

Conclusions

In general, if standard compounds cannot be obtained, to identify a structure it is necessary to fractionate the mixture and obtain rather pure compounds for characterization by NMR, MS, IR, etc. Preparative separations of mixtures are difficult and time-consuming, so identification of structures is troublesome. It was not necessary to obtain pure compounds for LC-NMR. NMR spectra were obtained easily by LC-NMR and structural information derived by analysis of the data. In this study, we demonstrated that the two regio-isomers of commercial ethyldimethylpyrazine were directly identified using LC-NMR. 2D on-flow mode analysis provided ^1H -NMR data for each isomer clearly without the need for a general preparation method. Fraction loop mode analysis gave not only detailed ^1H -NMR data but also ^{13}C -NMR data indirectly by 2D HMQC and HMBC experiments. These data were useful for structure determinations. We believe that the use of LC-NMR for the rapid identification of constituents may be a useful tool for quality control of food additives.

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References

- Buchi G, Galindo J. 1991. Regioselective synthesis of alkylpyrazines. *Journal of Organic Chemistry* 56:2605–2606.
- Burdock GA, editor. 1997. *Encyclopedia of food and color additives*. Boca Raton, FL: CRC Press. pp 968–969.
- Goldman IM, Seibl J, Flament I, Gautschi F, Winter M, Willhalm B, Stoll M. 1967. Research on the aromas. On the aroma of coffee. II. Pyrazines and pyridines. *Helvetica Chimica Acta* 50:694–705.
- Iwasa K, Kuribayashi A, Sugiura M, Moriyasu M, Lee D-U, Wiegrebe W. 2003. LC-NMR and LC-MS analysis of 2,3,10,11-oxygenated protoberberine metabolites in *Corydalis* cell cultures. *Phytochemistry* 64:1229–1238.
- Ohta A, Ohta M, Lgarashi Y, Saeki K, Yuasa K, Mori T. 1987. Ethylation of pyrazines using alkylmetals, such as triethylaluminum, diethylzinc and triethylborane. *Heterocycles* 26:2449–2454.
- Smallcombe SH, Patt SL, Keifer PA. 1995. WET solvent suppression and its application to LC-NMR and high-resolution NMR spectroscopy. *Journal of Magnetic Resonance Series A* 117:295–303.
- Sohda K, Minematsu T, Hashimoto T, Suzumura K, Funatsu M, Suzuki K, Imai H, Usui T, Kamimura H. 2004. Application of LC-NMR for characterization of rat urinary metabolites of zanampanel monohydrate (YM872). *Chemical and Pharmaceutical Bulletin* 52:1322–1325.
- Waridel P, Wolfender J-L, Lachavanne J-B, Hostettmann K. 2004. *ent*-Labdane glycosides from the aquatic plant *Potamogeton lucens* and analytical evaluation of the lipophilic extract constituents of various *Potamogeton* species. *Phytochemistry* 65:945–954.
- Wolfender J-L, Ndjoko K, Hostettmann K. 1998. LC-NMR in natural products chemistry. *Current Organic Chemistry* 2:575–596.
- Wolfender J-L, Ndjoko K, Hostettmann K. 2001. The potential of LC-NMR phytochemical analysis. *Phytochemical Analysis* 12:2–22.

Note

Standard Infrared Absorption Spectrum of Betaine and Optimal Conditions for its Measurement

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The infrared absorption (IR) spectrum is often used as a standard reference in identification tests of food additives in Japan. In the case of betaine, many different IR spectra have been reported and, therefore, it is necessary to establish an IR spectrum that is reproducible and reliable enough to be used as a standard for identification. In the present study, suitable conditions to obtain a standard IR spectrum were examined from various viewpoints, including pretreatment, selection of method, and measuring technique. The KBr disk method, which has generally been used to identify betaine, was found to be humidity-dependent, and there was also an interaction between betaine and KBr. A reproducible IR spectrum suitable as a standard could be obtained by drying betaine at 105°C for 3 hours over phosphorus pentoxide, and then measuring the IR spectrum by the liquid paraffin (Nujol) paste method.

