

Simultaneous Determination of Ethanol and Carbon Dioxide by Two-Order Microdiffusion Analysis and Its Application to Analysis of Alcoholic Fermentation¹⁾

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(Received February 12, 1971)

A procedure and an apparatus of two-order microdiffusion analysis have been devised and applied to the simultaneous determination of ethanol and carbon dioxide. The conditions and the limitation of the assay method are as follows:

1. Amount of outer chamber solution; 3 ml
2. pH of outer chamber solution; 1.3—8.7
3. Range of determination and value of coefficient of variation; ethanol, less than 23 mg, 2%
carbon dioxide, less than 14 mg, less than 6%
4. Diffusion temperature; 30—37°
5. Diffusion time; either more than 24 hr at 37° or 30 hr at 30°
6. Rate of recovery; ethanol, 99% (pH 1.3), carbon dioxide 100% (pH 1.3)

The assay method was also applied to simultaneous determination of ethanol and carbon dioxide evolved in the process of alcoholic fermentation.

The microdiffusion analysis was established by Conway and his coworkers³⁾ and, up to this time, the method has been used only for the estimation of one component released from a sample solution. However, in chemical or biochemical reactions, there often exist more than two volatile components in the reaction media. Such situation imposes some restrictions on the applicability of this method. Therefore, it is desirable to be able to estimate simultaneously more than two volatile components using only one unit operation. From this standpoint, the authors devised a novel microdiffusional procedure by which more than two volatile components can be simultaneously estimated. We applied this procedure to a simultaneous determination of carbon dioxide and ethanol, both of which are products of alcoholic fermentation. Using this technique, we also investigated the effect of dyes on the rate of the fermentation. The purpose of the investigation was to check the dye which inhibit alcoholic fermentation. In the new procedure the conventional methods for the determination of ethanol and carbon dioxide were somewhat modified. To the colorimetric determination of ethanol, we applied Ozawa's modified procedures⁴⁾ of Conway's method with further improvement. Among several simplified methods for the determination of carbon dioxide, we selected Kawabata's chelate-titration method,⁵⁾ and used it with some modifications. This selection is based on the fact that the interference of volatile organic acids formed during fermentation can be excluded.

Experimental

Apparatus—Conway-Ishizaka's semi-micro unit (the outer chamber: inner diameter 90 mm, inner height 17 mm; the inner chamber: outer diameter 65 mm, inner diameter 60 mm, inner height 8.5 mm),

- 1) The work was presented at the Meeting of Tokai Branch, Pharmaceutical Society of Japan, Nagoya, Feb. 1967 and a part of this work was presented as a preliminary communication; H. Ishihara and O. Ishizaka, *Chem. Pharm. Bull.* (Tokyo), **16**, 2524 (1968).
- 2) Location: *Tanabe-dori, Mizuho-ku, Nagoya.*
- 3) E. J. Conway, "Microdiffusion Analysis & Volumetric Error," Crosby Lockwood & Son Ltd., 1962.
- 4) M. Ozawa, T. Nagaoka, and K. Kakuno, *Eisei Kagaku*, **10**, 17 (1964).
- 5) M. Kawabata, H. Mochizuki, R. Kajiyama, M. Watanabe, M. Ishii, and K. Sasaki, *Bunseki Kagaku*, **11**, 192 (1962).

purchased from Shibata Chemical Apparatus MFG. Co., Ltd. is used with special accessory cups. The inner chamber contains two large cups for absorbents (inner diameter 22.3 mm, inner height 12 mm), and if necessary, an additional large cup or a small cup (inner diameter 12.5 mm, inner height 12 mm) is inserted in it: when test solution is less than 2 ml the test solution is inoculated, not into the outer chamber but into the third cup mentioned above, because the volume of the test solution decreases considerably owing to the strong desiccative effect of the acidic absorbent. The apparatus is shown in Fig. 1.

Reagents—1) 0.01 M EDTA-Mg Soln.: 7.5 g of disodium ethylenediaminetetraacetate was dissolved in 800 ml of H₂O, and the solution was adjusted to pH 7.0 with 1 N NaOH. 2.1 g of MgCl₂ was added to the solution and the solution was diluted to 1 liter with H₂O. The soln. was standardized against 0.01 M MgCl₂ standard soln.

2) 0.01M MgCl₂ Standard Soln.: To a suspension of 0.4032 g of MgO, previously dried, in 10 ml of H₂O was added dil. HCl to make a soln. Excess HCl and H₂O were evaporated to dryness on a water bath and the residue was dissolved in H₂O, the final volume being 1 liter.

3) Ammonia Buffer Soln.: 67.5 g of NH₄Cl was added to 570 ml of aq. NH₄OH (28%), and the solution was diluted to 1 liter with H₂O.

