Journal of Chromatography, 541 (1991) 265–272
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 989

Determination of glycerol in foods by high-performance liquid chromatography with fluorescence detection

TAKASHI HAMANO*, YUKIMASA MITSUHASHI, NOBUMI AOKI and SUSUMU YAMAMOTO
Public Health Research Institute of Kobe City, Minatozimanaka-machi, 4–6, Chuo-ku, Kobe 650 (Japan)
SUMIKO TSUJI and YOSHIO ITO
National Institute of Hygienic Science, Osaka Branch, Hoenzaka-cho 1–1, Chuo-ku 540 (Japan)
and
YOSHIKIYO OJI
Faculty of Agriculture, Kobe University, Rokkodai-cho 1–1, Nada-ku, Kobe 657 (Japan)
(First received August 23rd, 1990; revised manuscript received November 5th, 1990)

ABSTRACT

A high-performance liquid chromatographic method for the determination of glycerol in foods is described. The method involves the conversion of glycerol into formaldehyde by sequential enzymatic reactions (glycerokinase, glycerol-3-phosphate oxidase, catalase), followed by the derivatization of formaldehyde with 4-amino-3-penten-2-one. The calibration graph was linear in the range 0.1–4.0 μg/ml of glycerol. Many common ingredients of foods did not interfere. More than 90% of glycerol added at three levels was recovered from several foods. The method is simple and accurate. The detection limit was 1.0 μg/g when 5 g of sample were assayed.

INTRODUCTION

Glycerol (GL) is frequently added to various kinds of foods to increase the water-coating ability and it has also been used as a solvent for various food additives. Current regulations of the Ministry of Health and Welfare of Japan place no limit on the use of GL in foods. The use of the material and the absence of legal limits for its use initiated work on a quantitative method to meet demands, which are claimed by the consumer, to monitor excessive use.

Methods hitherto published for the determination of GL have involved gas chromatographic (GC) [1,2] high-performance liquid chromatographic (HPLC) [3–6] and enzymatic procedures [7]. The method of choice depends on the matrix in which GL is present, its relative concentration in the sample and the complexity of the latter.

Up to now, GL in foods has been determined chiefly by GC [2] or HPLC [5]. Both methods are very useful but require elaborate derivatization reactions and/or clean-up of the sample. In addition, the detection system in HPLC is not specific for GL, thus suffering interferences from other food ingredients when applied to some kinds of foods. Recently, an amperometric method has been applied successfully to the

0021-9673/91/$03.50

determination of GL in biological fluids [6], but the method has limitations and the instrument required is not commonly available in many laboratories.

GL can be converted by glycerokinase to glycerol-3-phosphate, which is then oxidized by second enzyme, glycerol-3-phosphate oxidase, to produce hydrogen peroxide. Preliminary experiments with use of existing techniques [8,9] resulted in the quantification of hydrogen peroxide with low presicion when working with certain kinds of foods, rich in reducing substances. Hydrogen peroxide can be determined as a suitable derivative by spectrophotometry and/or HPLC following the production of formaldehyde with a methanol–catalase system [10]. Formaldehyde is highly reactive toward reducing agents and/or proteins and cannot be detected directly when working with samples containing such substances. Therefore, the formation of suitable derivative is a prerequisite [11]. 4-Amino-3-penten-2-one (Fluoral-P) has been shown to be a specific derivatizing agent for formaldehyde in aqueous solution [12]. The purpose of this work was to develop a simple method for the determination of GL in foods with no special sample pretreatment or requirement for special instrumentation.

EXPERIMENTAL

Apparatus and conditions

The HPLC apparatus consisted of a Model 3A liquid chromatograph (Shimadzu, Kyoto, Japan) with a 20-μl loop injector (Rheodyne Model 7125), a fluorescence detector (Shimadzu Model RF-530) and a data processor (Shimadzu Model C-R3A). The chromatographic separation was carried out using a 25 cm × 4.6 mm I.D. reversed-phase column (Zorbax ODS, 5 μm) (Waters Assoc., Milford, MA, U.S.A.). The flow-rate of the mobile phase [acetonitrile–water (1:1)] was 1.0 ml/min. Separations were performed at ambient temperature (25°C).