Key words: betaine; seasoning; infrared spectrum; reference spectrum; anhydride; hydrate; identification

Introduction

Betaine (Fig. 1) occurs widely in nature, especially in beets, and is used as a food additive for seasoning or as a flavor-improving agent in Japan. An identification-test for betaine is described in "Voluntary Specifications of Existing Food Additives: 3rd Ed"¹⁾, which suggests identification of betaine on the basis of the wave numbers of the absorption bands in an infrared absorption (IR) spectrum measured by the KBr method, as well as retention time in HPLC, and the like. However, the criteria for identification employed in the test are not comprehensive; for example, the defined wave numbers do not include that of the absorption band of the COO⁻ group, which is one of the characteristic bands of betaine.

Apart from their use as food additives, betaine and related compounds have attracted much interest in terms of the relationship between IR spectra and struc-

ture, specifically, the influence of hydrogen bonding²⁾⁻⁵⁾. IR spectra of betaine are available from databases, for example, "The Sigma Library of FT-IR Spectra"⁶⁾, "Spectral Database for Organic Compounds, SDBS"^{*1} of the National Institute of Advanced Industrial Science and Technology, Japan (AIST), and "WebBook"^{*2} of the National Institute of Standards and Technology, USA (NIST). However, these IR spectra are very different from each other and hardly appear to represent the same compound.

In this study, we examined the influence of pretreatment, measuring method, and measurement conditions on the IR spectrum of betaine in order to establish a method to obtain a reliable and reproducible IR spectrum of betaine, to serve as a standard for an identification test.

Materials and Methods

1. Samples and reagents

Betaines (products of Nippon Beet Sugar Manufacturing Co., Ltd., and Danisco Japan Co., Ltd. Tokyo, Japan)

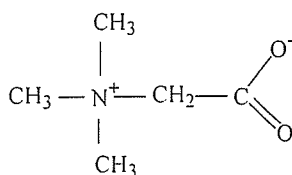


Fig. 1. Structure of betaine

^{*1} http://www.aist.go.jp/RIODB/SDBS/cgi-bin/direct_frame_top.cgi

^{*2} <http://webbook.nist.gov/cgi/cbook.cgi?ID=C590476&Units=SI&Mask=80>

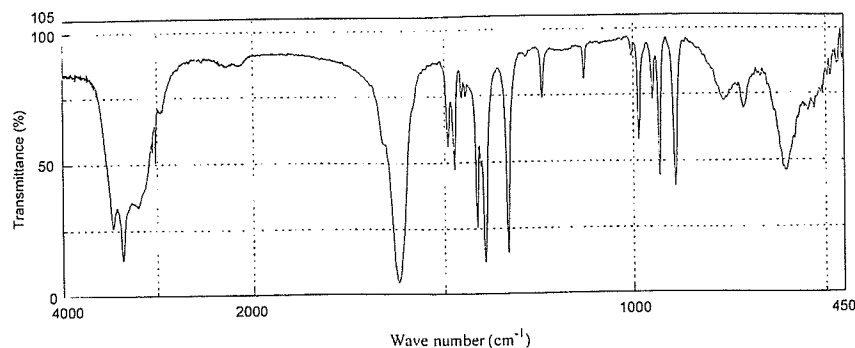


Fig. 2. IR spectrum of betaine dried over phosphorus pentoxide at 105°C for 3 hours (the KBr method)

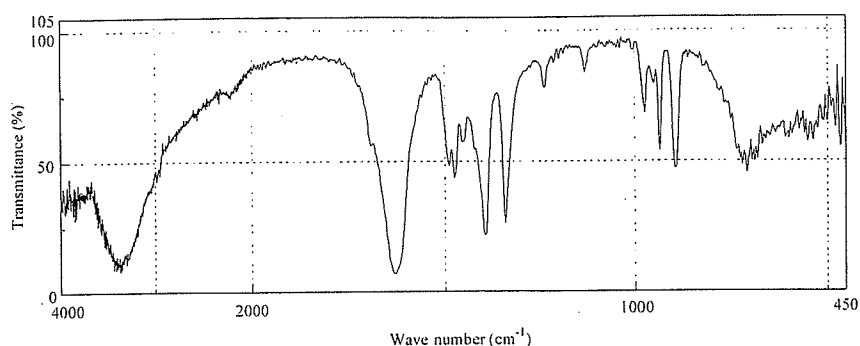


Fig. 3. IR spectrum of betaine dried over phosphorus pentoxide at 105°C for 3 hours (the KCl method)

were provided by Japan Food Additives Association (Tokyo, Japan) and used as received. Both betaines were in the form of white crystalline powders.

