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SIMPLE AND SENSITIVE DETERMINATION OF METHYLGLYOXAL IN BIOLOGICAL SAMPLES BY GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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SUMMARY

Methylglyoxal was allowed to react with 4,5-dichloro-1,2-phenylenediamine, and the 6,7-dichloro-2-methylquinoxaline formed was determined by gas chromatography with electron-capture detection. The standard curve of the quinoxaline was linear up to 160 pmol/ml. The recoveries of methylglyoxal from coffee and rat liver homogenate were 84.1 and 77.6%, respectively. This procedure was very selective and so sensitive that as little as 9 fmol of the quinoxaline could be measured in biological and food samples.

INTRODUCTION

Many methods for the determination of methylglyoxal (MG) have been published, using high-performance liquid chromatography (HPLC) [1,2], gas chromatography (GC) [1], spectrophotometry [3,4] and enzymic analysis [5]. When we began our studies of the metabolism and biosynthesis of MG, we applied the HPLC method of Moree-Testa and Saint-Jalm [1]. These authors determined MG in cigarette smoke using GC and HPLC. On applying this method to the determination of MG in biological samples, we encountered difficulties with overlapping peaks, but after exhaustive improvement, it was applicable to homogenates of several rat tissues as described in a previous report [6]. However, this improved method was still inadequate for determinations of MG in some foods and beverages, such as coffee, because of poor chromatographic resolution and low sensitivity. The method described here is a GC method with electron-capture detection (ECD), which is more simple, sensitive and specific than that given in the previous report (Fig. 1).

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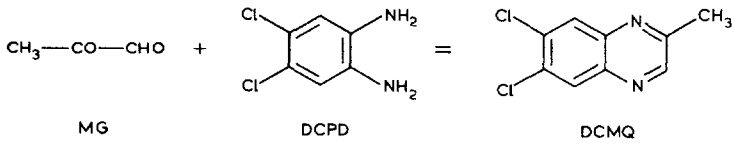

Fig. 1. Reaction of methylglyoxal (MG) with 4,5-dichloro-1,2-phenylenediamine (DCPD) forming 6,7-dichloro-2-methylquinoxaline (DCMQ).

EXPERIMENTAL

Chemicals

4,5-Dichloro-1,2-phenylenediamine (DCPD) and methylglyoxal dimethyl acetal were purchased from Aldrich (Milwaukee, WS, U.S.A.). 4-Chloro-1,2-phenylenediamine (CPD) was obtained from Tokyo Kasei Kogyo (Tokyo, Japan) and aldrin (standard-material grade) from Wako Pure Chemicals (Osaka, Japan). Benzene was of especially fine grade from Katayama (Osaka, Japan). 2-Nitro-4-trifluoromethylbenzenamine was kindly supplied from Daikin (Osaka, Japan). MG was prepared just before use by hydrolysis of the dimethyl acetal [7].

4-Trifluoromethyl-1,2-phenylenediamine (TFPD). 2-Nitro-4-trifluoromethylbenzenamine (5.4 g, 272 mmol) was reduced by 6.8 g of zinc dust in a mixture of ethanol (22 ml) and 20% sodium hydroxide (3 ml). The reaction mixture was stirred for 20 min under reflux, then filtered, and the filtrate was concentrated under reduced pressure. The residue was dissolved in 20 ml of water and extracted with three lots of 20 ml of diethyl ether. The ethereal layer was reextracted with three 20-ml portions of 2 *M* hydrochloric acid, and the combined aqueous extracts were evaporated under reduced pressure. The crystals were recrystallized from methanol containing a small amount of hydrochloric acid in a yield of 6.2 g (95.4%). Sublimation occurred from 150 to 177°C and carbonization from 250 to 300°C (in a sealed capillary). Calculated for $C_7H_9N_2F_3Cl_2$: C = 33.76; H = 3.64; N = 11.25. Found: C = 34.08; H = 3.63; N = 11.32. R_F values on silica gel, 0.15 (chloroform) and 0.77 (ethyl acetate).