Reagents

Fluoral-P was obtained from Wako (Osaka, Japan). Methanol and acetonitrile (both from Wako) were redistilled over dimedone prior to use. Adenosine 5′-triphosphate (ATP) was purchased from Wako and dissolved in cold distilled water to 1 mg/ml. A stock standard solution was prepared by diluting 1 g of guaranteed glycerol solution (Wako) with distilled water to 1 mg/ml. A working standard solution was prepared by diluting the stock solution with distilled water to 0.1–4.0 μg/ml.

Enzymes

Catalase (270 000 units/ml) was obtained from Boehringer (Mannheim, Germany). An enzyme solution was prepared by dilution with cold water to 1000 units/ml. This solution is stable for at least 3 weeks when kept refrigerated. Glycerokinase (500 units/ml) was also obtained from Boehringer and was diluted with cold distilled water to 10 units/ml. This solution can be used for more than 1 month without appreciable loss of activity. Glycerol-3-phosphate oxidase (from Boehringer) was dissolved in cold distilled water to give 100 units/ml. This enzyme solution should be prepared fresh daily.

Extraction

A 5-g sample of food was weighed accurately into a 50-ml test-tube and about 30

ml of distilled water were added, followed by 1 ml of 1% sodium hypochlorite solution. The sample was homogenized using a Polytron Model PT20 blender (Brinkmann, Westbury, NY, U.S.A.) for 1 min at maximum speed. After adjusting the pH to about 6 with 0.1 *M* HCl or 0.1 *M* NaOH, the contents were diluted to 50 ml with distilled water. Aliquots of 5 ml of the solution were filtered through a Millex-HA 0.45-μm filter (Millipore, Bedford, MA, U.S.A.). The filtrate is referred to as the sample solution.

Enzyme reaction

A 1-ml volume of sample solution was introduced into a 10-ml test-tube containing 3.35 ml of 0.4 *M* potassium phosphate buffer (pH 6.0; 0.1 m*M* $MgCl_2$), 0.1 ml of ATP solution and 0.25 ml of methanol. To this was added 0.1 ml of Fluoral-P (0.2 g/ml in acetonitrile), followed by 0.1 ml of catalase, 0.05 ml of glycerol-3-phosphate oxidase and 0.05 ml of glycerokinase. The reaction was carried out at ambient temperature for 20 min. Blank tests were performed by omitting glycerokinase. For clean-up of the reaction mixture, a Sep-Pak C_{18} cartridge column (Waters Assoc.) was used. The reaction mixture was charged on the cartridge column. After washing the column with a small volume of distilled water, the derivative of formaldehyde, formed by reaction with Fluoral-P, was eluted with 5 ml of acetonitrile–water (50:50). The eluate is referred to as the test solution.

HPLC analysis

A portion (20 μl) of the test solution was injected into the HPLC system. The eluent from the column was monitored with a fluorescence detector using emission and excitation wavelengths of 510 and 410 nm, respectively, because these conditions were optimum for detection of the formaldehyde derivative [12]. The peak height obtained was measured and the concentration of GL in the sample was calculated from a calibration graph prepared with working standards.

RESULTS AND DISCUSSION

Reaction conditions and clean-up

The reactions involved are illustrated in Fig. 1. In order to establish the optimum conditions for GL measurement, analyses were carried out at various pH values,

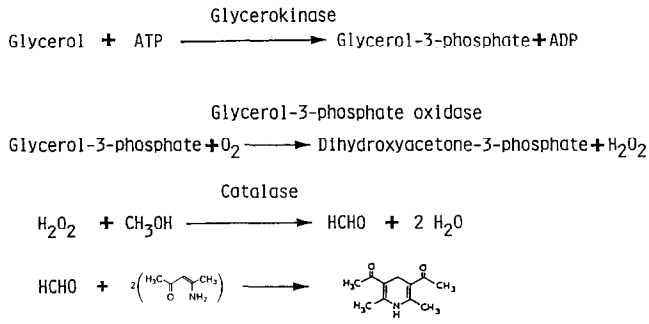


Fig. 1. Enzymatic and derivatization reactions by which glycerol was determined.

