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DETERMINATION OF NITRITE IN HUMAN, COW AND MARKET MILKS BY GAS–LIQUID CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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SUMMARY

A simple and practical method for the determination of very small amounts of nitrite in human, cow and market milks is described. It is based on the reaction of nitrite with 1-hydrazinophthalazine in acidic solution to form tetrazolophthalazine, a stable compound which can be extracted with an organic solvent and then determined by gas–liquid chromatography with electron-capture detection using a column of 3% OV-225 on Chromosorb W HP; the detection limit for nitrite was 2 ng/ml. The procedure for determining nitrite in milks involves extraction with solvent, followed by further clean-up by alumina column chromatography; the detection limit is about 4 ppb* and recovery in human, cow and market milks was satisfactory. The method makes possible a micro-assay for nitrite. The tetrazolophthalazine was identified by its elemental composition, melting point and by combined gas chromatography–mass spectrometry.

INTRODUCTION

The determination of nitrite in environmental samples at the trace level is of interest, in particular, because of the potential for *in vitro* production of nitrosoamines from the reaction of nitrite ion with secondary amines in the human stomach[1]. Nitrite is usually determined by coupling with the Griess reagent[2] and N-(1-naphthyl)-ethylenediamine and spectrophotometric measurement of the reaction products. This colorimetric method is limited by the fact that occasionally turbid and slightly coloured sample extracts can affect the colour of the azo dye and, consequently, the accuracy of the determination.

Recently, gas–liquid chromatography (GLC) has been employed for this purpose[3–5]. The method described in our previous paper[5] was suitable for routine use, but not for trace nitrite analysis. Other workers have made brief studies of nitration with nitrite and benzene[6] or 2,4-xylenol[7] in the presence of 80% sulphuric

* Throughout this article, the American billion (10^9) is meant.

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acid using an electron-capture detector (ECD); the procedure is complex, vigorous reaction conditions are required and these assays are non-specific for nitrite.

We have found that tetrazolophthalazine[8] can quantitatively be prepared by a reaction with nitrite and 1-hydrazinophthalazine[9] in acidic medium and can be extracted with several organic solvents over a wide range of pH (2–14). This specific reaction for nitrite and the favourable GLC properties permit quantification by an ECD, and is suitable for biological samples such as human milk and blood. On the other hand, the determination of nitrite in milks was briefly reported by Sukegawa and co-workers[10,11] who found it difficult to obtain good accuracy and sensitivity; they recommended use of another method. In the present study, deproteinized milks were analysed by GLC after reaction and clean-up by alumina column chromatography; recovery of nitrite added to several milks was satisfactory.

MATERIALS AND METHODS

Reagents and apparatus

Sodium nitrite was dried at 100°C under vacuum immediately before use. The stock nitrite solution was prepared by dissolving 0.493 g of sodium nitrite in 1000 ml of distilled water to give a concentration of 1 μg/ml of nitrite-nitrogen (NO_2-N). 1-Hydrazinophthalazine (Tokyo Kasei Kogyo, Tokyo, Japan) was of a special high grade and was used without further purification. A solution (0.1%, w/v) of it was prepared by dissolving 0.1 g in 100 ml of distilled water. The internal standard solution for GLC was prepared by dissolving 0.05 μg of ethyl *p*-nitrophenylthionobenzenephosphonate (EPN) in 1 ml of toluene. Deproteinizing solution A was prepared by dissolving 90 g of ammonium thiocyanate and 80 g of mercuric chloride in 1000 ml of distilled water; deproteinizing solution B was prepared by dissolving 125 g of zinc acetate in 500 ml of distilled water. All water used was triply distilled and deionized. The column-packing materials for GLC, *viz.*, Chromosorb W HP, DC-200, SE-30, QF-1, OV-1, OV-17, OV-225, OV-101 and XE-60, were of high purity and were obtained from Nishio (Tokyo, Japan). All other reagents and solvents were of high purity and were obtained from Wako (Osaka, Japan).

For identification of tetrazolophthalazine, a Shimadzu LKB-9000 combined gas chromatograph–mass spectrometer was used. Conditions for GLC; a glass tube (1.0 m × 2 mm I.D.) packed with 3% OV-225 on Chromosorb W HP (80–100 mesh); helium flow-rate 30 ml/min; column temperature 230°C. Conditions for mass spectrometry (MS): separator temperature 260°C; ion source temperature 290°C; trap current 60 μA; electron energy 70 eV; accelerating potential 3.5 keV.

