Note

Identification of 10-Hydroxy-12Z-octadecenoic and 10-Hydroxy-12Z,15Z-octadecadienoic Acids in Corn Silage[†]

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In our previous papers, the analyses of corn silage were reported in comparison with components of chopped corn before stuffing in the silo, the volatile and non-volatile parts obtained by steam distillation being analyzed.¹⁻⁶⁾ In the present study, components were surveyed by a solvent extraction method, in order to further clarify the changes taking place during fermentation. This paper deals with the isolation and elucidation of the structure of three fatty acids, their methyl esters and two 10-hydroxyalkenoic acids contained in an *n*-pentane soluble fraction from water containing a methanolic extract of corn silage.

A 5 kg corn (Newdent 105 days; medium maturing variety) silage sample was obtained from a silo at the Experimental Farm of the College of Dairying on February 15, 1982. The sample was extracted with 80% MeOH (18 liters) for 1 month and 70% MeOH (13 liters) for 5 days at room temperature. The filtrates were concentrated under reduced pressure to 1400 ml and successively extracted with *n*-pentane (500 ml × 5), ether (600 ml × 4), ethyl acetate (700 ml × 4) and 1-butanol (600 ml × 4). The *n*-pentane fraction (5.87 g) was a deep green liquid.

Five grams of the fraction were chromatographed over silica gel (Wako gel C-200, 200 g) using solvent systems of *n*-hexane-benzene (1:1, 2200 ml), benzene (2000 ml) 1% acetone in benzene (3000 ml), 2% acetone in benzene (1400 ml), 3% acetone in benzene (1600 ml) and 5% acetone in benzene (3000 ml). Fractions (Fr., 200 ml each) were collected and monitored by TLC using silica gel plates (No. 5715, Merck). Spots were made visible by

spraying with 10% H₂SO₄ and charring for several minutes. Fr. 4~10 (1.24 g) eluted with *n*-hexane-benzene (1:1) gave a pale yellow oil, which showed a single spot on TLC at *R*_f 0.48 with a benzene-*n*-hexane (1:1) solvent system and was designated as P-1. Fr. 9 and 10 of benzene and Fr. 1~5 of 1% acetone in benzene (1.24 g) gave a pale yellow oily solid, which showed a single spot at *R*_f 0.40 (benzene-acetone = 10:1) and was designated as P-2. Fr. 6~15 (0.33 g) of 5% acetone in benzene gave a colorless oily solid, which showed a single spot at *R*_f 0.34 (benzene-acetone = 5:1) and was designated as P-3.

The IR spectrum of P-1 showed a characteristic ester band at 1740 cm⁻¹. GLC analysis on a column of 1.5% Silicone OV-17 (2 m) at 240°C revealed that P-1 consisted of three compounds. Their MS spectra gave M⁺ at *m/z* 270, 294 and 292, and M⁺ - OCH₃ fragments at *m/z* 239, 263 and 261, respectively. The base peaks were observed at *m/z* 74, 67 and 79, respectively. The results of IR and MS spectral analyses indicated that P-1 comprised a mixture of methyl palmitate, methyl octadecenoate and methyl octadecadienoate, the relative concentration of these three compounds being 21.6%, 41.5% and 29.9%, respectively. Retention times of these compounds coincided with those of authentic methyl palmitate, methyl linoleate and methyl linolenate. P-1 showed a dark brown spot on HPTLC plate RP-8 F₂₅₄ s at *R*_f 0.40 and 0.48 when the plate was developed with acetonitrile-methanol-water-formic acid (95:2:3:0.5). *R*_f values coincided with those of authentic methyl linoleate and methyl linolenate.

The methyl esters seem to have been formed as the result of an ester interchange reaction which took place during the extraction. When a small amount of corn silage was extracted with 1% acetone in benzene, these methyl esters were not detected.

