Journal of Chromatography, 204 (1981) 429–435
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,353

DETERMINATION OF BETAINE IN SUGAR AND WINE BY LIQUID CHROMATOGRAPHY

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SUMMARY

The analysis of betaine by high-performance liquid chromatography presents some difficulties owing to the quaternary ammonium moiety. This problem was investigated on polar stationary phases such as silica gel and amine-bonded silica gel. A method for titration is described and examples of betaine analysis in commercial beet sugars and wine are given. Pre-treatment of samples is required prior to the quantitative determination of betaine by liquid chromatographic analysis. Under particular conditions, it would be possible to detect or to determine the amount of sugar added to wines in order to increase their ethanol content.

INTRODUCTION

In France, as in some other countries, the addition of sugar to wines in order to increase their ethanol content is prohibited in some wine-growing areas, whereas in some other areas it is permitted in a given range. Saccharose is generally used for this purpose, and more especially refined saccharose from beet sugar. Because of the rapid hydrolysis of saccharose in wine a standard analysis of saccharose is not a convenient method for controlling either the fraudulent or the permitted addition of sugar to wines.

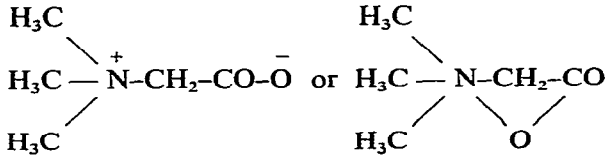
As betaine is a permanent component of beet sugar and as the initial content of betaine in wines is very low, the determination of betaine in wines could be a method determining the addition of saccharose.

Titration methods for the determination of betaine involved long and tedious chemical steps, and the first alternative chromatographic separations were carried out by thin-layer chromatography (TLC)[1–3]. Subsequently, by derivatization in order to obtain a volatile compound from the thermal degradation of the butyric ester of betaine, Dubois and Simand[4] made use of the great thermal sensitivity of gas chromatography (GC). Liquid chromatographic methods have been reported[5,6]. Because of the poor precision of TLC and the complexity of GC, which necessitates deriva-

0021-9673/81/0000–0000/$02.50

tization of the betaine, it was of interest to develop an improved method based on high-performance liquid chromatography (HPLC).

Betaine is the anhydride of carboxymethyltrimethylammonium hydroxide. It exists with either an open or an internally linked structure:



Owing to the presence of the trimethylamino group, it has the characteristics of a nitrogenous base. On the basis of its molecular structure, a method of separation similar to that used to separate amino acids[7,8] by ion-pair partition chromatography on a non-polar stationary phase was tried. However, the elution of betaine could not be properly controlled.

Considering the polarity of the molecule, we then performed the separation on polar stationary phases: firstly amino-bonded silica gel and secondly silica using chromatographic conditions derived from those used by Greving *et al.*[9] for the separation of quaternary ammonium compounds. Both methods are described in this paper.

EXPERIMENTAL

The pump was a Model 380 Chromatem (Touzart et Matignon, Vitry, France) and the detector an Optilab Multiref 902 interferometer (Touzart et Matignon), monitored in all experiments at a flow-rate of 1 cm^3/min.

Stainless-steel columns of length 15 or 5 cm and I.D. 4.7 mm were packed using the balanced density slurry method. According to the chromatographic method used, they were packed with silica (Partisil, 5 μm; Whatman, Ferrieres, France) or NH_2-bonded silica (LiChrosorb NH_2, 10 μm; Merck, Paris, France).

Samples were injected by means of a Rheodyne 7010 high-pressure valve (Touzart et Matignon) fitted with a 20-μl sample loop.

All solvents were of HPLC grade, purchased from various suppliers.

pH and potential values were measured by means of a Minisis millivoltmeter (Solea-Tacussel, Lyon, France), a working glass electrode (Solea-Tacussel) and a home-made Ag/AgCl/acetonitrile–methanol (97:3) saturated with KCl/$LiClO_4$ electrode as reference electrode. Using these electrodes, linearity of the calibration graph (potential *versus* pH) was verified with perchloric acid solutions in acetonitrile–methanol (97:3) solvent. The titration graphs for the various acids dissolved in the organic solvent were plotted using tetrabutylammonium hydroxide solutions as titrant. The potential of the half-neutralization point was related to the equivalent potential of the calibration graph and the corresponding pH value gave the pK_a value of the acid.

