Journal of Chromatography, 347 (1985) 89–97
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 041

EVALUATION OF INTERNAL STANDARDS AND EXTRACTION SOLVENTS IN THE GAS CHROMATOGRAPHIC DETERMINATION OF THIAMINE

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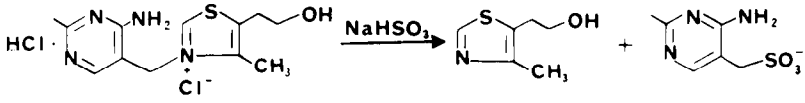
(First received May 14th, 1985; revised manuscript received July 16th, 1985)

SUMMARY

A previously developed gas chromatographic method for determining thiamine has been improved by using a pre-extraction internal standard to reduce the amount of reagents and degree of skill needed to perform the procedure. Three compounds, 3-(3-pyridyl)-1-propanol, 2-anilinoethanol and 6-methyl-2-(2-hydroxyethyl) pyridine, out of more than thirty studied, have been found to be suitable pre-extraction internal standards. Extraction characteristics of three solvents, chloroform, butanone and dichloromethane, have been compared. The improved procedure has been tested by analyzing standard thiamine solutions, corn flakes, vitamin formulations and pork chops.

INTRODUCTION

Gas chromatography (GC) has been used to quantitate thiamine, a water soluble vitamin, in various substances[1]. The procedure involves using sodium bisulfite[2] to cleave thiamine into 2-methyl-4-amino-5-methylenepyrimidine sulfonic acid (II) and 4-methyl-5-(2-hydroxyethyl)thiazole (I), a volatile compound which can be chromatographed. The quantitation depends on the total recovery of thiamine from several steps that require great patience and skill. The reported GC method uses 150 ml of chloroform, 10 g of sodium bisulfite, 10 g of sodium chloride, and a large volume of sodium hydroxide, whereas the standard fluorometric method[3,4] uses small amounts of reagents, including a volume of isobutanol (up to only 25 ml) as the extracting solvent.



I II

The present investigators have tested more than 30 compounds from a variety of classes (including pyrroles, pyridines, piperidines and substituted anilines) for their possible use as pre-extraction internal standards. This paper details results obtained

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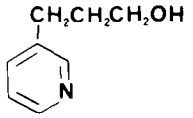
for the three compounds, 3-(3-pyridyl)-1-propanol (III), 2-anilinoethanol (IV) and 6-methyl-2-(2-hydroxyethyl)pyridine (V), that were found to be suitable pre-extraction internal standards. Also reported are some performance characteristics of five of the other compounds, 2-(2-hydroxyethyl)pyridine, N-benzyl-N-methylethanolamine, 4-(dimethylamino)phenylethyl alcohol, 2-(N-ethyl-*M*-toluidino)ethanol and N,N-(dimethylamino)phenylethyl alcohol, that were rejected. The study of pre-extraction standards was undertaken in an effort to reduce the working volume to approximately 25 ml with a concomitant reduction in time and reagents.



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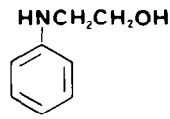

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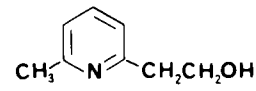

V

Quantitative results of the extraction properties of three solvents, chloroform, butanone and dichloromethane, have been compared. Butanone and dichloromethane were investigated as alternatives to using chloroform for extraction.

EXPERIMENTAL

Materials

Standard solutions in concentrations of 15 ppm and 30 ppm of the internal standards, 4-methyl-5-(2-hydroxyethyl)thiazole and thiamine hydrochloride, were prepared. Pork chops, corn flakes (cereal) and vitamin formulations were purchased.

Instrumentation

A glass-lined Perkin-Elmer Sigma I gas chromatograph, equipped with a dual nitrogen-phosphorus detector, was used for the analyses. The column was a 6 ft. × 2 mm I.D. coiled glass, packed with either 5% OV-17 or OV-1 on Chromosorb W. For the internal standard extractions, the instrument was run isothermally for 5 min at a temperature beween 180°C and 205°C (depending on the standard) with the injector and detector set at 225°C. For foodstuffs the temperature was programmed up to 260°C for 7 min.