methanol concentrations, enzyme concentrations and reaction times. The effect of reaction pH was studied first. Given that the optimum pH of the three enzymes (glycerokinase, glycerol-3-phosphate oxidase and catalase) is around or above 7.0 [13–15] and that the maximum rate of reaction of formaldehyde with Fluoral-P is obtained around pH 2.4, a compromise pH range for the determination of GL would be expected to be 3.0–7.0. The peak height at various pH values was measured using 100 units of catalase, 5 units of glycerol-3-phosphate oxidase and 1 unit of glycerokinase. As shown in Fig. 2, the optimum pH for the determination of GL was 6.0. The reaction was also time dependent (Fig. 3). Considering the speed of analysis and optimum reaction rate, we selected 20 min for routine analysis.

The variables of glycerol-3-phosphate oxidase, glycerokinase and catalase were optimized individually. We first evaluated the amount of glycerol-3-phosphate oxidase, a crucial enzyme in this study, for optimization of the enzyme reaction. As shown in Fig. 4, the peak height increased significantly up to 3.0 units of glycerol-3-phosphate oxidase, after which it remained constant. Accordingly, 5 units of this enzyme were used routinely. The optimum amount of glycerokinase required was then determined and found to be >0.5 unit. Hence 1 unit of this enzyme was adopted in routine analysis. The optimum amount of catalase required was also determined and found to be >50 units. Because the enzyme is inexpensive, an excess of catalase (100 units) was used in routine analysis.

The fluoral-P concentration is a critical factor in the determination of GL by the proposed method. The optimum concentration was found to be $>0.3\%$, as is clear from Fig. 5. Accordingly, 0.1 ml of a 20% solution was used in routine analysis (final concentration, 0.4%).

An additional variable that required optimization was the concentration of methanol, and the optimum was found to be 3–10%. Methanol concentrations $>10\%$

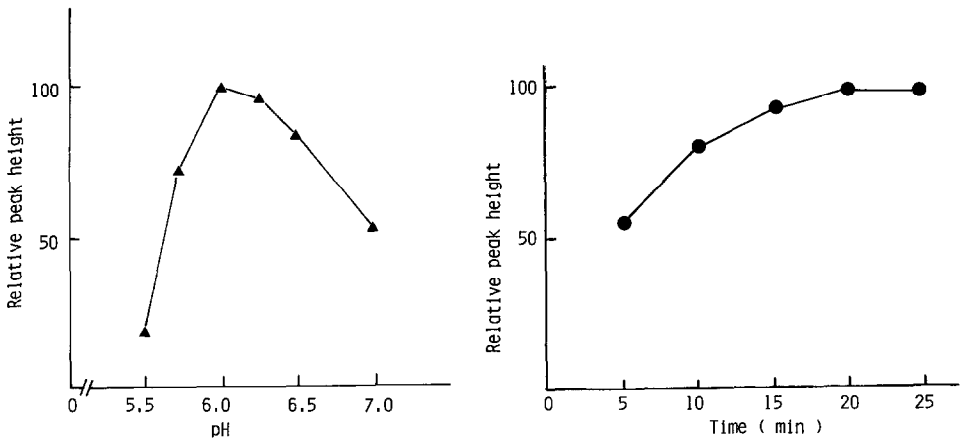
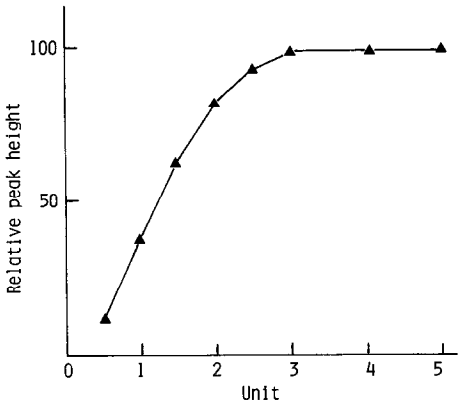


Fig. 2. Effect of pH on the determination of glycerol. A 2 μg/l standard glycerol solution was assayed at various pH values. Other conditions: glycerokinase, 5 units; glycerol-3-phosphate oxidase, 1 unit; catalase, 100 units; Fluoral-P, 0.4%; methanol, 5%; reaction time, 20 min; temperature, ambient.