The extraction method was described many years ago by various authors[10,11]. However, the extraction conditions are defined here in order to ensure a good recovery of betaine. Pre-treatment of natural samples was carried out using glass columns of

length 15 cm and I.D. 1.8 cm: column I was packed with a cation-exchange resin (Dowex 50W-X8, 20–50 mesh) and column II with 1:2 (v/v) mixture of a cation-exchange resin (Amberlite IRC 50) and an anion-exchange resin (Dowex 1-X4, 20–50 mesh) (Rhône Alpes Chimie, Lyon, France).

For the determination of betaine in commercial refined sugar, a known volume of the solution to be analysed is, if necessary, purified on charcoal and evaporated in order to concentrate it to about 200 cm^3, the pH is adjusted to 3 with sulphuric acid and the solution is applied to column I (H^+ form). The column is then eluted with a large excess of distilled water in order to achieve a neutral pH of the eluate and to eliminate part of the sugar content of the sample. The betaine and amino compounds retained on the resin are then eluted using about 300 cm^3 of 4% ammonia solution and about 100 cm^3 of distilled water. The eluate is evaporated to dryness under vacuum and the residue is dissolved in 5 cm^3 of the mobile phase. This procedure was also used for the determination of betaine of commercial beet saccharose.

For the determination of betaine in wines, 1 l of wine is treated as described above on column I. The eluate is then concentrated under vacuum to about 100 cm^3 and adjusted to pH 7 by addition of 0.1 *N* hydrochloric acid. The solution is passed through column II. The betaine is eluted under these conditions whereas the main part of the amino compounds are retained. The column is then rinsed with 100 cm^3 of distilled water. The eluate is collected and evaporated to dryness under vacuum and the residue is dissolved in 5 cm^3 of the mobile phase.

RESULTS AND DISCUSSION

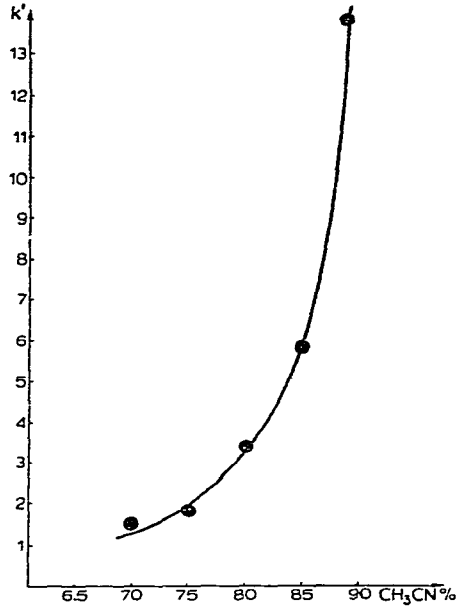
Chromatographic separation of betaine on amine-bonded silica

This stationary phase has already been used for sugar analysis[12] and has been mentioned for the chromatography of betaine in natural mixtures in the sugar beet industry[5]. The eluent is a binary mixture of acetonitrile and water. The dependence of the retention on the mobile phase composition is illustrated in Fig. 1. Fig. 2 shows the separation of a mixture of various oses and betaine. The peak corresponding to betaine was collected and identified by mass spectrometry. It should be noted that under the chromatographic conditions used, the betaine is eluted before the oses, which is different from the results obtained previously[5].

Chromatographic separation of betaine on silica

Quaternary ammonium compounds are eluted on silica by ion pairing with inorganic acid anions such as ClO_4^- and Br^-, and with methanol as the mobile phase[9]. With such a chromatographic system, the betaine shows a broad, tailing peak with a very long retention time. The addition of an inorganic acid allows the elution of the betaine as a sharp peak in a short period of time. However, the addition of counter ions does not improve the retention. In order to increase the retention time it was necessary to use a less polar mobile phase. Good results were obtained with acetonitrile–methanol (97:3).