Extraction of the internal standard and 4-methyl-5-(2-hydroxyethyl)thiazole (I)

An amount of 30 μg (1 ml of 30 ppm) of the desired pre-extraction internal standard and 30 μg of the thiazole I were added to flasks containing 15, 20, or 25 g of sodium chloride. To each flask were added 100 ml of water, after which the pH was adjusted to 12–13 with 15 *M* sodium hydroxide. A single extraction of a given solution was made with 50 ml of chloroform, 2-butanone, or dichloromethane. Each extraction was carried out in multiples of 5. The extracting solvent was evaporated at 40°C in a 100-ml cone-shaped flask on a roto evaporator. The residue was taken up with two rinsings of 1.5–2 ml of methanol. To this mixture 30 μg of a second internal standard was added to check the amounts of pre-extraction standard and

thiazole I recovered. The solution was allowed to evaporate to approximately 0.5–1 ml in a dry bath at 50°C to 60°C. The GC analysis was carried out and the extraction ratio of the thiazole I to the pre-extraction internal standard, along with the total extracted thiazole, was calculated (Tables I and II).

The procedure was repeated using 5 g of salt and 25 ml of extracting solvent in a large test tube (25 × 200 mm) with a PTFE-lined cap. After shaking with the solvent, the mixture was poured into a 125-ml separatory funnel to separate the layers. When butanone was used as the solvent, an additional evaporation using 10–15 ml of absolute ethanol was used to azotrope the last traces of water before taking up the residence with methanol.

Determination of thiamine standard

Amounts of 30 μg of thiamine and 15 μg of the internal standard were added to a test tube containing 2.5 g of sodium bisulfite and 25 ml of water. The solution was heated at 100°C for 1 h. The pH was adjusted to 12–13 with sodium hydroxide and 25 g of sodium chloride were added. The solution was extracted with 25 ml of butanone, chloroform, or dichloromethane. After evaporation, the thiazole I was determined and converted to micrograms of thiamine (Table III).

Determination of thiamine in cereal

Corn flakes (375 μg/ounce reported thiamine) were pulverized and stored for further use. Samples of 2 g were blended with 1 ml of 15 ppm (15 μg) standard and approximately 100 ml of 0.1 *M* hydrochloric acid. The emulsion was centrifuged, and the supernatant decanted (without additional rinses). Sodium bisulfite (2.5 g) was added, and the resulting solution was heated at 100°C for 1 h. [When using the solvents chloroform or dichloromethane, the addition of 2–3 ml of 50% trichloroacetic acid (TCA) is required, followed by an additional 15 min of heating.] The solution was cooled and filtered. After adding 3 g of sodium chloride, the solution was extracted with 25 ml of chloroform or dichloromethane. When butanone was used as the extracting solvent, the TCA and filtering steps were omitted.

Determination of thiamine in vitamin formulations

Ten tablets (12.5 mg/tablet) were dissolved and diluted to 100 ml in 0.1 *M* hydrochloric acid to give a concentration of 1250 ppm. A volume of 3 ml of this solution was diluted to 100 ml and used as the stock solution. The determination followed the procedure for the standard thiamine solution determination.

Determination of pork chops

Lean portions of pork chops were ground into sausage form. An amount of 5 g was added to 1 ml of a 30-ppm standard (30 μg) and blended with 25 ml of 0.3 *M* hydrochloric acid (pH maintained less than 1). The mixture was heated for at least 1 h at 100°C. After cooling, the pH was adjusted to 5 with sodium acetate, and the coagulated solids were removed by centrifugation. After pouring the supernatant into a large test tube, 0.5 g of mylase and 0.5 g of papain were added. The resulting mixture was allowed to stand overnight at room temperature. Sodium bisulfite (2.5 g) was added; the pH was readjusted to 3–4 with hydrochloric acid (only when standard IV was used); the solution was heated for 1 h at 100°C; 2 ml of TCA (50%)

TABLE I

EXTRACTION RATIO

III = 3-(3-pyridyl)-1-propanol, IV = 2-anilinoethanol, V = 6-methyl-2-(2-hydroxyethyl)pyridine.

