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Quantitative Gas Chromatography of Nonvolatile Organic Acids: Evaluation of the Internal Standard Method

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The six organic acids oxalic, fumaric, succinic, malic, *trans*-aconitic, and citric were separated as their methyl esters by means of linear temperature-programmed gas chromatography. The acids were determined quantitatively by means of the internal standard method using the methyl ester of myristic acid as internal standard. The equation of linear regression for each test compound was computed using W_a/W_{std} as the abscissa value and A_e/A_{std} as the ordinate value in which W_a and W_{std} are the weights of the free acid and standard and A_e and A_{std} are the areas of the peaks of the ester and standard, respectively. Furthermore, the standard deviation of the regression coefficient and the square of correlation coefficient were determined. The results well indicated a linear regression, except for *trans*-aconitic and oxalic acid. In addition, the limitations of the internal standard method for quantitative analysis by GLC are evaluated.

Gas liquid chromatography (GLC) has proved to be a very suitable method for identifying nonvolatile organic mono-, di-, and tricarboxylic acids, including the acids of the TCA cycle.[1–23] However, owing to the low vapor pressure of the acids, they cannot be chromatographed as free acids, but instead they must be altered to suitable derivatives. Derivatives which have been used include ethyl esters,[4,15] trimethylsilyl derivatives,[21] but most commonly methyl esters.[1–3, 6, 8, 12, 13, 15–18, 20, 23] Methyl esters have been prepared by different reagents: methanol-sulfuric acid,[16, 20] diazomethane,[1, 2, 6, 12, 15, 20, 23] methanol-boron trifluoride,[15, 18] methanol-hydrochloric acid,[15] and methanol-hydrochloric acid-thionyl chloride.[17] These methyl esters have generally been chromatographed either in polyester columns[3, 4, 12, 13, 16–18, 20, 23] or silicon columns,[3, 6, 12, 15] and the regulation of temperature has been either isothermal[1–3, 6, 12, 13, 15, 20, 23] or programmed.[12, 17, 18, 20]

To the quantitative determination of organic acids by GLC attention has been paid in few studies. Quin and Hobbs [2] determined the contents of certain acids in cigarette smoke. These determinations were based on standard curves showing the relation between the area of the peak and the amount of methyl

ester of the acid. They reported that the duplicate determinations generally agreed within 5—10 % and the relation between the area of the peak and the amount of ester varied from direct proportionality to slight nonlinearity. Mirocha and DeVay [4] observed in determining fumaric acid in biological material that the height of the peak, rather than the area, is better correlated with the amount of ester. The correlation between area of the peak and the quantity of ester has been studied in other investigations.[13, 23]

The present study deals with the quantitative gas chromatographic determination of six organic acids: oxalic, fumaric, succinic, malic, *trans*-aconitic, and citric. The internal standard method [24, 25] was used with the methyl ester of myristic acid as internal standard.

EXPERIMENTAL

Reagents. Oxalic acid (Merck *p.a.*), fumaric acid (Fluka, purum), succinic acid (Merck *p.a.*), malic acid (Fluka, purum), *trans*-aconitic acid (Fluka, puriss.), and citric acid (Merck *p.a.*). Methyl myristate (The Hormel Institute, purity > 99 %). Anhydrous methanol (Fluka, puriss. *p.a.*). Thionyl chloride (Merck, LAB). An anhydrous 8 % solution of hydrogen chloride in methanol was prepared by passing anhydrous hydrogen chloride gas into anhydrous methanol.

Apparatus. Chromatographic separations were carried out with a Wilkens Aerograph 200 using linear temperature programming and dual flame ionization detection.

The recorder was a Honeywell 1 mV potentiometer, to which was coupled a Disc integrator. The sample was injected by means of a Hamilton 10 μl syringe.