4) Indicator Soln.: 0.5 g of Eriochrome Black T and 4.5 g of hydroxylamine-HCl were dissolved in 100 ml of MeOH.

5) 0.03 M BaCl₂ Soln.: 7.32 g of BaCl₂·2H₂O was dissolved in CO₂-free H₂O, and the solution was made up to 1 liter.

6) 2 N K₂Cr₂O₇-H₂SO₄ Soln.: 9.808 g of K₂Cr₂O₇ was dissolved in 10 N H₂SO₄, and the solution was made up to 100 ml with 10 N H₂SO₄.

7) Henneberg's Culture Medium: A mixture of (5 g of CaCO₃, 2 g of MgSO₄·7H₂O, 5 g of KH₂PO₄, 5g of peptone and 150 g of sucrose in 1 liter of H₂O) is sterilized in an autoclave. This medium is adopted only for cultivation of yeast strain.

8) Culture Medium for Assay (C.M.A.): Modified Henneberg's medium is adopted; sucrose content is decreased to 0.1 M (34.23 g/liter).

9) Culture Medium for Blank Test (C.M.B.): A medium in which sucrose is omitted from C.M.A. is adopted.

Sake Yeast Strain—*Saccharomyces sake* var. the Brewing Institute No. 6 was used throughout this work. It was inoculated on the Henneberg's slant agar-agar culture medium and incubated at 30° for 4 days.

Preparation of Yeast Cell Suspension—Pipetted yeast cells at the exponential phase were suspended in sterilized soln. of 0.5% KH₂PO₄ and the suspension was centrifuged at 3000 rpm for 10 min. The procedure was repeated further three times. The precipitated cells were resuspended in 0.5% KH₂PO₄. The volume of the suspension was adjusted so as to give a cell count approximately 5 × 10⁷ per ml. Count of yeast was measured by Thoma's blood cell counter. The suspension was assayed, after one day's standing at room temperature.

Procedure—1. General Manipulation and Diffusion: One ml of test soln. (or solvent for blank test) is inoculated in the outer chamber, and to each of large cups (a) and (b) is pipetted 1 ml of 2 N K₂Cr₂O₇·H₂SO₄ and 1 ml of 1 N KOH soln., respectively. Then 2 ml of 0.1 N H₂SO₄ is introduced into the outer chamber as a releasing agent through a pipet. The unit is covered immediately with a lid smeared with liquid paraffine, closed airtightly with a steel spring clip, and left standing at 30–37°. After the lapse of time the lid is detached from the unit and the contents are assayed.

2. Determination of Carbon Dioxide: The large cup (b) is transferred into a beaker of 100 ml contents. Ten ml of 0.03 M BaCl₂, 10 ml of ammonia buffer, 30 ml of MeOH and 5 drops of indicator soln. are added successively as fast as possible. When test soln. contain more than 12 mg of carbon dioxide, 15 ml of 0.03 M BaCl₂ is added. The cup is taken out from the beaker, washed with a small amount of H₂O, and the washings combined with the contents of the beaker. The combined contents are titrated with 0.01 M EDTA-Mg standard soln. under continuous stirring. Blank unit is treated simultaneously as mentioned above.

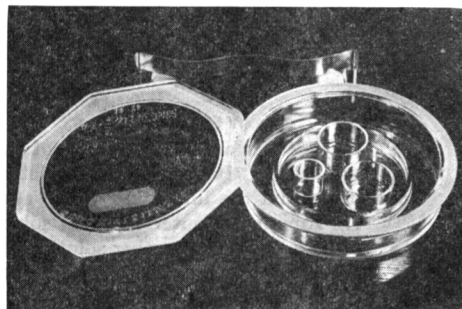


Fig. 1. Apparatus of Two-Order Micro-diffusion Analysis

$$\text{CO}_2 \text{ mg/outer chamber soln.} = (B - A) \times 0.44 \times f$$

B: ml of titration for blank unit

A: ml of titration for assay unit

f: factor of 0.01M EDTA-Mg soln.

3. Determination of Ethanol: The contents of large cup (a) is transferred into a measuring flask of 10 ml contents with an aid of H_2O , and made up to 10 ml with addition of H_2O . The optical absorbance⁶⁾ is checked under the comparison with blank test soln., and ethanol concentration calculated from the calibration curve.

4. Assay of Alcoholic Fermentation: The manipulation is performed as fast and aseptically as possible, following the steps (*cf.* procedure 1) as mentioned below.

1) One ml of 2 N $K_2Cr_2O_7-H_2SO_4$ is pipetted into a large cup (a).