	Chloroform (g)			*Dichloromethane (g)*			*Butanone (g)*			*25 ml/5 g sodium chloride*		
	15	*20*	*25*	*15*	*20*	*25*	*15*	*20*	*25*	*Chloroform*	*Dichloromethane*	*Butanone*
III	1.34	1.24	1.20	1.20	1.06	1.14	0.97	0.95	1.08	1.18	1.02	1.00
IV	1.00	1.28	1.30	1.82	1.61	1.35	1.40	1.30	1.35	1.24	1.24	1.25
V	1.26	1.20	1.25	0.85	0.90	0.95	0.80	0.85	0.85	1.20	0.87	0.78

were then added with an additional 15 min of heating. The solution was cooled (5–10°C) and filtered. The rest of the procedure follows the above procedure for corn flakes.

RESULTS AND DISCUSSION

Compounds chosen for the initial testing for possible internal standards were those with characteristic functional groups similar to the thiazole I. In order for a compound to be used as a pre-extraction internal standard, the solubilities of the compound and the thiazole I in the extraction solvent should be about the same. The boiling point of the compound should be high enough to resist evaporation and yet low enough to be chromatographable at a reasonable temperature (180–200°C).

To test solubilities of the internal standards at different ionic strengths, extractions with 50 ml of solvent were performed on 100 ml of an aqueous solution of a given standard and thiazole I with different salt concentrations. Extractions with 25 ml of solvent from 25 ml of an aqueous solution of standard and thiazole were used to simulate the actual extraction of thiazole in a thiamine-determination procedure. These extractions were used to deduce the extraction ratios (Table I) used in calculating thiamine concentrations. The ionic strength (salt concentration) of the actual mixture was manifested by a combination of sodium chloride, sodium sulfite, and sodium trichloroacetate. It is important that the ratio of thiazole to standard extracted be relatively constant over a range of ionic strengths because of the difficulty that would be involved in maintaining an exact salt concentration for extraction.

A high sodium hydroxide concentration (15 *M*) was used to keep the overall volume to a minimum at a pH of 12–13. No significant difference in a given extraction ratio was observed at a pH of 11–13.5.

The results in Table I show that the extraction ratios using chloroform, dichloromethane and butanone are essentially the same for salt concentrations of 20–25 g/100 ml, except for dichloromethane and standard IV, whose ratio varies from 1.82 to 1.35. When dichloromethane is used for extraction from 25 ml of solution, the extraction ratio is lower than that for extraction from 100 ml of solution. When analyzing standard thiamine solutions, this lower ratio proves to be the correct one to use.

The thiamine concentration of a given solution is determined and calculated by adding a given quantity (15–30 μg) of the internal standard at the beginning of the determination. Since the standards (which are amines) and thiamine must be kept acidic until the final extraction, they exist as soluble salts (protonated); and losses, therefore, that may occur through handling do not affect the overall reading of the thiazole concentration because the standard and the thiamine (or thiazole) are equally lost. The following steps are involved in calculating the thiamine concentration:

$$\text{Extraction ratio} = \frac{\text{conc. of standard extracted}}{\text{conc. of thiazole extracted}}$$

$$\text{Total thiazole} = \text{extracted thiazole} \cdot \text{extraction ratio}$$

$$\text{Total thiamine/unit} = \frac{\text{mol.wt. of thiamine} \cdot \text{total thiazole/unit}}{\text{mol.wt. of thiazole}}$$

Chloroform, dichloromethane and 2-butanone were used as solvents. Chloroform and dichloromethane have similar characteristics, including being carcinogen-suspecting agents. The reported toxicity levels for humans are 10 ppm and 500 ppm inhalation for chloroform and dichloromethane, respectively[5]. Both are included because many scientists have personal preferences between the two solvents. Butanone has a b.p. of 80°C and a toxicity level of 200 ppm inhalation[6], but it is not a carcinogen-suspecting agent.

The amounts of thiazole extracted with each solvent using different salt concentrations are given in Table II. Chloroform extracts over 50% more thiazole than dichloromethane, but the latter (b.p. 40°C) is easier to evaporate. Butanone and chloroform extract approximately the same amount of thiazole. Butanone has the advantage of not being as hazardous as chloroform, but it is more difficult (b.p. 80°C) to evaporate and requires an extra step of using ethanol to remove the last traces of water. Although 2-butanol is used in the standard fluorometric procedure for determining thiamine, it was found to carry over too much salt and water, along with being too difficult to evaporate. The thiazole was not soluble enough in ether or hexane for them to be useful as extraction solvents.