Esterification.[17] 10 ml of anhydrous hydrogen chloride-methanol solution was added to the acid mixture containing 20, 30, or 40 mg each one of the acids (oxalic, fumaric, succinic, malic, *trans*-aconitic, and citric). To this solution 0.1 ml thionyl chloride was carefully added along the walls of the flask. The flask was provided with a vertical condensor provided with a $CaSO_4$ tube and held on a boiling water bath for exactly 10 min. The mixture was allowed to cool to room temperature and the methanol and excess reagent were evaporated under vacuum at 20°C. 10 ml of methanol was added to the dry residue and evaporation was repeated until all the solvent had disappeared. Quantitative extraction of the esters from the dry residue was achieved with 5 × 0.7 ml methanol.

Internal standard. Methyl myristate was added to the ester extract as internal standard. One ml of the methanol solution of methyl myristate (15 mg/ml) was added to the extract and it was diluted with methanol to 5 ml. 1 μl of this solution was chromatographed. So the amount of standard was always 3 μg.

Chromatographic conditions. The stationary phase was 5 % ethylene glycol succinate (EGS) on Chromosorb W HMDS (60—80 mesh). The phase was packed into a copper tube with dimensions of 1.5 m × 1/8". The carrier gas was nitrogen and its flow rate was 40 ml/min in both columns. The flow rate of hydrogen was 30 ml/min and that of air about 300 ml/min in both detectors.

The temperature was in the injector 190°C and in the detector oven 200°C. The temperature of the column oven was linear-programmed (4°C/min) from 60 to 180°C. A period of 30 min was required for one run. The attenuation was 4/10 and the chart speed 0.5 cm/min.

Calculations. The areas of the peaks were determined from the chromatograms as Disc units. By means of computor, the equation of the linear regression of each acid was calculated from the experimental results and transferred to a coordinate system whose ordinate is the relative area A_e/A_{std} and abscissa the relative composition W_a/W_{std}, in which A_e and A_{std} are the areas of the peaks of the ester and standard, and W_a and W_{std} are the weights of the free acid and standard, respectively. For each regression analysis the ordinate values corresponding to the three different abscissa values were determined. At each abscissa value the chromatographic runs were repeated 6 times. Those 6 determinations comprised two sets of triplicate analyses made from two separate

esterifications. Thus the regression analysis of each acid is based on 18 determinations. In addition to the regression equation the standard deviation (s) of the regression coefficient and the square of correlation coefficient (R^2) were computed.

RESULTS AND DISCUSSION

Fig. 1 shows the chromatogram obtained. The esters in order of their elution are: dimethylester of oxalic acid, dimethylester of fumaric acid, dimethylester of succinic acid, methylester of myristic acid (internal standard), dimethylester of malic acid, trimethylester of *trans*-aconitic acid, and trimethylester of citric acid. Since the product of fumaric acid esterification,

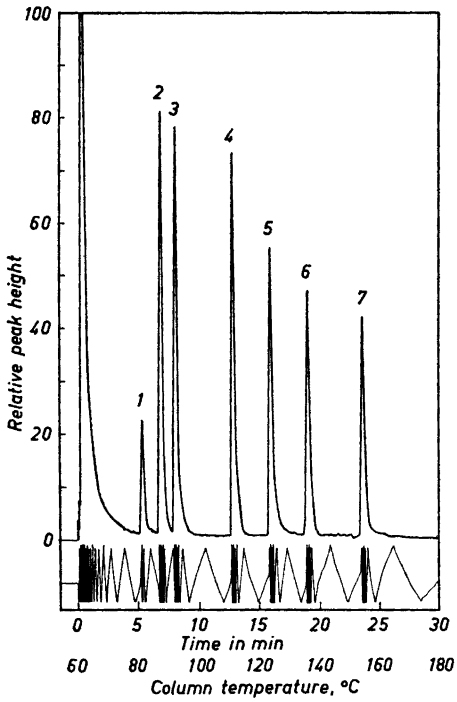

Fig. 1. Linear temperature-programmed gas chromatogram of methyl esters of the organic acids.
Column size, 1.5 m × 1/8 in. Column packing, 5 % EGS on Chromosorb W HMDS (60–80 mesh). Column temperature, 60°–180°C programmed 4°C/min. Nitrogen flow rate 40 ml/min. Esters: 1, oxalic acid dimethylester; 2, fumaric acid dimethylester; 3, succinic acid dimethylester; 4, myristic acid methylester; 5, malic acid dimethylester; 6, *trans*-aconitic acid trimethylester; 7, citric acid trimethylester.