2-Methyl-6(7)-trifluoromethylquinoxaline (MTFQ). MG was prepared from the dimethyl acetal (0.28 g) in 5% (v/v) sulphuric acid at 100°C for 25 min [7] and allowed to react with TFPD (0.5 g in 18 ml of methanol) at room temperature for 22 h. After adjustment to pH 2 with 0.2 *M* sodium hydroxide, the reaction mixture was extracted with three 30-ml portions of diethyl ether. The extract was dried over sodium sulphate, and the diethyl ether was evaporated. The residue was sublimed twice and then recrystallized from methanol and water as white flakes (m.p. 74–76°C, in a sealed capillary). ^{1}H NMR (C^2HCl_3, δ): 2.83 (3H, s, CH_3), 7.88 (1H, dd, J = 9 Hz and 2.5 Hz, H-7), 8.15 (1H, d, J = 9 Hz, H-8), 8.37 (1H, d, J = 2.5 Hz, H-5), 8.83 (1H, s, H-3); *m/z* 212 (M^+). R_F value was 0.35 on silica gel (chloroform). Because the compound was volatile and incombustible, the results of several elemental analyses were inconsistent.

6-Chloro-2-methylquinoxaline (6-CMQ) and 7-chloro-2-methylquinoxaline (7-

CMQ). The dimethyl acetal (2.4 g) was hydrolysed as described above. The hydrolysate was cooled and CPD (1.4 g) in 30 ml of ethanol was added. The mixture was allowed to stand at room temperature for 12 h. The reaction mixture was neutralized with sodium hydrogen carbonate, and after addition of ammonium sulphate, it was extracted with two 3-ml portions of benzene. The benzene layer was dried over sodium sulphate and evaporated under reduced pressure. The residue was recrystallized from a mixture of benzene and *n*-hexane and then a mixture of methanol and water. Two types of crystal were obtained: pale pink flakes, m.p. 125–128°C, reported to be 6-CMQ (m.p. 131°C) [8], (m.p. 129°C) [9]; pale pink flakes, m.p. 81–86°C, reported to be 7-CMQ (m.p. 91°C) [8]. For the former, ^{1}H NMR (C^2HCl_3, δ): 2.79 (3H, s, CH_3), 7.67 (1H, dd, J=9 Hz and 2.5 Hz, H-7), 7.97 (1H, d, J=9 Hz, H-8), 8.08 (1H, d, J=2.5 Hz, H-5), 8.75 (1H, s, H-3); *m/z* 178 (M^+); ratio of $M^+/M^+ + 2 = 3:1$. Calculated for $C_9H_7N_2Cl$: C=60.50; H=3.92; N=15.69. Found: C=60.27; H=3.88; N=15.63. For the latter, ^{1}H NMR (C^2HCl_3, δ): 2.78 (3H, s, CH_3), 7.64 (1H, dd, J=9 Hz and 2.5 Hz, H-6), 8.01 (1H, d, J=9 Hz, H-5), 8.02 (1H, d, J=2.5 Hz, H-8), 8.72 (1H, s, H-3); *m/z* 178 (M^+); ratio of $M^+/M^+ + 2 = 3:1$. Found: C=60.34; H=3.88; N=15.66. Total yield 1.5 g (84%); R_F value of both isomers was 0.40 on silica gel (chloroform).

6,7-Dichloro-2-methylquinoxaline (DCMQ). DCPD (1.77 g) in 80 ml of ethanol was added to MG, which had been prepared from the acetal as described above. After standing at room temperature for 18 h, sodium hydrogen carbonate (2.5 g) was added and the reaction mixture was extracted with 30 ml of *n*-hexane followed by two 30-ml portions of benzene. The organic layers were combined and dried over sodium sulphate. After evaporation, the residue was recrystallized from benzene and then methanol as red plates (m.p. 147–147.5°C) in a yield of 1.6 g (75%). ^{1}H NMR (C^2HCl_3, δ): 2.79 (3H, s, CH_3), 8.15 and 8.20 (each 1H, each s, H-5 and H-8), 8.74 (1H, s, H-3); *m/z* 212 (M^+); ratio of $M^+/M^+ + 2/M^+ + 4 = 6.9:4.7:1$. Calculated for $C_9H_6N_2Cl_2$: C=50.70; H=2.82; N=13.15. Found: C=50.65; H=2.78; N=12.97. R_F value was 0.55 on silica gel (chloroform).

Gas chromatography

A Shimadzu gas chromatograph Model GC-4CMPFE (Shimadzu, Kyoto, Japan), equipped with a ^{63}Ni electron-capture detector, was used for the determination of DCMQ. The glass column (2 m×3 mm I.D.) was packed with 1.5% Silicone OV-17 on Shimalite W, 80–100 mesh (Shimadzu). The temperature of the detector and injector block was 280°C, and the column was at 180°C. The nitrogen gas flow-rate was 50 ml/min. The electron-capture detector was operated at a pulse frequency of 10 kHz, and the electrometer was set at an attenuation of 10^2.