TABLE II

CONCENTRATIONS OF THIAZOLE EXTRACTED

Amount of sodium chloride	*Chloroform*	*Dichloromethane*	*Butanone*	*Ether*	*Hexane*
15 g**	15.5 ± 1.7	10.9 ± 0.7	15.5 ± 1.7	ND*	ND
20 g	19.2 ± 2.0	12.5± 1.0	17.9 ± 1.8	ND	ND
25 g	18.7 ± 1.8	13.2 ± 0.8	18.0 ± 1.9	<5	<5
5:25:25***	19.4 ± 2.0	13.5 ± 1.7	18.6 ± 1.9	ND	ND

* Not determined.
** From 100 ml of water and 50 ml of solvent.
*** 5 g of sodium chloride, 25 ml of water, and 25 ml of solvent.

Since dichloromethane extracts about 40% of the available thiazole, materials with low thiamine content are better extracted with one of the other solvents. It is desirable for the total thiamine in the sample to be on the order of 30 μg, which will yield 13 μg of thiazole. Approximately 8.6 μg and 5.6 μg of the thiazole are extracted, respectively, with chloroform and dichloromethane. When reduced to a working volume of 0.25 ml, the thiazole will be 20 to 35 ppm. Good quantitation of the complex mixture requires an effective minimum concentration of 20–25 ppm.

It was observed upon evaporation of butanone that many of the compounds tested for standards would give a wide variation in value for the ratio of standard/thiazole. After re-examination of most of the compounds tested, they were eliminated as possible standards because of their large losses in the evaporation process compared to the loss of the thiazole. Because of their relatively large losses during evaporation, compounds with boiling points less than 225°C could not be used as standards, even though their extraction ratios, in many instances, were similar to the ratios for the three compounds chosen as standards.

The relative rates of evaporation were measured by allowing 30 μg (1 ml of 30 ppm) of the desired standard and 30 μg of the thiazole to evaporate at room temperature until 30–50% of the thiazole had evaporated. An amount of 30 μg of an alternate standard was then added, and the amounts of standard and thiazole lost were determined. Standards III and IV evaporated at approximately the same rate as the thiazole I, and standard V evaporated at a rate 15% faster than the thiazole. Other ideal compounds (with ideal extraction ratios), such as 2-(2-hydroxyethyl)-pyridine (b.p. 114–116°C/9 mmHg) and N-benzyl-N-methylethanolamine (b.p. 95–105°C/2 mmHg), evaporated 85–90% faster than the thiazole.

In the analyses of standard 30 ppm thiamine solutions and food materials, 4-(dimethylamino)phenylethyl alcohol and N-benzyl-N-methylethanolamine gave thiamine concentrations higher than 30 ppm for the standard solutions and abnormally high thiamine concentrations in the foods. Internal standard IV, 2-anilinoethanol, gave excessive amounts of thiamine when added to buffered solutions in the analyses of pork chops (also when thiamine was added). These results suggested a possible decomposition of the standards. Accordingly, 30 μg of a standard and 30 μg of the thiazole at a given pH were allowed to react overnight with sodium bisulfite at 100°C. The decompositon rates for 2-anilinoethanol and N-benzyl-N-methylethanolamine were less than 0.5%/h at a pH of 5; no significant breakdown was evident at pH values of 2–3. 2-(N-ethyl-*m*-toluidino)ethanol and N,N-(dimethylamino)phenylethyl alcohol, under the same conditions, decomposed at a rate greater than 2%/h. In a solution buffered to pH 5 with sodium acetate, the decomposition rates of these two standards were over 5%/h.

The above decomposition is assumed to be dealkylation of the amine, with the results showing that N,N-dialkylaromatic amines are destroyed faster than alkylamines or secondary amines. Because of this decomposition, when standard IV is used with pork chops (meats or solutions using enzymes) the buffering action is overridden by adding hydrochloric acid; and the reaction time of 1 h is strictly ad-