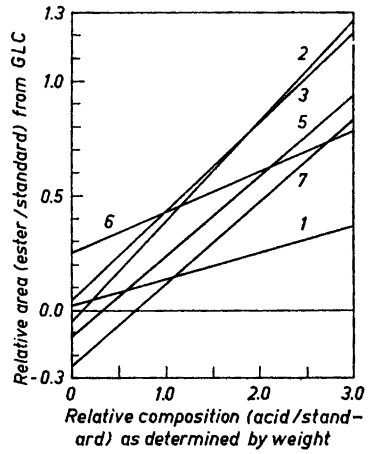

Fig. 2. Regression lines for oxalic (1), fumaric (2), succinic (3), malic (5), *trans*-aconitic (6), and citric (7) acid.

dimethyl fumarate, was eluted as a single peak at 83°C and the dimethylester of maleic acid was found to elute under the experimental conditions as a single peak at 98°C, the isomerization of the *trans* configuration to the *cis* configuration (or *vice versa*), observed by Luke *et al.*,[12] was not found to occur in this case (*cf.* Refs. 20, 23). On the other hand, the trimethyl ester of both *cis*- and *trans*-aconitic acid were found to elute at the same temperature, 136°C. This agrees with the studies of McKeown and Read, [20] who conclude that the trimethyl ester of *cis*-aconitic acid is unstable and isomerizes spontaneously to the *trans* form. Luke *et al.*[12] have reported, however, that *cis* $\rightleftharpoons$ *trans* isomerization occurred with the trimethyl esters of both *cis* and *trans* aconitic acid, and they separated the isomers by gas chromatography.

Fig. 2 shows the regression lines of six organic acids, determined as described above under Calculations. Table 1 gives the results of regression analyses. These results indicate that, except for *trans*-aconitic and oxalic acids, linear regression is well confirmed.

Table 1. Results of regression analysis.

Acid	Equation of linear regression $y = a + bx$	Standard deviation of regression coefficient (s)	Square of correlation coefficient (R^2)
oxalic	$y = 0.014 + 0.120x$	0.016	0.7896
fumaric	$y = -0.058 + 0.442x$	0.008	0.9953
succinic	$y = 0.030 + 0.395x$	0.014	0.9815
malic	$y = -0.112 + 0.351x$	0.009	0.9895
aconitic	$y = 0.257 + 0.177x$	0.028	0.7274
citric	$y = -0.252 + 0.362x$	0.028	0.9162

$y = A_e/A_{std}$ (relative area ester/standard)
$x = W_e/W_{std}$ (relative composition by weight ester/standard)

Use of internal standard. When the internal standard method is used for quantitative analysis by GLC it is possible in certain instances by means of known standards to determine the so called relative detector response K of the compound being studied:

$$K = \frac{A_x/A_{std}}{W_x/W_{std}} \tag{1}$$

In this equation W_x and W_{std} are the weights of the compound and the internal standard, respectively, and A_x and A_{std} are the areas of their peaks in the chromatogram. By means of the K value thus obtained, the amount W_x' of compound in an unknown chromatographed sample, to which has been added a quantity W_{std} of internal standard, can be calculated as follows:

$$W_x' = \frac{A_x' \times W_{std}}{K \times A_{std}} \tag{2}$$

In this equation A_x' and A_{std} are the areas of the peaks produced by the compound x and the internal standard in the chromatogram. This mathematical K value method, however, gives correct results only if the standard curve obtained from the measurements is linear throughout the required area in the coordinate system and it continues through the origin, *i.e.* if the equation of the standard curve has the form $y = bx$, in which $b = K$. If the standard curve is linear but its continuation does not pass through the origin, *i.e.* if its equation is $y = \pm a + bx$, the use of the coefficient b as being equal to K leads to systematic errors in calculating the value of W_x'. The extent of this error is