Extraction of samples

Determination of MG in water. An aliquot (10–100 μl) of the aqueous solution of MG (100 μg/ml) was made up to 1 ml with water or 0.5 *M* perchloric acid. After addition of 0.2 ml of 1% DCPD ethanol solution, the mixture was allowed

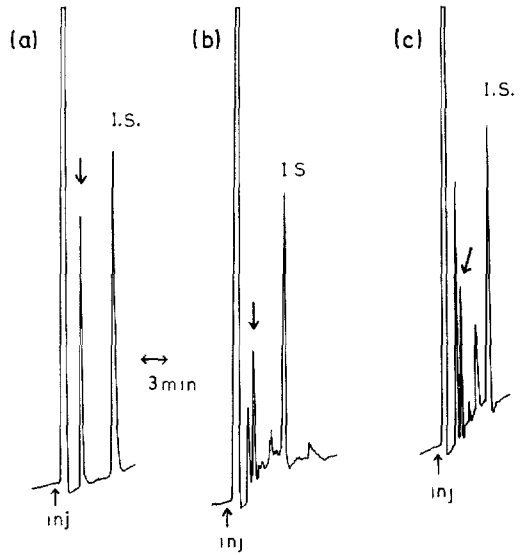

Fig. 2. Gas chromatograms of authentic DCMQ (a), and that obtained from instant coffee (b) and rat liver homogenate (c). (a) DCMQ (140 pmol) was dissolved in 1.0 ml of benzene containing aldrin (0.025 μg/ml), and 2 μl of that were subjected to GC-ECD. (b) Instant coffee (1 g of Maxwell Blendy in 100 ml of water) was treated with DCPD, and the DCMQ formed was extracted into 4 ml of benzene containing aldrin (0.025 μg/ml). After diluting the extract with benzene 50 times, 2 μl were subjected to GC-ECD. (c) Rat liver was homogenized with two volumes of 0.5 *M* perchloric acid. The homogenate was centrifuged, and 1 ml of the supernatant was treated with DCPD and treated as described above.

to stand at 25°C for 1 h. The reaction mixture was made alkaline with 0.5 *M* sodium hydroxide, and extracted with 4 ml of benzene containing 50 pg/ml aldrin as internal standard. The benzene layer was further diluted with benzene to appropriate concentration for the GC analysis.

Determination of MG in coffee. A 1-g sample of instant coffee was dissolved in 100 ml of cold water. Samples of ordinary coffee were prepared from beans as follows: 8 g of coffee beans were well or moderately roasted, finely ground in a manually operated home coffee mill, extracted with 100 ml of boiling water and filtered through coffee filter paper. Raw coffee beans (8 g) were also ground and treated in a similar manner as above. Aliquots of the extracts from the well and moderately roasted beans were evaporated under reduced pressure and dried over phosphorous pentoxide. The dried residues weighed 0.9 and 1.1 g from each 100-ml extract of the well and moderately roasted beans, respectively. The coffee extracts were allowed to react with DCPD and were extracted in the same manner as described under *Determination of MG in water.* After appropriate dilution of the benzene layer with benzene, an aliquot (2 μl) was subjected to GC. Fig. 2b shows a chromatogram of DCMQ from coffee.

Determination of MG in rat organs. Male Wistar albino rats were decapitated, and the organs were removed immediately, washed in physiological saline and homogenized in two volumes of 0.5 *M* perchloric acid using a Potter-Elvehjiem homogenizer with a PTFE pestle for spleen, brain and kidney, and a Waring

blender (at 10 000 rpm for 5 min) for liver, muscle and heart. After centrifugation at 6000 *g* for 30 min, a 1-ml aliquot of the supernatant was treated with DCPD, extracted and assayed as described above.

Fasting rats. Male Wistar albino rats aged five weeks were fasted for three days but allowed to drink water freely. The control group received normal solid food. The body weight after three days was 215 ± 21.8 g ($n=5$) for the control group and 158 ± 12.9 g ($n=6$) for the fasting group.

Alloxan diabetic rats. Male Wistar albino rats were injected with alloxan in physiological saline (200 mg/kg, intraperitoneally) and controls were given the same volume of physiological saline. Each group was maintained on normal solid food and water ad libitum for three days. On the last day the weights were 222 ± 11.5 g ($n=5$) for the control groups and 173 ± 24.4 g ($n=6$) for the diabetic group, and blood glucose concentrations were 187 ± 44.1 and 416 ± 177 mg/100 ml, respectively.

