usually time-consuming and may easily cause errors in the experiment.[17-18]

Headspace gas chromatography (HS GC), usually based on the partition of analyte(s) between vapor and liquid phase, can avoid the penetration of non-volatile species into the GC system and therefore is widely used for the determination of volatile species in complex matrices, including electrolytes and solids.[19] However, the conventional HS GC is a highly sample matrix dependent technique and cannot use a simple external calibration in ABE sample analysis.[19] Although an on-line GC technique based on headspace sampling from a fermentor[20] has been developed, it suffers from the problems associated with method calibration because the partition coefficients of the species are highly related to the process conditions, e.g., the temperature and matrices of the samples. The drawbacks found in the above methods can be overcome by the full evaporation headspace GC technique (FE-HS GC).

In the previous work, several FE-HS GC methods were developed for the determination of a number of volatile organic compounds in pulping liquor,[21-22] polymer matrix[23-24] and alcohol.[21,25] Using a similar FE-HS GC technique, we believe that the major products in ABE samples can be also determined. The challenge will be the interference caused by the matrix substances originated from either thermochemical pretreatment or enzymatic hydrolysis.

In the present work, we report on an FE-HS GC method designed to rapidly and simultaneously determine acetone, butanol and ethanol in the butanol fermentation broth. The research focus has been mainly on the optimization of the conditions for these species in the headspace equilibration for GC measurement.

EXPERIMENTAL

Chemicals

All chemicals used in this work were of reagent grade from commercial sources. Standard solutions for GC analysis consisted of equal volumes of ethanol, acetone and butanol by adding different volumes of solvents into 100 mL distilled water.

Samples

The samples were obtained from the fermentation process for cassava dreg hydrolysate with a concentration of 45 g/L. The cassava dregs were hydrolyzed with 50 filter paper units (FPU)/g substrate of cellulose (Cellic® CTec2, Novozymes A/S, Bagsveard, Denmark) at 50 °C, pH 4.8 and 150 rpm for 72 h. Then the hydrolysate was fermented by *Clostridium acetobutylicum* (GIM 1.165, Microbial Culture Collection Centre, Guangdong Institute of Microbiology, Guangzhou, China) at 37 °C, pH 7.5.

Apparatus and operation

For standard solutions or fermentation liquors, a 1-100 μL aliquot was injected into a sealed vial and heated in an HP-7694 Automatic Headspace Sampler (DANI, Italy). Samples were equilibrated in headspace at a temperature of 105 °C, vial pressurization time of 0.2 min, sample loop fill time of 0.2 min, and loop equilibration time of 0.05 min. The GC system (Agilent Technologies, Santa Clara, US) operating conditions were as follows: HP-5 capillary column at 34 °C and a nitrogen carrier gas flow rate of 1.1 mL/min. A flame ionization detection (FID) was employed with hydrogen and air flow rates of 40 and 400 mL/min, respectively. If not stated otherwise, all data reported herein were the means of triplicate measurements with standard derivation as error.

RESULTS AND DISCUSSION

Headspace analysis of acetone, butanol and ethanol in butanol fermentation broth

Fig. 1 shows typical headspace gas chromatograms from butanol fermentation broth. The volatile products were eluted in an order of ethanol (1.13 min) < acetone (1.19 min) < butanol (2.034 min), whose characteristic peaks are sufficiently separated. The overall analytical time for a sample in GC is less than 3 min, which is neglectable compared to the several-day fermentation process.

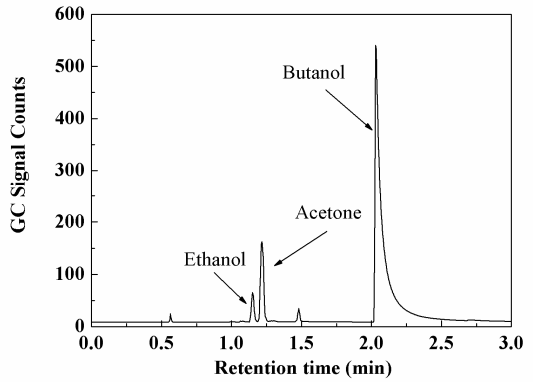

Figure 1: HSGC chromatogram from a butanol fermentation sample

Conditions for FE headspace analysis

It is crucial in FE headspace analysis to achieve a complete mass transfer of analytes from the liquid phase to vapor phase in a short period of time in the HS GC analysis. In this work, the effect of major experimental parameters on mass transfer is investigated.