Preparation of tetrazolophthalazine

A suitably diluted solution of nitrite was placed in a 50-ml centrifuge tube, 4 ml of 1 *M* hydrochloric acid solution and 1 ml of 0.1% 1-hydrazinophthalazine solution were added and the mixture was heated at 70°C in a water-bath with occasional shaking for 20 min. After cooling to room temperature, the reaction mixture was transferred to a 25-ml separating funnel and 5 ml of toluene were added. The mixture was shaken for 3–5 min, and the operations were repeated. The combined toluene solutions were then evaporated in a stream of nitrogen at 40°C. The residue was dissolved in 2 ml of internal standard solution and dried with 1 g of anhydrous

sodium sulphate. A 2-μl volume of the final solution was injected into the gas chromatograph.

Gas–liquid chromatography

A Shimadzu GC-4BM gas chromatograph with an ECD was used for all analyses. The column was a glass tube (1.0 m × 2 mm I.D.) packed with 3% OV-225 on Chromosorb W HP (80–100 mesh) and was conditioned at 230°C; the detector and injector temperatures were 250°C. The flow-rate of nitrogen carrier gas was 50 ml/min, and the electrometer range was 10^2 MΩ × 0.08 V.

Calibration graph

A series of standard nitrite solutions was prepared by diluting the stock solution. Aliquots were placed into 50-ml centrifuge tubes. After addition of 1-hydrazinophthalazine and hydrochloric acid solution and subsequent reaction, the mixtures were extracted with toluene and the solvent removed by evaporation according to the procedure described above. The residues were dissolved in 2 ml of internal standard solution and 2-μl aliquots of the resulting solutions were injected into the GLC column. The concentration range of the nitrite-nitrogen standard was 2–40 ng/ml. As shown in Fig. 1, the retention time of tetrazolophthalazine relative to that of EPN was 0.68. The peak height ratios of tetrazolophthalazine to EPN were plotted against the amount of nitrite analysed; a typical standard graph is shown in Fig. 2.

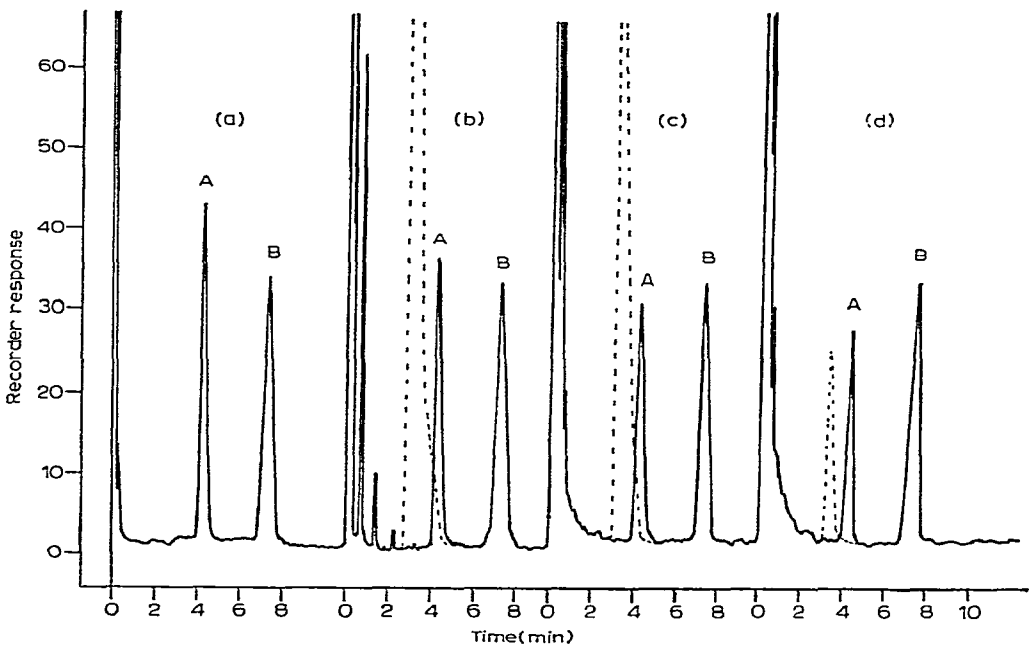

Fig. 1. Gas chromatograms of toluene extracts of a standard reaction mixture (a), to which nitrite was added at the level of *ca.* 0.07 ppm, human milk (b), cow milk (c) and market milk (d). The sample size was 2 μl. Peaks: A = tetrazolophthalazine; B = EPN. The dotted lines show the shape of chromatograms obtained for samples before clean-up by alumina column chromatography.