Liquid paraffin (Nujol[®]) and potassium bromide (KBr) for IR spectroscopy were purchased from Merck AG (Germany) and JASCO Co., Ltd. (Hachioji, Japan), respectively. Potassium chloride (KCl) for IR spectroscopy was from JASCO Co., Ltd. (Japan).

2. Measurements of IR spectra

Measurements were carried out by the KBr method and the paste method using liquid paraffin as described in the 7th Ed. of Japan's Specifications and Standards for Food Additives⁷⁾ for measuring a solid sample. A KBr disk without any sample and a KBr optical plate were used as references in the KBr method and the paste method, respectively. The resolution was about 4 cm^{-1} (32 or 64 scans). The Fourier transform (FT)-IR device used was an Impact 400 FT-infrared spectrophotometer (Nicolet Co., Madison, Wis., USA) which could nominally measure up to 400 cm^{-1} , though the practical limit was about 450 cm^{-1} . The measurement was conducted in a room used exclusively for IR spectral measurement, where the humidity and temperature were controlled to 30–40% and 23°C, respectively. KBr disks and pastes were usually prepared in the controlled room, but sometimes in an ordinary laboratory. Measurements using the KCl disk or paste method were carried out under the same conditions.

3. Recommended procedure for measurement of a standard IR spectrum of betaine

Betaine as a sample was dried over phosphorus pentoxide at 105°C for 3 hours. The IR spectrum was measured by using the paste method as described in the General Methods of the 7th Ed. of Japan's Specifications and Standards for Food Additives⁷⁾. As a reference, a KBr optical plate was used.

Results and Discussion

1. Examination by the disk method

In the measurement by the KBr disk method, commercial betaine was used after having been dried at 105°C for 3 hours according to the "Loss on Drying Test" in the Voluntary Specifications of Existing Food Additives: 3rd Ed.¹⁾ The dried betaine was first subjected to measurement by the KBr method according to the General Methods of the 7th Ed. of Japan's Specifications and Standards for Food Additives⁷⁾. The IR spectrum obtained is shown in Fig. 2. The IR spectrum agreed well with that described in SDBS, but differed from those described in The Sigma Library (the paste method), NIST (the paste method) and reference 2 cited above (the KBr method), suggesting the importance of moisture absorption during disk preparation or an interaction between betaine and KBr. To examine whether or not an interaction between betaine and KBr exists, a betaine disk was prepared using KCl, and the IR spectrum was measured. The result is shown in Fig. 3. It is clear from Figs. 2 and 3 that the spectrum of betaine in the KBr disk is quite different from that in the KCl disk throughout the entire region measured.

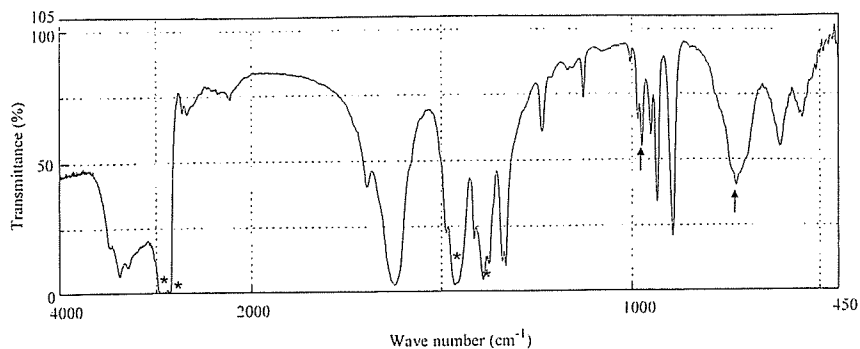


Fig. 4. IR spectrum of a 1 : 10 mixture of dried betaine and KBr (the paste method)
*Bands due to liquid paraffin.
↑ extra bands observed by the paste method.