TABLE III

THIAMINE ANALYSIS

Standard	*Standard solution (μg/ml)*			*Vitamin (mg/tablet)*		
	Chloroform	*Dichloromethane*	*Butanone*	*Chloroform*	*Dichloromethane*	*Butanone*
III	28.5 ± 2.6	28.0 ± 0.5	25.0 ± 0.2	12.5 ± 1.0	12.0 ± 0.8	10.9 ± 1.5
IV	30.6 ± 2.0	30.2 ± 1.9	30.1 ± 0.8	12.7 ± 0.3	12.6 ± 0.2	12.6 ± 0.5
V	31.4 ± 3.1	29.6 ± 2.9	29.8 ± 2.0	13.4 ± 1.8	13.5 ± 1.0	13.5 ± 1.0
	Corn flakes (μg/g)			*Pork chops (μg/g)*		
	Chloroform	*Dichloromethane*	*Butanone*	*Chloroform*	*Dichloromethane*	*Butanone*
III	13.0 ± 2.3	10.8 ± 1.9	13.8 ± 2.0	9.9 ± 1.3	10.2 ± 1.0	10.6 ± 1.5
IV	14.2 ± 1.0	15.1 ± 2.0	14.2 ± 0.9	9.7 ± 1.8	8.8 ± 1.4	10.0 ± 2.0
V	15.9 ± 1.8	11.5 ± 1.5	11.5 ± 1.0	ND*	ND	ND

* Not determined.

hered to. When standard III or V is used, the reaction time can be based on convenience.

Table III gives analyses of materials using internal standards, reduced volumes, and one extraction. The calculated or theoretical values are 30 μg/ml for the standardized thiamine solution, 12.5 mg/tablet for the vitamin, 13.3 μg/g for corn flakes, and 8.5 μg/g for pork chops based on a total extraction. Each number in Tables III and II is an experimental mean of ten trials reported at the 95% confidence level.

When extracting with butanone, TCA is not used to facilitate the separation of the butanone and aqueous layers. The solubility of the salt of TCA in the butanone layer renders TCA useless in this extraction. The time of analysis of corn flakes and pork chops is reduced by separating the solid and liquid phases by centrifugation instead of filtration. No additional rinsing is required.

In the analyses, OV-17 (polar) and OV-1 (non-polar) liquid phases were used. An example of the chromatographic resolution obtained with each liquid phase is given in Fig. 1. The last peak in the chromatograms is 2-(N-ethyl-*m*-toluidino)ethanol, which was used as an added marker to check such things as total recovery or losses from a given step (note discussion on decomposition). Standard V cannot be used with OV-1 because of the closeness of its retention time to that of the thiazole. When analyzing pork chops, standard V is also unsuitable with OV-17 because of interferences from unknown peaks. If so desired, the analysis can be carried out using two internal standards to double check the results.

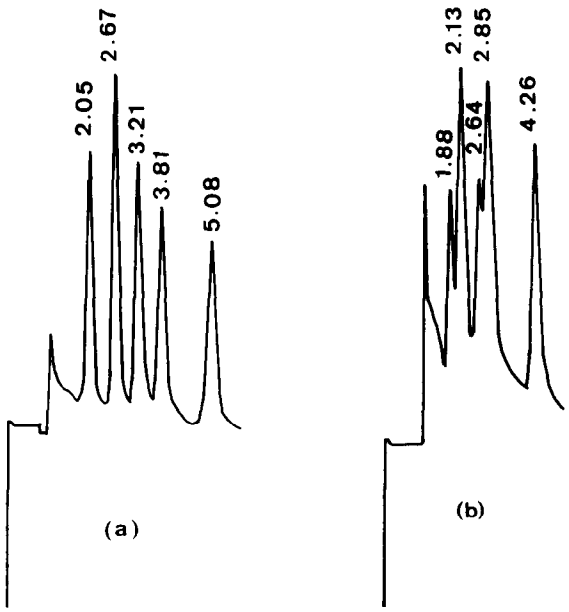

Fig. 1. Chromatographic resolution of standards and the thiazole derivative: (a) OV-17 at 190°C, (b) OV-1 at 190°C. The first, third, and fourth peak over which the retention times (in min) appear are, respectively, standards V, III and IV. The second peak is the thiazole derivative. The last peak is that of a fifth compound (see text) that was used to check such things as total recovery or losses from a given step in the procedure.

CONCLUSIONS

The results show that adding an internal standard at the beginning of the procedure simplifies the quantitation and reduces by three fourths the amounts of reagents needed. Of the compounds tested for use as an internal standard, 3-(3-pyridyl)-1-propanol was found to be the best overall standard becaused it placed no restrictions on the method. By using pre-extraction internal standards, the need for total recovery is eliminated and the data are more consistent. Data from the solvent study show that dichloromethane and butanone can be used as extracting solvents, but chloroform is better.

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