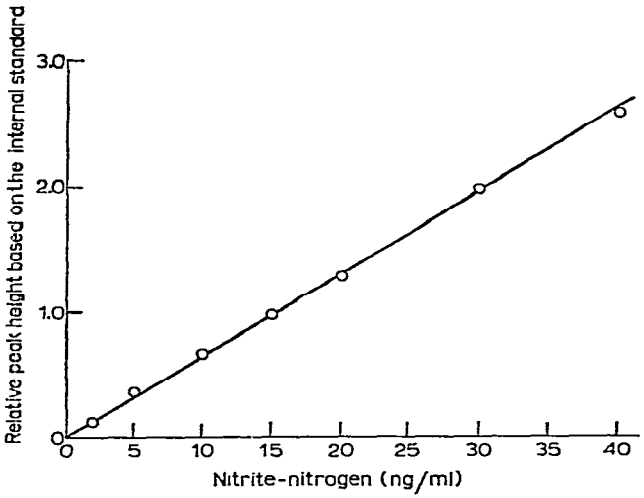

Fig. 2. Calibration graph for nitrite in the reaction mixture. Reaction: 70°C for 20 min. Sample size for GLC: 2 μl. Column temperature: 230°C. Nitrogen flow-rate: 50 ml/min.

Preparation of milk extracts and their determination

The accurately weighed sample (generally 5 g) of milk was placed in a 10-ml test-tube (11.5 × 1.5 cm). A 1-ml volume of each deproteinization reagent A and B was added, followed by 3 ml of distilled water, and the mixture was shaken for 1 min. The contents of the test-tube were then filtered. Five millilitres of the filtrate were placed into a 50-ml centrifuge tube and 1 ml of 0.1 % (w/v) 1-hydrazinophthalazine solution and 4 ml of 1 *M* hydrochloric acid solution were added. The mixture was allowed to react as described above. The interfering substances from milk were removed by alumina column chromatography as follows. A 10 cm × 10 mm I.D. column was prepared from 4.0 g of activated alumina topped by 0.3 g of anhydrous sodium sulphate suspended in toluene. After damping the contents of the column with toluene, 5 ml of the concentrated solution were passed through the column. Twenty millilitres of hexane and 15 ml of acetone–toluene (1:1, v/v) were added successively and eluted at a flow-rate of 1.5–2.0 ml/min. The first 2–3 ml fraction of the effluent was discarded and subsequent effluent was collected. The effluent was transfered to a test-tube (18 cm × 15 mm I.D.) and the solvent was evaporated with dry nitrogen gas at 40°C. To the residue was added 2 ml of internal standard solution. A 2-μl volume of the final solution was injected into the gas chromatograph.

RESULTS AND DISCUSSION

Standard assay

As shown in Fig. 2, the calibration graph was rectilinear for 2–40 ng of nitrite-nitrogen per ml of reaction mixture. The average standard deviation of five determinations was 3.0% for 5 or 10 ng of nitrite-nitrogen and 4.3% for 30 ng, and the reproducibility was considered satisfactory.

Conditions for reactions

pH. 1-Hydrazinophthalazine reacts with nitrite to form tetrazolophthalazine

in an acidic medium. The reaction was studied at various pH by mixing 0.1 μg of nitrite-nitrogen and 1 ml of 1-hydrazinophthalazine solution followed by heating at 70°C for 20 min. The results are shown in Fig. 3. In the range pH 1.5–3.0, a constant peak height is obtained, but above pH 3.5 the amount of tetrazolophthalazine decreased. In our procedure, if 4 ml of 1 *M* hydrochloric acid solution are added, the pH of solution is about 2.0.