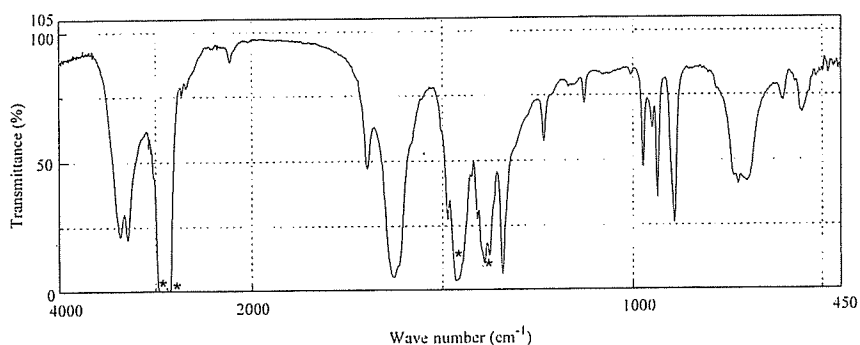


Fig. 5. IR spectrum of a 1 : 10 mixture of dried betaine and KCl (the paste method)
*Bands due to liquid paraffin.

Thus, there was an interaction between betaine and KBr during the procedures of mixing and grinding, or compressing, which resulted in a change of the IR spectrum. In the KBr method, it is well known that a sample may form a solid complex with KBr⁸⁾, or may interact with KBr through hydrogen bonding or ion-exchange^{9), 10)}. To elucidate the causes of the altered spectrum, the IR spectrum of betaine was measured by the paste method using a paste prepared by mixing betaine and alkali metal halide (KBr or KCl) at the mixing ratio of about 1 : 10 (betaine : alkali metal halide), and grinding in agate mortar. The measurement was carried out using a portion of the resultant paste, with a greater thickness than usual. The results are shown in Figs. 4 and 5.

The IR spectrum measured by the paste method in the presence of KBr (Fig. 4) and that of the KBr method (Fig. 2) are in good agreement in the region between 3,500 and 3,000 cm^{-1} , and almost in agreement in the region of 1,000–800 cm^{-1} although extra bands (marked with \uparrow) exist. From these results, it was presumed that the spectrum of betaine is easily changed even by very mixing and grinding betaine with KBr. When the KBr disk was prepared by very brief grinding, the IR spectrum of betaine was again different from that shown in Fig. 2.

It has been reported that the spectrum of betaine measured by the KBr method is influenced by the relative humidity in the measuring room²⁾. It has also been reported that the IR spectrum of betaine anhydride,

which was obtained by crystallization from ethanol, coincided with that of betaine hydrate when measurement was conducted by the KBr method under the condition of 12% or higher relative humidity²⁾. We measured the IR spectrum at a humidity level as low as possible (23°C, relative humidity 30%), but could not obtain the IR spectrum of betaine anhydride. The relative humidity varies greatly within Japan, and also among the cold districts (Hokkaido, North Europe, etc.) where betaine is mainly produced. Such regional differences in humidity may be linked to the variations observed in the IR spectra provided by the manufacturers in different districts, since test laboratories are not necessarily environmentally controlled. It was therefore considered that the KBr method does not necessarily give an IR spectrum with good reproducibility.

On the other hand, the IR spectrum measured by the paste method in the presence of KCl (Fig. 5) is in agreement with that measured by the KCl disk method (Fig. 3) in the region between 1,000 and 800 cm^{-1} . In addition, these IR spectra are almost in agreement with the IR spectrum of betaine hydrate measured by the paste method described below, and, also are similar to the IR spectrum provided by NIST. These facts indicate that the KCl method is preferable, and that betaine tends to absorb moisture to form a hydrate. Therefore, further investigation into the IR spectrum was conducted by the paste method, which was hardly affected by humidity.