Equilibration temperature and completeness of solvent mass transfer

An equation for calculating the traction yield in the full evaporation technique was provided by Kolb and Ettre,[19] i.e.,

$$Y\% = \frac{100}{KVs/(Vt - Vs) + 1} \quad (1)$$

where Y is the extraction yield, K (=1/ H) is the partition coefficient, and Vs and Vt are the sample and sample vial volume, respectively. The partition coefficient depends on the absolute temperature and is given by Eq. (2):

$$\ln K = \frac{A}{RT} - B \quad (2)$$

At 80 °C, the partition coefficient of butanol is 98.9,[26] the extraction yield is 92% when spiking 20 µL samples to a 22 mL headspace vial. It can be found in Eq. (2) that K decreases with the increasing absolute temperature, therefore it is necessary to raise the temperature to equalize the two phases of the system. In Fig. 2, it can be seen that when the sample is subjected to headspace at a volume of 20 µL for 5 min, a two-phase (gas-liquid) equilibrium is achieved at 85 °C, which is in accordance with the theoretical calculation.

Combined with the partition coefficient (K=207) of the ethanol-water system at 80 °C,[26] the extraction yield drops to 84%, when the sample and sample vial volume is 20 µL and 22 mL, respectively. According to Eq. (2), raising the oven temperature can lead to reaching near-complete evaporation. In Fig. 2, it can be seen that 105 °C is the equilibrium temperature of ethanol, which agrees with the result in Li's report.[25] The mass transfer of the acetone form condensate phase to a gaseous phase reaches equilibrium at 55 °C. In conclusion, 105 °C was selected as the headspace equilibration temperature.

To evaluate the completeness of the analytes' mass transfer to the vapor phase, a 20 µL sample was added on a piece of filter paper, which was then placed in a closed vial for the first headspace GC measurement at the selected equilibration temperature of 105 °C and equilibration time of 5 min. Then the second HSFE measurement was performed under the same conditions, and employed the filter paper transferred from the first vial. The GC response of the first measurement is significantly higher than that expressed in the second measurement. The mass transfer quantity (i.e., efficiency) of solvent from the liquid phase to the vapor phase can be calculated by the known peak areas (A), i.e.,

$$Eff(\%) = \left(1 - \frac{A_2}{A_1}\right) \times 100 \quad (3)$$

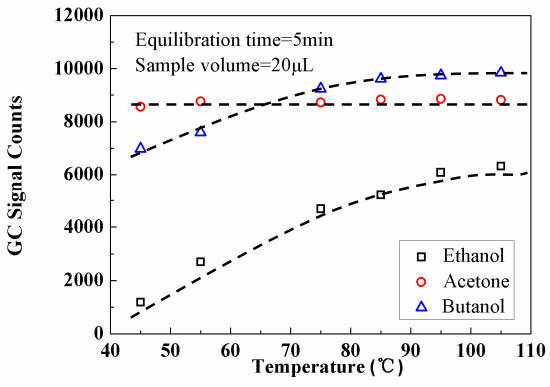

Figure 2: Effect of equilibration temperature

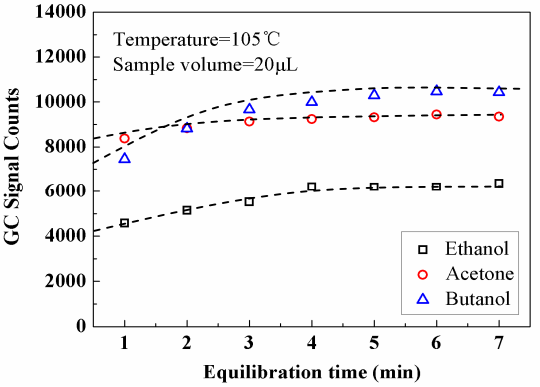

Figure 3: Effect of equilibration time

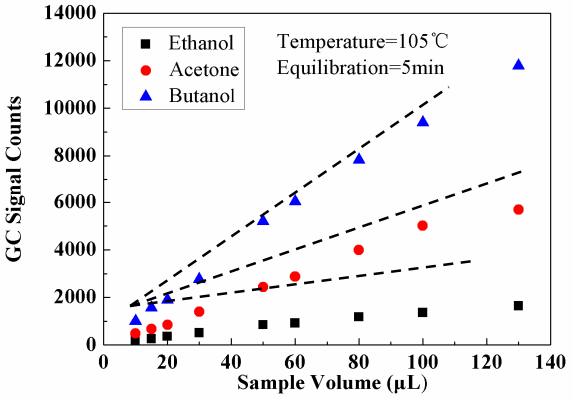

Figure 4: Effect of sample volume

The results show that the efficiency of the first equilibration for ethanol, acetone and butanol is 96.13%, 99.36%, 97.59%, respectively, experimentally proving that near-complete evaporation has been achieved under the given conditions.

Equilibration time

At the given temperature (105 °C), for the purpose of increasing sample injection accuracy and reducing potential condensation,[25] a 20 μL sample volume was chosen to investigate the effect of equilibration time of the gaseous solvent transferred from a fermentation sample. As shown in Fig. 3, the vapor–liquid equilibrium of acetone, ethanol and butanol can be achieved within 2, 4 and 5 min respectively. The equilibration time is dependent on the saturated vapor pressure at a certain temperature. The results indicate that acetone is the most volatile species followed by ethanol and butanol. Thus, 5 min is an appropriate equilibration time to establish the GC signal counts of the three solvents and maximize the solvent in the vapor in the closed sample vial.