RESULTS

Reaction conditions

The optimum reaction conditions for the preparation of methylquinoxaline from *o*-phenylenediamine and MG have been reported previously [6]. This provided a guide for the present study using DCPD, and to find the most suitable reaction conditions, MG (1 μg) in 1 ml of 0.5 *M* perchloric acid was allowed to react with 0.1 ml of 1% ethanol solution of DCPD at 0, 30 and 50 °C for 15 to 120 min. The reaction yield was between 82.5 and 92.2%, except at 0 °C for 15 min (75%). Thus, the reaction at 25 °C for 60 min was adopted in the analysis.

Standard curve

The standard curve of DCMQ was linear up to at least 160 pmol/ml. For an injection of 2 μl of DCMQ in benzene, the determination limit was 9 fmol.

Recovery test of MG

From aqueous solution, various amounts of MG (1, 3, 5, 7 and 10 μg/ml in water), allowed to react with DCPD, gave recoveries of 85.7, 84.7, 95.4, 93.5 and 89.9%, respectively.

Various amounts of MG were added to 1 ml of instant coffee and the total amount of MG was measured. The results are shown in Table I, indicating good recovery and the presence of considerable amounts of MG in instant coffee.

Various amounts of MG were added to 1 ml of the supernatants of the rat liver homogenate and determined as described above. The recovery was $77.6 \pm 7.42\%$ ($n=4$).

MG in coffee

As shown in Table II, MG contents in four kinds of instant coffee and six kinds of coffee prepared from two kinds of coffee beans were analysed using the present method. The table shows that instant coffee contains less MG than ordinary

TABLE I

RECOVERY OF MG ADDED TO COFFEE

MG added		MG found		Recovery (%)
μg/ml	nmol/ml	μg/ml	nmol/ml	
0	0	0.66	9.2	
1	13.9	1.46	20.3	80.0
3	41.7	3.38	46.9	90.6
5	69.4	4.78	66.4	82.5
10	138.9	8.97	124.6	83.1
Mean ± S.D.				84.1 ± 4.6%

coffee prepared from roasted ground beans, and ground raw beans also contain appreciable amounts of MG.

MG in fasting and diabetic rats

MG in several organs of fasting and alloxan diabetic rats was determined. Table III shows that MG levels are elevated in hearts of both fasting and diabetic rats, and the level in plasma of diabetic rats is also raised. However, MG concentrations in liver and skeletal muscle of diabetic rats were decreased by the two procedures. The table also indicates that MG levels are relatively low in kidney and plasma.

Identification of peak of DCMQ

In order to identify the peak of DCMQ on the gas chromatogram, the following samples were treated as described under *Extraction of samples*: liver, heart, muscle and blood cells of normal rat; hearts of alloxan diabetic and fasted rat; ordi-

TABLE II

MG CONTENT IN COFFEE

	MG content (μg per 100 ml)	
Instant coffee		
Maxwell Blendy (Ajinomoto General Foods, Tokyo, Japan)	66	
Nescafe Coffee Excella (Nestle, Kobe, Japan)	125	
Nescafe Gold Blend, freeze-dried (Nestle)	144	
Nescafe Gold Blend, decaffeinated (Nestle)	134	
Coffee beans	*Colombia*	*Blue Mountain*
Raw	37	N.D.*
Moderately roasted	190	502
Well roasted	336	436
Stored for a week after roasting beans well	271	581

*N.D. = non-detectable.

TABLE III

MG CONTENTS IN VARIOUS RAT TISSUES IN UNUSUAL STATES

Tissue	Control (μg/g wet weight)	Fasted (μg/g wet weight)	Alloxan diabetic (μg/g wet weight)	$P<0.05$
Heart	0.44±0.06	0.83±0.20	—	↑
Liver	0.77±0.16	0.52±0.23	—	—
Skeletal muscle	0.90±0.13	0.86±0.20	—	—
Heart	0.46±0.29	—	0.85±0.47	↑
Liver	0.83±0.27	—	0.24±0.13	↓
Skeletal muscle	1.14±0.28	—	0.78±0.13	↓
Kidney	0.19±0.05	—	0.31±0.14	—
Blood cell	1.05±0.45	—	1.26±0.87	—
Plasma	0.14±0.05	—	0.22±0.19	↑

nary coffee. The benzene layer obtained was analysed by gas chromatography–mass spectrometry. Fig. 3 gives the mass spectra for the GC peaks of the reaction product from coffee and of authentic DCMQ. Mass fragments of authentic DCMQ are in good agreement with those of the corresponding peaks from coffee, and the parent peak appears at *m/z* 212 in both mass spectra. The peaks on the chromatograms of other samples were also identified as DCMQ.