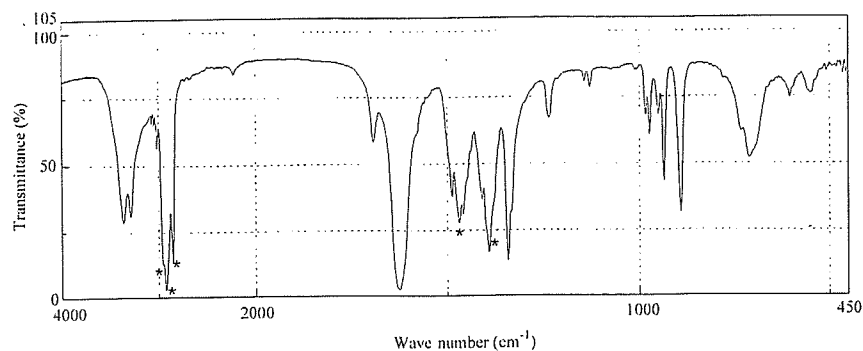


Fig. 6. IR spectrum of commercial betaine (the paste method)
*Bands due to liquid paraffin.

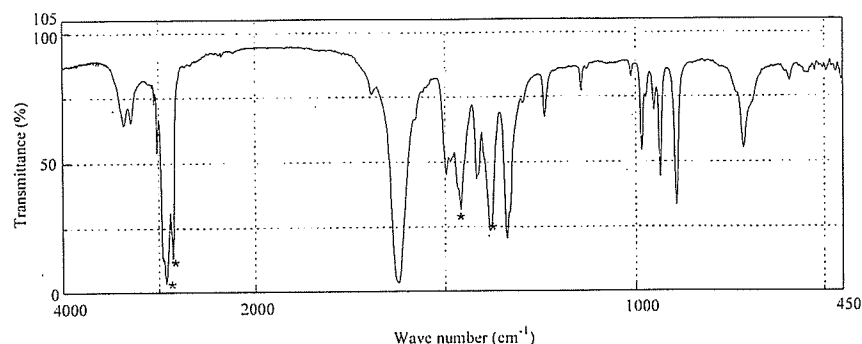


Fig. 7. IR spectrum of betaine dried over phosphorus pentoxide at 105°C for 3 hours (the paste method)
*Bands due to liquid paraffin.

2. Examination by the paste method

In the paste method, a target substance is coated with liquid paraffin when preparing a paste for measurement. Therefore, the IR spectrum should be little affected by humidity, even if the substance is hygroscopic. Figure 6 shows the IR spectrum of a betaine product (commercially available bulk powder) measured by the paste method without drying treatment. The high similarity of the IR spectrum in Fig. 6 to that of betaine hydrate in The Sigma Library⁶⁾ indicates that the bulk powder contained predominantly hydrate. Because the water content generally varies from one betaine product to another, the IR spectrum of a single product cannot serve as a standard IR spectrum. Another bulk powder also gave a similar IR spectrum to that shown in Fig. 6.

We then examined suitable drying conditions for obtaining the anhydride in a reproducible manner. Drying commercial betaine at 105°C for 3 hours did not affect the IR spectrum according to the General Method of "Loss on Drying Test" in the Voluntary Specifications of Existing Food Additives: 3rd Ed.¹⁾ Thus, drying was conducted over phosphorus pentoxide at 105°C for 3 hours. The IR spectrum of this dried betaine is shown in Fig. 7. The IR spectrum in Fig. 7 agreed with that of betaine anhydride provided by The Sigma Library⁶⁾. Further, the spectrum in Fig. 7 was in agreement with that of betaine anhydride measured by the KBr method²⁾, except that the former also contains absorption bands due to liquid paraffin.

When drying was conducted at different tempera-

tures, 120, 140, 170 or 200°C, over phosphorus pentoxide for three hours, the IR spectra of the dried betaine samples were identical. We, therefore, concluded that the IR spectrum shown in Fig. 7 could be available as a standard IR spectrum. Needless to say, when this spectrum is used as a standard in the identification of a betaine product, the spectra must be compared in the region other than that containing the absorption bands marked by "*", which are due to liquid paraffin.