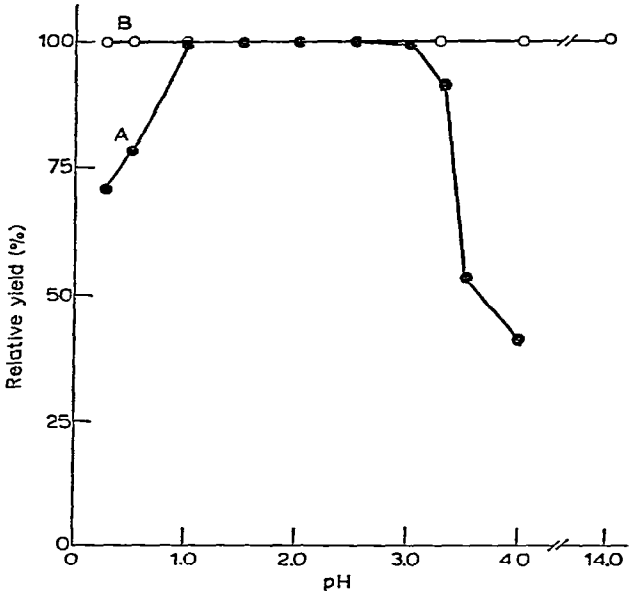

Fig. 3. A, Effect of pH on the completeness of the reaction with nitrite and 1-hydrazinophthalazine. To 0.1 μg of nitrite-nitrogen was added 1.0 ml of 0.1% 1-hydrazinophthalazine solution at 70°C for 20 min. B, Effect of pH on the extraction of tetrazolophthalazine.

Amount of 1-hydrazinophthalazine. If we assume that 1 mol of 1-hydrazinophthalazine reacts with 1 mol of sodium nitrite, then 1.14 μg of 1-hydrazinophthalazine are required for 0.49 μg of sodium nitrite (0.1 μg of NO_2-N). However, the relative yields of tetrazolophthalazine for various amounts of 1-hydrazinophthalazine added to 0.49 μg of sodium nitrite in a total volume of 5 ml of solution were 46.5% for 50 μg of 1-hydrazinophthalazine, 62.0% for 100 μg, 82.9% for 500 μg, 100% for 1000 μg and 100% for 5000 μg at 70°C for 20 min. After reaction, the product was determined by GLC as described for the preparation of tetrazolophthalazine. Therefore, addition of 1-hydrazinophthalazine in excess gave reasonable results, and in practice 1 ml of 0.1% reagent solution was used.

Temperature. The production of tetrazolophthalazine at several temperatures was studied by mixing 0.1 μg of nitrite-nitrogen, 1 ml of 0.1% 1-hydrazinophthalazine and 4 ml of 1 *M* hydrochloric acid solution. After reaction, the mixture was analysed by GLC according to the described procedure. The relative yields obtained after reaction for 20 min were 67.6% at 30°C, 73.2% at 40°C, 89.9% at 50°C, 100% at 60 or 80°C and 99.4% at 100°C. Therefore, 70°C was adopted.

Time. The optimum reaction time was investigated by mixing 0.1 μg of nitrite-nitrogen, 1 ml of 0.1% 1-hydrazinophthalazine and 4 ml of 1 *M* hydrochloric acid solution. The production of tetrazolophthalazine with time is shown in Fig. 4. A reaction time of at least 10 min at 70°C is required, and in practice 20 min was used.

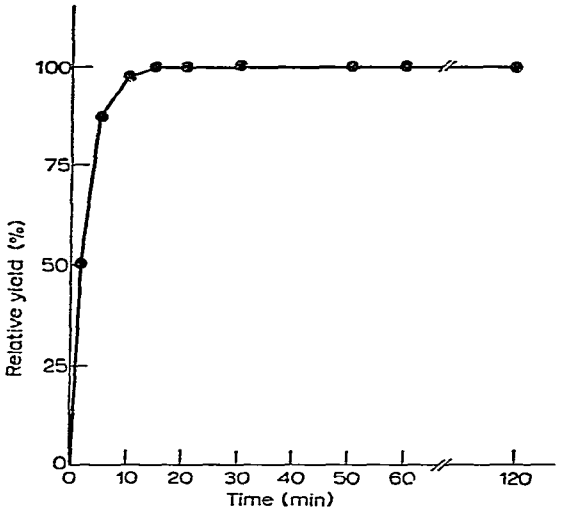

Fig. 4. Time course of tetrazolophthalazine formation on reaction of 1-hydrazinophthalazine and nitrite. To 0.1 μg of nitrite-nitrogen was added 1.0 ml of 0.1% 1-hydrazinophthalazine solution at 70°C. After the reaction the product was analysed by GLC according to the described procedure.