Sample volume

Larger sample volumes contribute to the sensitivity improvement of the headspace measurement, especially those metabolites accumulated in a small concentration. Increasing sample injection volume is unfavorable for the analytes in a closed vial to achieve near-complete evaporation, even in a longer equilibration time or at a higher temperature.

Fig. 4 shows the effect of sample volume on the full evaporation of the three solvents of the fermentation broth sample. The results suggest that the GC response signals were linearly proportional to the sample volumes of the fermentation products when the sample volume was in a certain range. The linear relationship indicates a near-complete mass transfer of the

solute from the liquid phase to the vapor phase is consistently achieved. When the sample volume of ethanol, butanol and acetone is respectively more than 50 μL, 80 μL and 100 μL, there is no linear relationship between the GC response and sample volume. The reason is probably that some of the analyte could be trapped in the condensate phase or the headspace conditions were not sufficient to achieve a truly full evaporation. Therefore, 10-50 μL is the optimum range that can be used for an accurate determination of the three solvents in the fermentation broth. It can be seen that the maximum sample volume for ethanol, butanol and acetone is 50 μL (the same as Li's result),[25] 80 μL and 100 μL, and in the previous work the maximum sample volumes of the analytes are also different.[23-26] According to Eq. (1), the maximum sample volumes are directly determined by the partition coefficient and headspace vial sample under the conditions of complete full evaporation.

Method calibration, precision and validation

External standard calibration was used in the present FE-HS GC method. The calibration was based on adding into the headspace sample vials 20 μL of standards with different solute concentrations. The following standard calibration curves for acetone, butanol and ethanol were obtained and can be expressed as:

$$A=a(\pm\Delta a)+b(\pm\Delta b)\times c \quad (4)$$

where A and c represent the integrating area of the GC response of the solute and its concentration (mg/L or ppm) in the headspace sample vial, respectively. The regression coefficients (R^2) were 0.9991, 0.9993 and 0.9997 (n = 6) for ethanol, acetone and butanol, respectively.

The precision of the present method was studied. The results show relative standard deviations of ethanol, acetone and butanol in six measurements to be less than 1.69%, 1.23 % and 0.76%.

Table 1
Parameters of the calibration equation

Species	a	Δa	b	Δb	R^2
Ethanol	163.9	27.21	609.5	6.91	0.9994
Acetone	52.89	36.60	1037	8.99	0.9996
Butanol	209.8	50.39	1029	13.01	0.9992

Table 2
Method validation

Species	Sample No.	Concentration (mg/L)		Recovery (%)
		Added	Measured	
Ethanol	1	578	590	102.1
	2	891	872	97.9
	3	2368	2354	99.4
	4	6476	6591	101.8
	5	8550	8649	101.2
Acetone	1	547	539	98.5
	2	843	832	98.7
	3	2044	2098	102.6
	4	5814	5739	98.7
	5	8343	8276	99.2
Butanol	1	625	613	98.1
	2	1063	1095	103.0
	3	2584	2511	97.2
	4	6551	6420	98.0
	5	10010	10221	102.1

The validation of the FE-HS GC method was performed by accurately adding 500 μL standard solutions with different concentrations into 5 mL butanol fermentation broth. The original

fermentation broth (i.e., without added solvents) contained 330 mg/L, 752 mg/L and 1644 mg/L ethanol, acetone and butanol respectively in a proportion of 1:1.9:4.2. Therefore, the net contribution of the added solvents for make-up samples could be determined by the present method. The concentration of the volatile species, i.e. butanol, in the sample can be calculated by Eq. (3).

The measured and added concentrations of the three species in the fermentation broth were summarized in Table 2, covering a detection concentration from about 500 to 11000 ppm. The recoveries from these measurements were of 100±1.78%, 99.6±1.74% and 99.7±2.68% for ethanol, acetone and butanol, respectively. This indicates the method can provide consistently high and accurate data for the ABE process.

CONCLUSION

An FE HS-GC technique for the determination of acetone, butanol and ethanol in a butanol fermentation broth has been successfully developed. The near-complete mass transfer of the analytes from the liquid phase to the vapor phase can be achieved by injecting less than 50 μL of sample volume into a sealed sample vial for 5 min, at the selected temperature of 105 °C. The relative standard deviations (RSD) of ethanol, acetone and butanol in this method were of 1.7%, 1.2% and 0.76%, with a recovery of 99.7±2.68%, 100±1.78% and 99.6±1.74%, respectively, indicating high accuracy and precision. The present method requires no sample pretreatment, so it is simple, practical, automated, and suitable for acetone-butanol-ethanol determination simultaneously on both industrial and laboratory scale.

ACKNOWLEDGEMENTS: The financial support of the National Natural Science Foundation of China (No. 31170549) and the Major State Basic Research Development Program (No. 2010CB732206) are gratefully acknowledged.

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