A study of the elution with different acids showed that the acids that can lead to a good elution of betaine are weakly dissociated in the mobile phase. In order to establish their dissociation in such an organic medium (acetonitrile–methanol, 97:3),



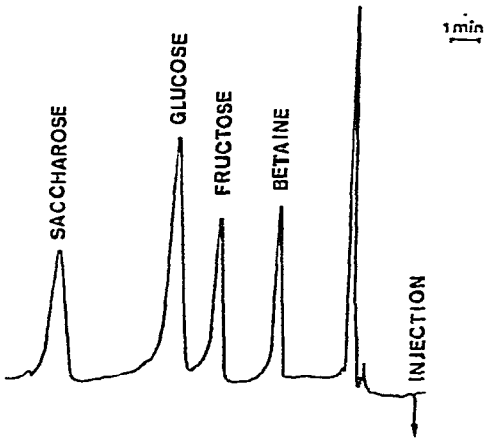


Fig. 1. Dependence of k' on the mobile phase composition. Column: 15 × 0.47 cm I.D. packed with NH_2-bonded silica; particle size, 10 μm. Eluent: acetonitrile–water.

Fig. 2. Separation of betaine and oses. Column: 15 × 0.47 cm I.D. packed with NH_2-bonded silica; particle size, 10 μm. Eluent: acetonitrile–water (75:25).

their pK_a values were measured by the classical potentiometric method. The measured pK_a values increase in the order 11 $\approx$ CH_3–COOH $\approx$ $CHCl_2$–COOH $<$ CCl_3–COOH $<$ HNO_3 $<$ HBr $<$ H_2SO_4 $=$ 3.2 $\ll$ $HClO_4$.

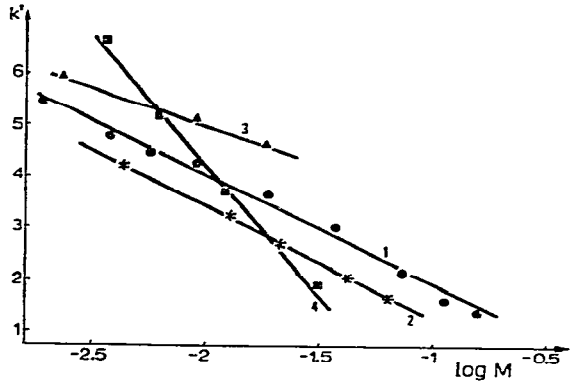
Perchloric acid, which is totally dissociated in the organic medium, elutes betaine without any retention whereas acetic acid is too weak to give a satisfactory elution.

Fig. 3 shows the influence of the acid concentration on the retention of betaine. Sulphuric and nitric acids have a similar influence and give the largest variation in k'. Trichloroacetic acid results in a broad and leading peak whereas hydrobromic acid, in spite of a suitable retention, corrodes parts of the chromatograph. Fig. 4 shows the variation of the retention with the composition of the mobile phase for sulphuric and nitric acids.

Detection level and betaine calibration graph

The use of the Optilab differential refractometer allows a considerable increase in sensitivity over other refractometric detectors. It was possible to detect down to a few tens of nanograms of betaine, and by optimizing the operating parameters it should be possible to lower the detection limit to a few nanograms.

Calibration graphs for betaine are drawn using the external calibration method. The linearity of the detector response (betaine peak height) *versus* concentration of betaine was determined over the entire working range, from 40 ng to 10 μg. The relative standard deviation of the calibration factor was less than 4%.



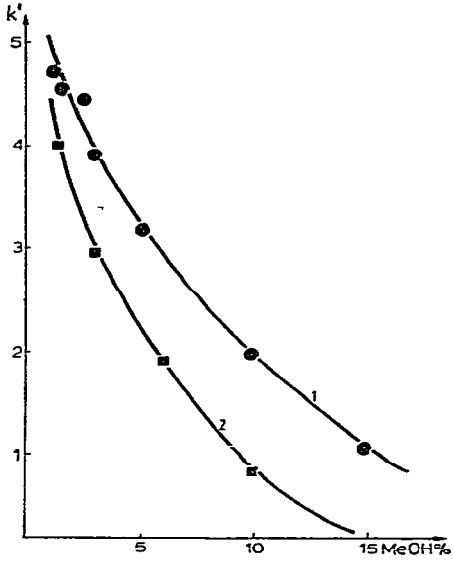


Fig. 3. Dependence of k' on acid concentration. Column: 5 × 0.47 cm I.D. packed with Partisil; particle size, 5 μm. Eluent: acetonitrile–methanol (97:3). 1 = H_2SO_4; 2 = HNO_3; 3 = HBr; 4 = CCl_3COOH.