Extraction

Tetrazolophthalazine can be extracted into toluene over the range pH 2–14 as shown in Fig. 3. However, 1-hydrazinophthalazine can be extracted into an organic solvent at pH values above 7.0. Therefore, above pH 7.0, the excess of reagent is extracted together with the tetrazolophthalazine and its peak overlaps with that of tetrazolophthalazine on the gas chromatogram. Fortunately, as the optimum pH range of the reaction is the same as that of the extraction, as shown in Fig. 3, adjustment of the pH of extraction is not necessary.

Various extraction solvents were tried, as shown in Table I. When benzene, toluene, xylene, ethyl acetate, butyl acetate or methyl isobutyl ketone were used the extraction yield of tetrazolophthalazine was higher than with hexane or cyclohexane. Toluene was preferred because it did not cause co-extraction from the milks and did not form an emulsion with water. As a shaking time of 1 min is the minimum for complete extraction, mechanical shaking for 2 min was used in this method.

Gas chromatography

Sensitivity. Columns containing DC-200 (5%, w/w), SE-30 (3%, w/w), QF-1 (3%, w/w), OV-1 (5%, w/w), OV-17 (2%, w/w), OV-225 (3%, w/w), OV-101 (2%, w/w) and XE-60 (3%, w/w) on Chromosorb W HP were tested. Our initial objection to the tetrazolophthalazine analysis centred on the poor peak shape of tetrazolophthalazine when subjected to GLC. When polar phases such as OV-225 and XE-60

TABLE I

OPTIMUM SOLVENT FOR EXTRACTION OF THE TETRAZOLOPHTHALAZINE PRODUCT AFTER REACTION OF 1-HYDRAZINOPHTHALAZINE AND NITRITE

Reaction and GLC conditions as in Fig. 2. The reaction mixture contained 0.1% 1-hydrazinophthalazine (1.0 ml) and nitrite-nitrogen (0.1 μg).

Solvent	*Relative peak height (%)*
Toluene	100
Buthyl acetate	100
Xylene	100
Ethyl acetate	86.0
Benzene	85.0
Methyl isobutyl ketone	82.5
Cyclohexane	3.0
Hexane	2.5

were used, symmetrical peaks were obtained on the chromatogram. Peak tailing was observed if non-polar phases such as OV-1 and OV-101 were used. Particularly good peak characteristics and sensitivity were achieved with OV-225 under the conditions described above. The retention time of tetrazolophthalazine relative to that of the internal standard was 0.68.

After reaction, the tetrazolophthalazine should be injected into the gas chromatograph as soon as possible; the sample was stable for 15 h, but after 24 h the content of tetrazolophthalazine had decreased to 81.4%. The response of the ECD to various compounds is often markedly temperature dependent. The optimal detector temperature for the determination can be ascertained from a plot of $\ln AT^{3/2}$ *versus* $1/T$, where A = peak area for a constant mass of tetrazolophthalazine and T = absolute temperature of the detector oven. Plots of this type can also provide valuable insight into the mechanism of the electron-capture process[12]. For tetrazolophthalazine, as shown in Fig. 5, above 260°C the response of the ECD decreased with increasing temperature, indicating a non-dissociative mechanism of electron attachment. The maximum detector response was obtained at a detector oven temperature of 250°C.

Influence of interferences. Analysis of the sample digest by GLC after the formation of tetrazolophthalazine is shown Fig. 1. Beside the peak for tetrazolophthalazine, these was another peak, the retention time of which relative to that of the internal standard was 0.45. The intensity of this peak was sufficient to interfere in the determination of tetrazolophthalazine. However, the clean-up procedure on the alumina column was effective in removing this interfering substance. Tetrazolophthalazine in 5 ml of the toluene extract was not separated on 2.0 g of alumina. However, on 4.0 g of alumina, although tetrazolophthalazine was not found in the first 2–3 ml fraction of the effluent, it was present in the subsequent 15 ml of effluent. In practice, after the toluene extract as described above was passed through the alumina column, 20 ml of *n*-hexane and 15 ml of effluent were then added successively to the column. In conclusion, the interfering peak was eliminated by adding *n*-hexane as shown in Fig. 1.