Fig. 3. Effect of reaction time on the determination of glycerol. A 2 μg/ml standard glycerol solution was assayed with various reaction times. Other conditions as in Fig. 2.



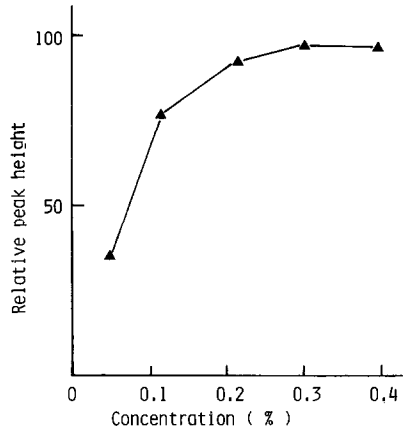


Fig. 4. Effect of amount of glycerol-3-phosphate oxidase on reaction rate. A 2 μg/ml standard glycerol solution was assayed with various amounts of glycerol-3-phosphate oxidase. Other conditions as in Fig. 2.

Fig. 5. Effect of Fluoral-P concentration on reaction rate. A 2 μg/ml standard glycerol solution was assayed with various concentrations of Fluoral-P. Other conditions as in Fig. 2.

decreased the peak height, probably owing to the retardation of the enzymes used and/or the derivatization reaction. Therefore, we used 0.25 ml of methanol, corresponding to 5% in the reaction mixture (5 ml).

Particles and/or proteins in the sample should be removed prior to HPLC, because they otherwise result in a deterioration of the column efficiency. We therefore used a Sep-Pak C_{18} cartridge column for clean-up of the reaction mixture, which effectively remove particles and proteins.

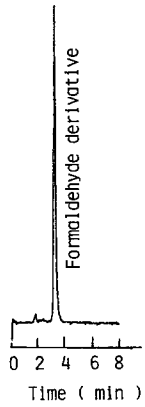


Fig. 6. Typical chromatograms of the formaldehyde derivative with Fluoral-P. Chromatographic conditions: column, Zorbax ODS C_{18} (250 mm × 4.6 mm I.D.); mobile phase, acetonitrile–water (1:1); flow-rate, 1 ml/min. A 2 μg/ml standard solution of glycerol was assayed according to the procedure described under Experimental.

Conditions for HPLC

Fig. 6 shows a typical HPLC trace obtained with the derivative of formaldehyde. The concentration of acetonitrile in the mobile phase was very important for a sharp resolution of the formaldehyde derivative on a reversed-phase column. At acetonitrile concentrations $<30\%$, the derivative was retained on the column, whereas it was eluted from the column without retention using $>70\%$ acetonitrile. The presence of an ionic strength modifier such as phosphate buffer had no effect on the retention time and peak shape of the derivative. Accordingly, acetonitrile–water (1:1) without any buffer was adopted in this study.

The relationship between the peak height and the amount of GL was evaluated over the range of 0.1–4 μg/ml in water. The relationship between peak height (y; μV s) and the amount of GL injected (x; ng) was obtained by the least-squares method: $y = 0.004x + 50$ ($n = 3$, correlation coefficient $r = 0.994$). The limit of detection for GL was 0.4 ng per 20-μl injection under the conditions adopted (signal-to-noise ratio = 3). This corresponds to a value of 1.0 μg/g in the sample when 5 g of sample were assayed. The reliability of the proposed method was evaluated by repeated analyses of standard GL solution (2 μg/ml in water). For five assays, the peak heights were found to be within 98% agreement.

Interference studies

The effect of possible interfering substances, particularly analogues of GL, was examined. The results are given in Table I. The relatively high specificity of two of the enzymes, glycerokinase and glycerol-3-phosphate oxidase, and the derivatization reaction make it unlikely that interferences will be encountered in most types of foods. Any interference resulting from reducing substances could be removed by using hypochlorite solution in the extraction step. Hypochlorite itself did not affect the proposed method. Fig. 7a shows the HPLC profile of fish sausage spiked with 20 μg/g of Gl. The peak detected was considered not to be derived from an interfering substance, as judged from Fig. 7b.