Fig. 4. Dependence of k' on mobile phase composition. Column: 5 × 0.47 cm I.D. packed with Partisil; particle size, 5 μm. Eluent: acetonitrile containing different amounts of methanol. 1 = With H_2SO_4, $9.0 \cdot 10^{-3}$ M; 2 = with HNO_3, $5.6 \cdot 10^{-3}$ M.

Recovery and accuracy of betaine measurements

In order to test the recovery of betaine, standard solutions of betaine in water in the range 3–6 mg/l were prepared. They were treated according to the above procedures by using either column I or columns I + II. The chromatographic analysis of the residue was then performed on amino-bonded silica. The betaine was determined and the recoveries are given in Table I. In all experiments, the recovery of betaine was greater than 95%.

TABLE I

RECOVERY FOLLOWING PRE-TREATMENT OF BETAINE SOLUTIONS

Betaine content of standard solution (mg/l)	*Betaine recovered (mg/l)*	*Recovery (%)*	*Columns used for treatment (see text)*
6.0	5.7	95	I
6.0	5.9	98	I
3.0	2.9	97	I
3.4	3.5	103	I + II
6.05	6.1	101	I + II

Application to quantitative analysis of betaine in natural media

Betaine is a nitrogenous component in many natural media such as beet sugar, fruit juices and wine. Despite its low concentration in these complex mixtures, the

sensitivity of previously described analytical methods would theoretically allow the detection of betaine by the direct analysis of these liquids or solutions without any pre-treatment or pre- or post-column derivatization. However, these samples are extremely complex mixtures in which betaine is only a minor constituent and it will often be necessary to extract and concentrate the betaine prior to its chromatographic analysis in order to avoid interferences from compounds eluted with identical retention times.

Therefore, the samples are treated according to the described clean-up procedures and the betaine content of the residue is determined by chromatographic analysis using one of the previously described methods. As the residue of the samples studied is not always completely soluble in the acetonitrile–methanol mixture but is soluble in acetonitrile–water (75:25), the analyses were carried out with the latter mixture as the mobile phase and with amino-bonded silica as the stationary phase.

The determination of betaine in three different samples of commercial beet saccharose was performed. A 37-g amount of sugar was dissolved in 250 cm^3 of distilled water. The solutions were treated on column I and the chromatographic analysis gave values from 30 to 37 mg/kg of betaine in the sugar. This range is normal as it is well known that the betaine content of beet and consequently beet sugar widely varies from year to year and with the district of growth[5,13].

Three samples of wine were analysed. Sample A was a reference sample, *i.e.*, without any sugar added prior to the fermentation step. In samples B and C, the amounts of sugar had been added in order to increase the ethanol content of the wine. The betaine contents of samples A, B and C were found to be 0.8, 2.9 and 5.7 mg/l, respectively. The initial betaine content of wine depends on the wine-growing area and during the past few years there has been some controversy about this subject[14].

The particular kind of wine that we studied, as is shown with the analysis of sample A, contains a small amount of betaine and the addition of sugar to samples B and C is accompanied by increases in the betaine content of the wine of 2.1 and 4.9 mg/l, respectively. Hence, it is possible to determine the amount of added sugar in wine by analysing the corresponding commercial beet sugar used to increase the ethanol content of the wine.

CONCLUSION

The use of HPLC with amino-bonded silica as the stationary phase and water–acetonitrile as the mobile phase for the analysis of betaine in refined sugar and wine gave satisfactory precision and accuracy and the use of an interferometer as a detector could allow a considerable decrease in the detection level to a few nanograms of betaine.

The correlation between the betaine content in a particular wine and the corresponding amount of sugar added in order to increase the ethanol content is critical. Indeed, the betaine content of white beet sugar may widely vary[5] according to the year and the district of growth. Likewise, the betaine content of wine has given rise to controversy[14]. Thus, a knowledge of the betaine content can allow the determination of the amount of added sugar in a wine only if the amounts of betaine in the must on the one hand and in the sugar used to increase the ethanol content of the wine on the other are known.

The addition of sugar to wine is prohibited in some wine-growing areas, especially in France, but at present there is no means of measuring the amount added. In the absence of a rigorous method and without any blank allowing an exact determination, the method described here would allow fraudulent additions of sugar to wine to be disclosed when the betaine content in the wine is abnormally high. Obviously, many experiments would be necessary in order to determine precisely the betaine content in beet sugar and in wines according to the year and the growing area, and perhaps a statistical average content could be determined. Such a method could be a means of disclosing fraudulent additions of sugar in wine.

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