During the extraction procedure, or after column chromatography, the toluene or toluene–acetone solution containing tetrazolophthalazine is evaporated. In order

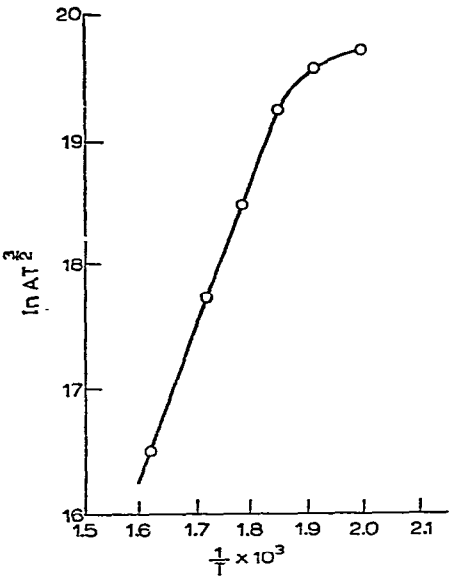

Fig. 5. Plot of ln $AT^{3/2}$ *versus* $1/T$ for tetrazolophthalazine. The positive slope indicates a non-dissociative mechanism of electron capture.

to examine possible losses in this step, 1.2 μg of tetrazolophthalazine (*ca.* 0.1 μg as NO_2-N) were dissolved in 10 ml or 15 ml of the solvent, and the solution was analysed during or after evaporation of the solvent at 40°C; no loss was observed.

Application and recovery

Nitrite added to various deproteinized milks (5 g) was determined after reaction and clean-up as described. Five determinations were carried out at each concentration and the results are presented in Table II. The mean recovery varied between 95.8 and 99.0%, the detection limit being 4 ppb.

TABLE II

PERCENTAGE RECOVERIES OF NITRITE ADDED TO VARIOUS MILKS

Each result is the average of five determinations.

Sample	*Amount of nitrite-nitrogen added (µg)*		
	0.05	*0.1*	*1.0*
Human milk	95.8	97.6	97.4
Cow milk	96.3	98.4	97.8
Market milk	96.7	98.7	99.0

Identification of tetrazolophthalazine

To obtain the tetrazolophthalazine, to 4.8 g of 1-hydrazinophthalazine and 2.1 g of sodium nitrite (molar ratio 1:1) were added 2 ml of 2 *M* acetic acid and 5 ml of distilled water, and the mixture was gently heated at 70°C with occasional shaking

for 20 min; when reaction was complete, the mixture was cooled and the crystals were collected on filter-paper and washed with distilled water. The product was recrystallized from 50% ethanol as pale brown needles (m.p. 207°C; reference value[8] 209–210°C). The elemental composition of the product was 56.95% C, 2.51% H and 40.12% N; for tetrazolophthalazine, $C_8H_5N_5$, the theoretical values are 56.15% C, 2.93% H and 40.92% N. In order to determine whether tetrazolophthalazine had decomposed during the GLC process, the identity of the product was confirmed by GLC–MS. The fragmentation pattern of the tetrazolophthalazine is shown in Fig. 6, with peaks at *m/e* 171 (M^+), 143 ($M^+ - N_2$), 115 ($-N_2$), 88 (—HCN) and 76 (—C). The parent peak (*m/e* 171) for the tetrazolophthalazine correspond to its molecular weight.

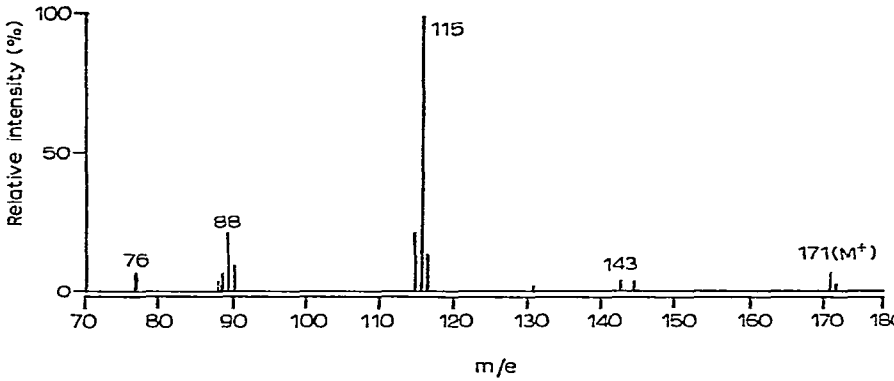

Fig. 6. Mass spectrum of tetrazolophthalazine.

CONCLUSION

The determination of trace levels of nitrite in biological samples such as human milk and blood has posed many difficulties. In this study, nitrite was determined as tetrazolophthalazine by GLC–ECD after sample clean-up with alumina column chromatography. The method is simple, and suitable for micro-assay of nitrite. It has successfully been applied to the determination of nitrite in various milks.

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