2) Each 0.5 ml of C.M.A. or C.M.B. is settled into a small cup through Ostwald's pipet, the former adapts for test soln. and the latter for blank test.

3) One ml of 1 N KOH is inoculated into a large cup (b) through Bang burette, 2 ml contents. This procedure is omitted when the assay is aimed at ethanol contents determination only.

4) Yeast cell suspension (0.5 ml) is inoculated into a small cup through Ostwald's pipet and the unit is manipulated under the direction described in procedure 1. at 30° for the period of more than 30 hr. After the lapse of time, the large cups (a) and (b) are taken out from the inner chamber of the unit and the contents analysed.

At any intermediate stage of fermentation, addition of 0.2 ml of 18% trichloroacetic acid into the small cup through a slit stops fermentation only, while diffusion of carbon dioxide and ethanol is continued as usual. Slit formation is performed by horizontal moving of a lid under a caution that the manipulation should be done as fast as possible.

Result

1. Range of Assay

One ml of 2 N $K_2Cr_2O_7-H_2SO_4$ was sufficient to oxidize 0—23 mg of ethanol as indicated in Fig. 2. On the other hand, it was apparent from the result of recovery of carbon dioxide shown in Table I that the carbon dioxide must be less than 14 mg for an accurate measurement.

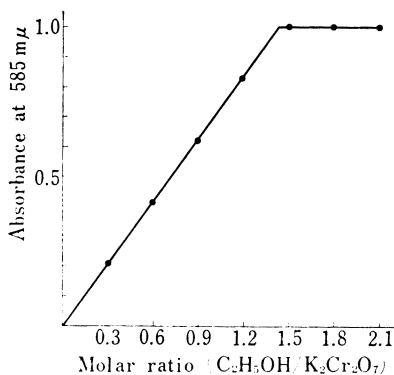


Fig. 2. Stoichiometry of the Oxidation of Ethanol

One ml of 2N $K_2Cr_2O_7-H_2SO_4$ and 0.1—0.7 ml of 1M C_2H_5OH were pipetted into a measuring flask of 10 ml volume, mixed and left standing at 30° for 120 min, then made up to 10 ml with H_2O and optical absorbance was measured against 2N $K_2Cr_2O_7-H_2SO_4$ diluted into 10-fold with H_2O .

in the presence of the dye it required more than 2×10^7 starting cells to attain the same fermentation (Fig. 5 (2)). In both cases the fermentation was markedly decreased or inhibited if the count diminished from the indicated values.

6. Fermentation Rate as a Function of Time

As shown in Fig. 6, the fermentation appeared to finish within 6 hr. As already described in 4, complete diffusion of ethanol and carbon dioxide required at least 30 hr at 30°.

2. Deviation and Recovery

The coefficient of variation and recovery in each standard series are described in Table I.

3. Consideration of Diffusion pH

As shown in Fig. 3, ethanol and carbon dioxide were almost completely recovered from a mixture of test and buffer solution at the range of pH 1.3—8.7

4. Effect of Diffusion Temperature

As shown in Fig. 4, diffusion completed at 30° after 30 hr or at 37° after 24 hr.

5. Relationship Between Yeast Cell Numbers and Fermentation Activities

Fermentation activities of yeast, inoculated in C.M.A. without or with 0.1% Rose Bengale, were investigated with preliminary examinations. As shown in Fig. 5 (1), without Rose Bengale, maximum fermentation was attained starting with the cells more than 1×10^7 , while

6) The absorbance was determined on a Hitachi Parkin-Elmer Spectrophotometer Model 139.

TABLE I. Deviation and Recovery

0.4M KHCO ₃ (ml)	CO ₂ (mg) ^{a)}	\bar{X} (ml)	σ/\bar{X} 100	Recovery %
0.1	1.76	3.07	6.0	92.5
0.2	3.52	8.03	3.3	100.3
0.3	5.28	10.97	1.4	91.5
0.4	7.04	16.10	1.3	100.6
0.5	8.80	19.69	0.35	98.4
0.6	10.56	24.40	0.29	101.7
0.7	12.32	28.20	0.56	100.7
0.8	14.08	31.70	1.2	99.1
0.9	15.84	33.50	1.6	93.1
1.0	17.60	36.60	0.85	91.5

0.4M C ₂ H ₅ OH (ml)	C ₂ H ₅ OH (mg) ^{a)}	\bar{X} (OD ₆₈₅)	σ/\bar{X} 100	Recovery %
0.1	1.84	0.086	1.8	108.1
0.2	3.68	0.162	1.1	101.9
0.4	7.36	0.325	2.0	102.0
0.6	11.04	0.506	0.89	101.2
0.8	14.72	0.672	1.6	103.4
1.0	18.40	0.832	1.1	104.0