The characteristic absorption bands of fatty acids at 1710 and 945 cm⁻¹ in the IR spectrum indicate that P-2 comprised free fatty acids. After methylation with diazo-methane using the standard procedure, the product was subjected to GLC on the same column already described. The product was separated into three peaks, with the *t*_R of each peak coinciding with those of the fatty acid methyl esters in P-1, and with the relative concentrations of the three esters at almost the same levels as those in P-1. P-2 showed a dark brown spot on HPTLC plate RP-8 F₂₅₄ s at *R*_f 0.52 and 0.60 when the plate was developed with the solvent already described. *R*_f values coincided with those of linoleic and linolenic acids. On the basis of these evidences, P-2 was confirmed to be a mixture of free palmitic, linoleic and linolenic acids.

P-3 was re-chromatographed on a silica gel column with 3% acetone in benzene (90 g) and 5% acetone in benzene (250 g). The fractions (each 3 g) were collected and monitored by TLC. The IR spectrum of Fr. 14~85 of 5%

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TABLE I. ^{13}C -NMR CHEMICAL SHIFT ASSIGNMENTS FOR METHYL 9,10-DIHYDROXY-12Z-OCTADECENOATE, METHYLATED COMPOUND A, METHYL 12-HYDROXY-9Z-OCTADECENOATE, METHYL 9,10-DIHYDROXY-12Z,15Z-OCTADECADIENOATE AND METHYLATED COMPOUND B (ppm)

Carbon	Me 9,10-Dihydroxy-12Z-octadecenoate*	Methylated Compound A	Me 12-Hydroxy-9Z-octadecenoate**	Me 9,10-Dihydroxy-12Z,15Z-octadecadienoate*	Methylated Compound B
1	173.1	173.9		174.3	173.9
2	33.8	34.1		34.1	34.0
3	24.8	24.9		24.9	24.9
4	29.8	29.6		29.5	29.5
5	29.1	29.2		29.1	29.0
6	29.0	29.2		29.1	29.0
7	25.5	25.7		25.7	25.6
8	33.6	29.4	27.4	33.6	29.3
9	73.9	36.8	125.5	73.9	36.8
10	73.9	71.4	133.0	73.8	71.3
11	31.7	35.3	35.5	31.7	35.3
12	133.6	133.2	71.6	131.1 ^a	131.1 ^a
13	124.6	124.9	37.0	125.3 ^b	125.2 ^b
14	27.4	27.4	25.8	25.7	25.6
15	29.3	29.4		126.8 ^b	126.6 ^b
16	31.5	31.5		132.2 ^a	131.9 ^a
17	22.5	22.5		20.6	20.5
18	14.1	14.0		14.3	14.2
OCH ₃		51.3			51.3

The spectra were taken in CDCl_3 with TMS as an internal reference.

^{a,b} Assignments may be reversed within each column.

* Methylate of the self-defensive substance in rice plant against rice blast disease.¹⁰⁾

** Methyl ricinolate.¹¹⁾

FIG. 1. The Structures of Compounds A and B.

acetone in benzene (0.15 g) showed characteristic bands of fatty acids at 1710 and 1216 cm^{-1} . After methylation with diazomethane, the product was subjected to GLC on the same column already described at 270°C. The product gave two peaks with relative concentrations of 55.8% and 44.2%. The IR spectrum showed characteristic absorption bands of hydroxy acid methyl esters ($\nu_{\text{max}}^{\text{CCl}_4}$: 3460 and 1740). The product showed a dark brown spot on HPTLC plate RP-8 F_{254} at R_f 0.35 and 0.43 when the plate was developed with methanol–water (9:1) and visualized already described. The product was chromatographed on a Lober column RP-8, size B (Merck) with methanol–water (9:1, 160 g). The fractions (each 2 g) were collected and monitored by HPTLC. Fr. 51~56 (21 mg)

were designated as methylated Compound B and Fr. 68~76 (34 mg) as methylated Compound A.