As mentioned above, the IR spectra of betaine hydrate measured by the KBr and paste methods were different. On the other hand, the IR spectrum of betaine anhydride measured by the KBr disk method at low humidity (relative humidity; less than 12%)²⁾ was in agreement with that of betaine anhydride measured by the paste method. Accordingly, when using the IR spectrum measured by the KBr method in the identification test of betaine, it is necessary to confirm that the spectrum is identical to the spectrum measured by the paste method in order to ensure the reliability of the test results. The identification method based on the IR spectrum has various advantages, including energy saving, and wide applicability, and is a convenient alternative to chemical identification methods. It is therefore important to ensure good reproducibility of the spectrum.

Conclusion

We examined suitable conditions and methods to obtain a reliable and reproducible IR spectrum applica-

ble to identification of betaine as a standard spectrum. It was found that a reproducible spectrum can be obtained by drying betaine at 105°C for 3 hours over phosphorus pentoxide and then measuring the IR spectrum by the paste method. The IR spectrum thus obtained is suitable to be used as a standard reference IR spectrum.

Acknowledgments

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References

- 1) Japan Food Additives Association Ed. "Voluntary Specifications of Existing Food Additives: 3rd Ed.", Japan Food Additives Association, Tokyo, Japan (2002).
- 2) Viertorinne, M., Valkkonen, J., Pitkanen, I., Mäthlouthi, M., Nurmi, J., Crystal and molecular structure of anhydrous betaine, $(\text{CH}_3)_3\text{NCH}_2\text{CO}_2$. *J. Mol. Struct.*, **477**, 23-29 (1999).
- 3) Toccalino, P. L., Harmon, K. M., Harmon, J., Hydrogen bonding: Part 26. Thermodynamics of dissociation and infrared spectra-crystal structure correlations for betaine monohydrate and trimethylamine oxide dehydrate. *J. Mol. Struct.*, **189**, 373-382 (1988).
- 4) Dega-Szafran, Z., Jakólski, M., Szafran, M., OHO hydrogen bond and electrostatic interactions in a complex of pyridine betaine with phenylacetic acid studied by X-ray diffraction, FTIR spectroscopy and PM3, DFT calculations. *J. Mol. Struct.*, **555**, 191-201 (2000).
- 5) Baran, J., Barnes, A.J., Engelen, B., Panthöfer, M., Pietraszko, A., Ratajczak, H., Sledz, M., Structure and polarised IR and Raman spectra of the solid complex betaine-trichloroacetic acid. *J. Mol. Struct.*, **550-551**, 21-41 (2000).
- 6) Keller, R. J., "The Sigma Library of FT-IR Spectra 1st Ed.", Sigma Chemical Co., Inc. (1986).
- 7) Ministry of Health, Labour and Welfare Ed. of "7th Ed. Japan's Specifications and Standards for Food Additives" published by The Ministry of Health and Welfare of Japanese Government (1999). This book can also be viewed at <http://www.ffcr.or.jp/zaidan/FFCRHOME.nsf/pages/spec.stand.fa>
- 8) Ketelaar, J. A. A., Hass, C., van der Elsen, J., Infrared absorption spectra of bifluorides in alkali halide disks. The formation of mixed crystals. *J. Chem. Phys.*, **24**, 624-627 (1956).
- 9) Pliskin, W.A., Eischens, R.P., Effect of using the pressed salt technique to obtain the spectrum of chemisorbed ammonia. *J. Phys. Chem.*, **59**, 1,156-1,159 (1955).
- 10) Famer, V. C., Effects of grinding during the preparation of alkali-halide disks on the infra-red spectra of hydroxylic compounds. *Spectrochim. Acta*, **8**, 374-389 (1957), see also Text Book for the IR spectroscopy, for example Jikken-Kagaku-Kouza Zoku Vol.10 "Infrared Spectra" in Japanese, Maruzen, Tokyo (1964).