Sensitivity of several quinoxalines on GC–ECD

Several halogen-containing quinoxalines were analysed by GC–ECD under the conditions described, except for the column temperature. The retention time was 1.2 min at 148°C for MTFQ, 4 min at 148°C for 6-CMQ and 7-CMQ, 1.8 min at

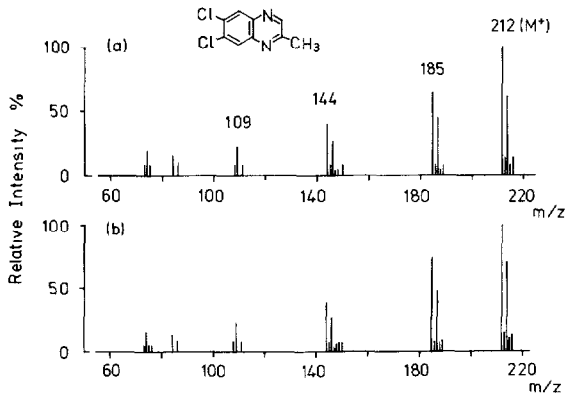

Fig. 3. GC–mass spectra of authentic DCMQ and the corresponding peak on the gas chromatogram formed from DCPD and MG in coffee. (a) Authentic DCMQ (0.47 μmol) in 1.0 ml of methylene chloride. (b) Coffee (1 ml) was treated with DCPD and extracted with 4 ml of methylene chloride. Column, 2% OV-17 (1 m×3 mm I.D.); oven temperature, 115°C; injection temperature, 240°C; separator temperature, 240°C; ion-source temperature, 250°C; carrier gas, helium at 38 ml/min; electron energy, 70 eV; mass spectrometer, Shimadzu LKB-9000.

190°C for DCMQ. The relative sensitivity was 1:10:10 for 6(7)-CMQ:MTFQ:DCMQ. Because of the volatility of MTFQ, DCMQ was adopted for the determination of MQ.

DISCUSSION

MG has been reported to be synthesized from L-threonine via aminoacetone [10–13], acetoacetate [14], dihydroxyacetone phosphate [15,16] and acetone [17]. On the other hand, MG is metabolized to pyruvate by glyoxalase [18], formaldehyde dehydrogenase [19], aldehyde dehydrogenase [20], α-ketoaldehyde dehydrogenase [21], L-glycol dehydrogenase [22], aldehyde reductase [23], methylglyoxal reductase [24] and carbonyl reductase [23]. However, it is still uncertain which is the main route, and what is the physiological significance of these routes. One possible cause of confusion could be the lack of specificity of methods for the determination of MG, which has in most cases been determined as 2,4-dinitrophenylhydrazone. We have found that triose and its phosphate derivatives, which have been thought to be substrates of MG, showed the same colour development as MG when they are treated with 2,4-dinitrophenylhydrazine at higher temperature or for many hours. We shall report this elsewhere. In our present procedure, the quinoxaline ring is formed specifically from MG and not from triose and its derivatives. The present method is superior to previous methods with respect to specificity, simplicity and sensitivity. In 1979, Nagao et al. [25] reported that coffee and instant coffee contain mutagens [25]. Three years later Kasai et al. [26] identified a mutagen in an extract of ground roasted coffee beans as MG: more than 50% of the total mutagenic activity of coffee was accounted for by MG. They reported that one cup of instant coffee contains 100–150 μg of MG and one cup of coffee prepared from ground coffee beans contains 470–730 μg. The data presented here indicate a somewhat lower MG content than reported by Kasai et al. It is generally thought that MG is produced during heating of biological samples [27–29]. However, it occurs fairly widely in biological substances, and it is not surprising that raw coffee beans contain MG.

In this paper, MG levels in several organs of fasted and alloxan diabetic rats were measured, following a suggestion that the rise of acetoacetate level leads to MG formation [14]. A detailed interpretation of the data will be given elsewhere. Studies of the biosynthesis and metabolism of MG are now under way, using the present determination method.

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