The EI-MS spectrum of methylated Compound A showed fragments at m/z 294 ($\text{M}^+ - \text{H}_2\text{O}$), 201 ($\text{CH}_3\text{OC}(\text{CH}_2)_8\text{CH}\cdot$) and 169 ($\text{O}=\text{C}=\text{CH}(\text{CH}_2)_7\text{CH}\cdot$)

formed by C–C cleavage after the hydroxy carbon with simultaneous loss of the elements of methanol⁷⁾ and 112 ($\text{CH}_3(\text{CH}_2)_4\text{CH}=\text{CHCH}_2 + \text{H}^+$).⁸⁾ The EI-MS spectrum gave fragments at m/z 313 ($\text{M}^+ + 1$), 295 ($\text{M}^+ - 17$) and 201. The EI-MS spectrum of the TMS derivative gave a base peak at m/z 273 ($\text{CH}_3\text{OC}(\text{CH}_2)_8\text{CH}\cdot$). The ^1H -NMR (100 MHz) spectrum gave signals at $\delta_{\text{TMS}}^{\text{CDCl}_3}$ 5.4 (2H, m,

$-\text{CH}=\text{CH}-$), 3.64 (3H, s, $-\text{COOCH}_3$), 2.28 (6H, $-\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_2-$ and $-\text{CH}_2-\text{COOCH}_3$), 1.30 (br.s, chain $-\text{CH}_2$) and 0.88 (3H, t, $-\text{CH}_2-\text{CH}_3$). The ^1H -NMR spectrum of Compound A gave a signal at $\delta_{\text{TMS}}^{\text{CDCl}_3}$ 3.6 (1H, br, $-\text{CH}-\text{OH}$).⁹⁾ The ^{13}C -NMR spectra are shown in Table I. Assignments were accomplished by comparing with the chemical shifts of synthetic methyl 9,10-dihydroxy-12Z-octadecenoate¹⁰⁾ and methyl 12-hydroxy-9Z-octadecenoate.¹¹⁾ The geometry of the double bond was determined by the chemical shifts of C-14 and C-11 (carbon between a double bond and a hydroxy group).¹¹⁾ On the basis of these evidences Compound A was identified as 10-hydroxy-12Z-octadecenoic acid (Fig. 1), which was optically active showing $[\alpha]_D^{24} = -1^\circ$ ($c = 1.0$, MeOH).

The MS spectrum of methylated Compound B showed fragments at m/z 201 and 169 in common with methylated Compound A, 292 ($\text{M}^+ - \text{H}_2\text{O}$) and 110 ($\text{CH}_3\text{CH}_2\text{CH}=\text{CHCH}_2\text{CH}=\text{CHCH}_2 + \text{H}^+$). The MS spectrum of the TMS derivative gave a base peak at 273, as it did in the case of methylated Compound A. The ^1H -NMR (100 MHz) spectrum gave signals at $\delta_{\text{TMS}}^{\text{CDCl}_3}$ 5.35 (4H, m, $2-\text{CH}=\text{CH}-$), 3.64 (3H, s, $-\text{COOCH}_3$), 2.76 (2H, t, $=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}-$), 2.28 (6H, $2-\text{CH}_2-\text{CH}=\text{CH}-$ and $-\text{CH}_2-\text{COOCH}_3$), 1.28 (br.s, chain $-\text{CH}_2$) and 0.94 (3H, t, $-\text{CH}_2-\text{CH}_3$). The ^{13}C -NMR spectra are shown in Table I. Assignments were accomplished by comparing with the chemical shifts of synthetic methyl 9,10-dihydroxy-12Z,15Z-octadecadienoate.¹⁰⁾ On the basis of these evidences, Compound B was identified as 10-hydroxy-12Z,15Z-octadecadienoic acid (Fig. 1).

These two 10-hydroxyalkenoic acids were prepared for the first time by anaerobic microbiological hydration of the 9Z double bond) when linoleic and linolenic acids were employed for substrates.¹²⁾ Recently, 10-hydroxy-12-octadecenoic acid has been found in human adipocere and linoleic acid was postulated as the substrate.⁸⁾ One of the authors has confirmed that in raw corn, these hydroxyalkenoic acids were not detected and a large amount of linoleic and linolenic acids occurred with palmitic acid.

In view of these observations, it is considered that 10-hydroxy-12Z-octadecenoic and 10-hydroxy-12Z,15Z-octadecadienoic acids are produced from linoleic and linolenic acids by anaerobic microbiological hydration during silage fermentation. Lactic acid bacteria may be concerned with that transformation.

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