Note

Identification of New Constituents of Cider Vinegar by ^{13}C -NMR Spectroscopic Study

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Recent developments in NMR spectroscopy allow us to obtain both qualitative and semi-quantitative structural information on multicomponent solutions. Our research interests have been focused on application of ^{13}C -NMR spectroscopy to extracts of tea^{1–3)} and fresh flush of plants.⁴⁾ We have already reported the isolation and identification of a new inositol glycoside, 2-*O*-(β -L-arabinopyranosyl)-*myo*-inositol, amounting about 0.8% of made tea²⁾ and a new 3-octulose, D-glucio-L-glycero-3-octulose, as a main constituent of *Laurus nobilis* flush.⁴⁾

In this paper we would like to describe the identification of new major chemical constituents of cider vinegar by application of ^{13}C -NMR spectroscopy.

Vinegar samples were analyzed by ^{13}C -NMR spectroscopy without any pretreatment or purification. The spectral features varied with the materials and manufacturing processes of the samples. In the course of complete signal assignment in the ^{13}C -NMR spectrum of each sample, we found several signals unassignable to any known constituents. Figure 1 shows the spectra of the cider vinegar samples (I–IV). Most of the signals were assigned to the major known components,⁵⁾ acetic and lactic acids, sorbitol, glycerol, ethanol, etc. by direct comparison with the spectra of authentic specimens. The signals with star marks, however, could not be assigned to any known vinegar components. In order to identify the unknown constituents, they were isolated guided by these characteristic signals.

A cider vinegar sample (IV, 2.7 liters) was extracted with 2.5 liters of ethyl acetate. The signals in question remained in the spectrum of the residual aqueous phase, which was concentrated and put on an activated carbon column (H_2O). The column was eluted with H_2O and then acetone- H_2O =0:1–1:0 with a linear gradient, and nine fractions were collected according to the TLC analysis (silica gel GF₂₅₄, EtOAc-MeOH-AcOH- H_2O =12:3:3:2). Further fractionation on a Toyopearl HW-40C (H_2O) column yielded two fractions (Frs. C and

Fig. 1. ^{13}C -NMR Spectra of Cider Vinegar Samples (I–IV), Authentic 1,3-Propanediol and Fr. D-6.

Spectra were measured in 0.5% D_2O solution containing 1% *t*-butanol as an internal standard on a JEOL FX-90Q FT-NMR spectrometer using a 10-mm cell under the following conditions: 22.5 MHz; spectral width 5000 Hz; 45° pulse; pulse repetition time 1 sec. Assignment of the signals due to known constituents was done as described previously.³⁾

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Fig. 2. ^{13}C -NMR Spectra of Authentic Mannitol, Fr. C2-A and a Mixture of Them.

Spectra were measured in *ca.* 0.5% D_2O solution on a JEOL JNM-GX400 FT-NMR spectrometer using a 5-mm cell (100 MHz; 45° pulse; acquisition time 0.682 sec; pulse delay 0.8 sec).

D) which contained the constituents showing the unassignable signals.

Fraction C was further chromatographed on a DEAE-Sephadex column (H_2O) to yield a fraction (Fr. C2-A) showing the signals in question, δ 66.0 (t), 72.1 (d), and 73.7 (d), in the ^{13}C -NMR spectra, which were assigned to mannitol by comparison of the spectra before and after addition of authentic one (Fig. 2).

Fraction D contained the other constituents showing unidentified signals. Repeated purification of Fr. D by Toyopearl HW-40SF column (H_2O) chromatography yielded a fraction (Fr. D-6) that showed a single spot on the TLC. This fraction gave six carbon signals at δ 19.3 (q), 20.3 (q), 36.4* (t), 61.2* (t), 73.5 (d), and 73.9 (d) as shown in Fig. 1. These signals with asterisks and others were ascribed to 1,3-propanediol (**1**) and 2,3-butanediol (**2**), respectively, by comparison with those of the authentic specimens.

p-Nitrobenzoylation of the mixture by the conventional method, followed by silica gel column chromatography (benzene-chloroform) gave *p*-nitrobenzoates, **3** [^1H -NMR (CDCl_3 , 400 MHz) δ 2.33 (2H, dt, 6.2, 6.2), 4.57 (4H, t, 6.2), 8.21 and 8.29 (4H each, d, 8.8)] and **4** [^1H -NMR (CDCl_3 , 400 MHz) δ 1.46–1.50 (6H, m), 5.45 (2H, m), 8.16–8.30 (8H, m)], which were identified with di-*p*-nitrobenzoyl derivatives of **1** and **2**, respectively.

The ^1H -NMR analysis showed that **4** was a dia-

stereomeric mixture, because the methyl and aromatic proton signals did not show clear couplings. The ratio of *meso*- versus DL-diastereomeric composition of **4** was calculated to be about 1:1 by HPLC analysis [silica gel (YMC SIL, S-5), hexane-tetrachloromethane (1:1, v/v); detection at 272 nm]. The di-*p*-nitrobenzoyl derivative of authentic 2,3-L-butanediol and the DL-mixture isolated from **4** showed $[\alpha]_D^{27} + 57.6^\circ$ ($c=1.0$, CHCl_3) and $[\alpha]_D^{29} - 45.4^\circ$ ($c=1.0$, CHCl_3), respectively. The enantiomeric excess and ratio of D- versus L- of **4** were deduced to be 80 and 9:1, respectively.

Although 2,3-butanediol has only been reported as an aroma constituent of vinegar,⁶⁾ we found that a diastereomeric mixture of 2,3-butanediol is contained about at 0.1% in the cider vinegar sample and is one of the main constituents.

Microbial production of D(-)-2,3-butanediol by *Bacillus* sp. is well known. Quite recently GLC analysis of chloroform extracts of wine using chiral stationary phase found that the L(+)-isomer is present in very small amounts besides *meso*- and D(-)-isomers in normal wines.⁷⁾ Our experimental results also showed that D(-)- and *meso*-isomers are much more abundant than L(+)-isomer (*ca.* 10:9:1) in the cider vinegar.

By this ^{13}C -NMR study, 1,3-propanediol was for the first time found to be present *ca.* 0.02–0.15% in **4** foreign cider vinegar samples analyzed this time.

Recently glycerol was found to be converted to 1,3-propanediol by *Klebsiella pneumoniae* and other *Clostridium* species.⁸⁾ Later on many other bacteria, were also found to produce 1,3-propanediol.^{9–11)} The 1,3-propanediol in the cider vinegars were, therefore, considered to be produced by the presence of any of these bacteria.

This new application of ^{13}C -NMR spectroscopic analysis suggests its usefulness for not only the classification and quality evaluation of vinegar but also for identifying their unknown constituents.

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