$$\pm \frac{a}{b} \times W_{std} \qquad (3)$$

According to the present studies (Fig. 2, Table 1), it appears from the above reasons that the use of the K value cannot be applied to the quantitative determination of all organic acids. For example, in the case of citric acid this method would cause an appreciable relative error particularly for small values of W_x'. In such cases, if W_x' is to be determined mathematically, the measurement results must be put into the equation of the linear regression and then solved. A second and perhaps easier means is a graphical solution by using the regression lines as standard curves. In this connection, reference can be made to the paper of Linning and Mandel,[26] in which a discussion is made on the evaluation of the precision of analytical methods involving linear calibration curves.

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REFERENCES

1. Nowakowska, J., Melvin, E. H. and Wiebe, R. *J. Am. Oil Chemists' Soc.* **34** (1957) 411.
2. Quin, L. D. and Hobbs, M. E. *Anal. Chem.* **30** (1958) 1400.
3. Ackman, R. G., Bannerman, M. A. and VandenHeuvel, F. A. *Anal. Chem.* **32** (1960) 1209.
4. Mirocha, C. J. and DeVay, J. E. *Phytopathology* **51** (1961) 274.
5. Agosta, W. C. *J. Org. Chem.* **26** (1961) 1724.
6. Lessard, J. R., Briggs, R. A. and Scalletti, J. V. *Can. J. Plant Sci.* **41** (1961) 507.
7. Spencer, S. F. *Facts & Methods* **3** (1962) 7.
8. Esposito, G. G. and Swann, M. H. *Anal. Chem.* **34** (1962) 1048.
9. Burchfield, H. P. and Storrs, E. E. *Biochemical Applications of Gas Chromatography*, Academic, New York 1962, p. 588.
10. Kowala, C., Kranz, Z. H. and Murray, K. E. *Australian J. Chem.* **54** (1962) 832; see Ref. 20.
11. Gehrke, C. W. and Goerlitz, D. F. *Anal. Chem.* **35** (1963) 76.
12. Luke, H. H., Freeman, T. E. and Kier, L. B. *Anal. Chem.* **35** (1963) 1916.
13. Sharpless, N. E. *J. Chromatog.* **12** (1963) 401.
14. Kuksis, A. and Vishwakarma, P. *Can. J. Biochem. Physiol.* **41** (1963) 2353; see Ref. 20.
15. Kellogg, H. M., Brochmann-Hanssen, E. and Svendsen, A. B. *J. Pharm. Sci.* **53** (1964) 420.

16. Rumsey, T. S., Noller, C. H., Burns, J. C., Kalb, D., Rhykerd, C. L. and Hill, D. L. *J. Dairy Sci.* **47** (1964) 1418.
17. Gee, M. *Anal. Chem.* **37** (1965) 926.
18. Alcock, N. W. *Anal. Biochem.* **11** (1965) 335.
19. Ferraz, F. G. P. and Relvas, M. E. *Clin. Chim. Acta* **11** (1965) 244; Ref. *Gas Chromatog. Abstr.* 1965, 717.
20. McKeown, G. G. and Read, S. J. *Anal. Chem.* **37** (1965) 1780.
21. Horii, Z., Makita, M. and Tamura, Y. *Chem. Ind. (London)* **34** (1965) 1494.
22. Hautala, E. *J. Assoc. Offic. Anal. Chemists* **49** (1966) 619.
23. Estes, F. L. and Bachmann, R. C. *Anal. Chem.* **38** (1966) 1178.
24. Ray, N. H. *J. Appl. Chem.* **4** (1954) 21.
25. Harvey, D. and Chalkey, D. E. *Fuel* **34** (1955) 191.
26. Linning, F. J. and Mandel, J. *Anal. Chem.* **36** (1964) 25 A.

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