\bar{X} : average of titer or absorbance σ : standard deviation
 a) theoretical

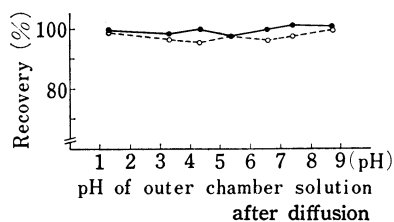


Fig. 3. Effect of pH on Simultaneous Recovery of C₂H₅OH and CO₂

Outer chamber solution contained 0.1 ml of 0.4M KHCO₃, 0.1 ml of 0.4M C₂H₅OH, 0.8 ml of H₂O and 2 ml of buffer. From pH 3.3 to 8.7, the buffer was 0.2M Na₂HPO₄-0.1M citrate. At pH 1.3, 0.1N H₂SO₄ was used.

---○---: C₂H₅OH ●---: CO₂

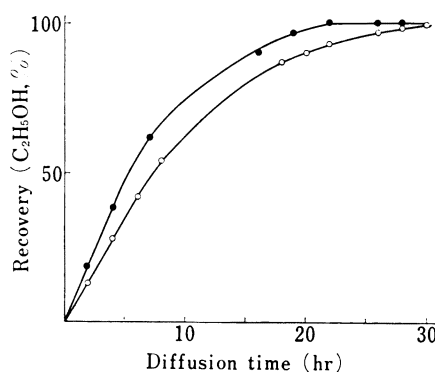


Fig. 4. Effect of Diffusion Time and Temperature on Recovery

A mixture of 0.5 ml of 0.4M KHCO₃, 0.5 ml of 0.4M C₂H₅OH and 2 ml of 0.1N H₂SO₄ were treated at 30° and 37° for the period of 2-30 hr.

---○---: diffused at 30° ●---: diffused at 37°

Therefore, the total incubation period was 36 hr. From the experimental data obtained, it was impossible to determine the order of production of carbon dioxide and ethanol.

7. Rate of Fermentation Under Addition of Dyes

Eight synthetic dyes were selected and investigated for their effect on fermentation. From the results shown in Table II, Phloxine and Rose Bengale remarkably inhibited the fermentation and the extents of the inhibition were 37% and 59%, respectively.

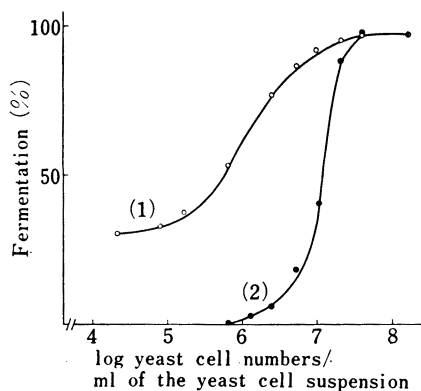


Fig. 5. Effect of Yeast Cell Numbers on the Fermentation

Into 0.5 ml of C.M.A. soln., 0.5 ml of the yeast cell suspension (ca. 10^4 – 10^8 cells per ml) was inoculated and incubated at 30° for 40 hr.

- (1) —○—: no addition
 (2) —●—: addition of Rose Bengale at the 0.1%

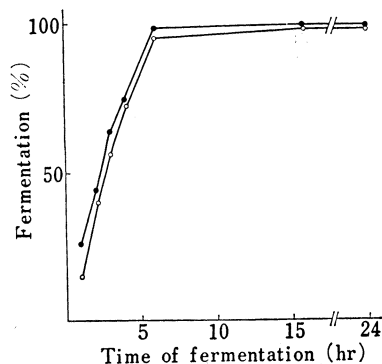


Fig. 6. Fermentation Rate as a Function of Time

At an arbitrary time of fermentation, 0.2 ml of 18% T.C.A. soln. was added into the small cup to stop the fermentation and the diffusion continued for further 30 hr.

- : C_2H_5OH —●—: CO_2

TABLE II. Influence of Dye on the Fermentation

Dye ^{a)}	Produced C_2H_5OH %	Dye ^{a)}	Produced C_2H_5OH %
Indigo carmine (90.7)	97.8	Phloxine (90.2)	63.0
Tartrazine (93.1)	95.6	Rose bengale (91.0)	41.3
Acid violet (86.6)	100.0	Acid red (94.4)	100.0
Erythrosine (91.3)	100.0	Control	100.0
Eosine (90.1)	103.2		

^{a)} Obtained from Sanei Kagaku Co., Tokyo.

(): purity %

Acknowledgement The authors are greatly indebted to Professor S. Tejima and Assistant Professor H. Ikezawa for their kind encouragement.