TABLE I

EFFECT OF POSSIBLE INTERFERING SUBSTANCES

A 50-μg amount of glycerol was assayed by the proposed method in the presence or absence of the substances indicated.

Substance	Amount (μg)	Relative peak height (%)
None		100
Propylene glycol	500	99.5
Ethylene glycol	500	101
Trimethylene glycol	500	100
Glucose	1000	100
Ethanol	100	99.7
Ascorbic acid	200	85.0 (99.8)[a]
Sulphur dioxide	50	43.6 (100)[a]

[a] The values in parentheses are results obtained by treatment with sodium hypochlorite prior to assay.

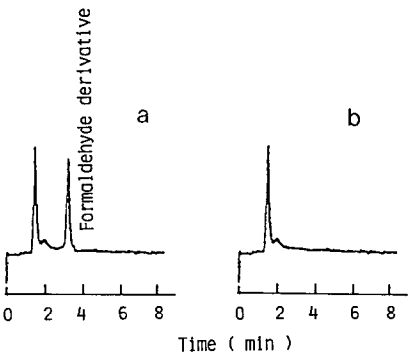


Fig. 7. HPLC elution profiles of fish sausage using the present method. Conditions as in Fig. 5. (a) Extract from fish sausage spiked with 20 μg/g of glycerol; (b) extract from fish sausage.

TABLE II

AMOUNTS OF GLYCEROL PRESENT IN SEVERAL FOODS AND RECOVERIES

Recoveries were determined in triplicate.

Sample	Amount present (μg/g)	Amount added (μg/g)	Amount recovered (μg/g)	Recovery (%)
White wine	1460	1000	2430	97.0
Red wine	1750	1000	2735	98.5
Fish sausage	N.D.[a]	20	19.4	97.0
Ice cream	140	100	95.7	95.7
Mayonnaise	N.D.	20	19.8	99.0
Salad dressing	N.D.	100	96.0	96.0

[a] N.D. = Not detected.

Recovery studies

Table II shows the recoveries obtained by spiking samples with GL at three levels prior to analysis. GL in water was added to the weighed samples in a test-tube and subjected to analysis as described under Experimental. Good recoveries were obtained in all instances, which demonstrated the accuracy (>95%) of the proposed method.

REFERENCES

1 G. E. Martin, R. H. Dyer and D. M. Figert, *J. Assoc. Off. Anal. Chem.*, 58 (1975) 1147.
2 M. Fujii (Editor), *Analytical Methods for Food Additives*, Kodansha, Tokyo, 1982, p. 584.
3 V. Kahle and K. Tesarik, *J. Chromatogr.*, 191 (1980) 121.
4 R. Pecina, G. Bonn, E. Burtscher and O. Bobleter, *J. Chromatogr.*, 287 (1984) 245.
5 G. Bonn, *J. Chromatogr.*, 350 (1985) 381.
6 S. V. Prabhu and R. P. Baldwin, *J. Chromatogr.*, 503 (1990) 227.
7 T. A. Kelly and G. D. Christian, *Analyst (London)*, 109 (1984) 453.
8 K. Kobayashi and S. Kawai, *J. Chromatogr.*, 245 (1982) 339.
9 M. Toyoda, Y. Ito, M. Iwaida and M. Fujii, *J. Agric. Food Chem.*, 30 (1982) 346.

10 H. U. Bergmeyer (Editor), *Methods of Enzymatic Analysis*, Vol. 2, Academic Press, New York, 1974, p 438.
11 K. Kobayashi, M. Tanaka and S. Kawai, *J. Chromatogr.*, 187 (1980) 413.
12 J. C. Compton and W. C. Purdy, *Anal. Chim. Acta*, 119 (1980) 349.
13 K. Kobayashi and S. Kawai, *J. Chromatogr.*, 245 (1982) 339.
14 S. Hayashi and E. C. C. Lin, *J. Biol. Chem.*, 242 (1967) 1030.
15 T. W. Esders and C. A. Michrina, *J. Biol. Chem.*, 254 (1979) 2710.