Direct GC Analysis of the Fatty Acid Compositions of Conjugated Linoleic Acid and Its \( \text{\textit{l}} \)-Menthyl Esters

Takashi Kobayashi\(^1\), Toshihiro Nagao\(^2\), Yomi Watanabe\(^2\) and Yuji Shimada\(^3\)

\(^1\)Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University (Oiwake-cho, Kitashirakawa, Sakyo-ku, Kyoto 606-8502, JAPAN)

\(^2\)Osaka Municipal Technical Research Institute (1-6-50 Morinomiya, Joto-ku, Osaka 536-8553, JAPAN)

\(^3\)Okamura Oil Mill, Ltd. (4-5 Kawahara-cho, Kashiwara, Osaka 582-0004, JAPAN)

Abstract: Commercially available conjugated linoleic acid (CLA) is a mixture of two main isomers. Fractionation of the two isomers was performed by a lipase-catalyzed esterification of CLA with \( \text{\textit{l}} \)-menthol. In this study, a GC analytical method was developed to simultaneously determine the degree of esterification and fatty acid (FA) compositions of CLA in the free fatty acid (FFA) and ester forms without separation of the FFA and the ester. The methylation of the oil phase of the reaction mixture was performed using trimethylsilyldiazomethane in a mixture of toluene/methanol. Only FFA was quantitatively methylated, whereas the other compounds were little changed. A GC analysis using a polar column was performed to simultaneously determine the degree of esterification and the fatty acid compositions.

Key words: CLA, direct GC analysis, lipase-catalyzed esterification, \( \text{\textit{l}} \)-menthyl ester, methylation

1 INTRODUCTION

Commercially available conjugated linoleic acid (CLA) mainly contains two structural isomers (\(9\)-\textit{cis},\(11\)-\textit{trans} and \(10\)-\textit{trans},\(12\)-\textit{cis}-CLA). It has been reported that each isomer has different physiological activities such as anticancer activity\(^1,2\) or an increase in energy expenditure\(^3\). To develop nutraceuticals containing the two CLA isomers at arbitrary contents, a fractionation process for the CLA isomers is needed. Lipase from \textit{Candida rugosa} (CRL) has high substrate specificity toward \(9\)-\textit{cis},\(11\)-\textit{trans}-CLA. By using this specificity, the fractionation of the two CLA isomers has been performed by esterification of CLA with lauryl alcohol\(^4,5\) or \(\text{\textit{l}}\)-menthol\(^6\).

For the fractionation processes, it is necessary to determine the degree of esterification and the fractionation efficiency (the ratio of one isomer versus the sum of the two isomers). Typically, they are separately determined by different procedures. To determine the efficiency, various steps are required, e.g., the separation of the oil phase of the reaction mixture into its ester and free fatty acid (FFA) fractions by hexane extraction followed by methylation of the FFA and ester, and, then, GC analysis. The degree of esterification can be determined by alkali titration of FFA in the reaction mixture. In this study, to simplify these procedures, we developed a one-step process to simultaneously determine the fractionation efficiency along with the degree of esterification by GC for the lipase-catalyzed esterification of CLA with \(\text{\textit{l}}\)-menthol.

2 EXPERIMENTAL PROCEDURES

2.1 Materials

CLA in the FFA form was a kind gift from Nisshin OilliO Group Co., Ltd. (Tokyo, Japan), the contents of which were 33% \(9\)-\textit{cis},\(11\)-\textit{trans}-CLA, 34% \(10\)-\textit{trans},\(12\)-\textit{cis}-CLA, 4.9% other CLAs, and 28% other fatty acids (FA). \(\text{\textit{l}}\)-Menthol, lauryl alcohol, and oleic acid were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). The lipase from \textit{Candida rugosa} (lipase-OF; CRL) was a gift from Meito Sangyo (Aichi, Japan). The CLA lauryl ester (CLA-Lau) was synthesized based on a previously reported method\(^4\), where saturated fatty acids, especially C18:0, were esterified only slightly because of the low substrate specificity toward them, and the CLA-Lau product contained only small amounts of C16:0 and C18:0.
2.2 Reactions and analysis

The CLA 1-menthyl ester (CLA-Men) was synthesized at 30°C by mixing CLA in the FFA form (28 g), 1-menthol (15.6 g), and an aqueous solution of CRL (11 mL, 200 or 3000 U/g-reaction mixture) for 40 h. The fractionation efficiency and degree of esterification were simultaneously determined by GC. Prior to the GC analysis, the reaction mixture was centrifuged at 8000 × g for 1 min to separate it into oil and aqueous phases. FFA in the oil phase then underwent methylation without separation of the oil phase into the FFA and ester fractions; about 5 mg of the oil phase were dissolved in 1 mL of toluene/methanol (3:1, v/v). To this solution, 50 μL of a trimethylsilyldiazomethane diethyl ether solution (2 mol/L; Aldrich, St. Louis, MO) were added, and then the mixture was stored for 1 min at room temperature. After methylation, the sample solution was analyzed with GC (6890N, Agilent Technologies, Santa Clara, CA, USA) connected to a DB-23 column (0.25 mm × 30 m; Agilent Technologies) containing 50% (cyanopropyl) methylpolysiloxane; this has been widely used for the analysis of fatty acid methyl esters (FAME). The column temperature was raised as follows: 150 to 200°C at 10°C/min, 200 to 210°C at 2°C/min, 210 to 230°C at 15°C/min, and, then, maintained at 230°C for 10 min. The temperatures of the injector and flame ionization detector (FID) were 245 and 250°C, respectively.

Because the sensitivities of 1-menthol, FAME, and CLA-Men toward FID were different, the correction factors for estimating the degree of esterification were subsequently determined. To determine these factors, the GC analysis was performed using different mixtures of 1-menthol, FAME, and CLA-Men at given compositions, in which the sensitivities of the various FAs were assumed to be the same.

The same analytical procedure was applied to the hydrolysis of CLA-Lau. One gram of CLA-Lau was hydrolyzed with 0.5 mL of water containing 9000 U of CRL at 30°C for 5 d. After the reaction, the reaction mixture was separated into the oil and aqueous phases, followed by methylation. The GC conditions for the reaction mixture after methylation were as follows: 150 to 200°C at 3°C/min, 200 to 240°C at 10°C/min, and, then, maintained at 240°C for 15 min.

3 RESULTS AND DISCUSSION

The GC chromatogram for CLA-Men after methylation contained groups of peaks (1-menthol, FAME, and CLA-Men), as shown in Fig. 1A. The components eluted are 1-menthol, the FAME group, and the CLA-Men group, with good separation. The FAME group in the chromatogram was considered to correspond to the methylated derivatives of FFA. The nearly complete separation of CLA in each group was also achieved, and isomerization of CLA did not occur. The order of elution for each group depended on the vapor pressure of the compounds, indicating that even a DB-23 column has the capability to separate each compound by the difference in vapor pressure. It can be considered that a chromatogram of the oil phase obtained using a nonpolar capillary column (e.g., 100% dimethylpolysiloxane column) gives three peaks rather than three groups of peaks. These facts show that a DB-23 column has the potential to recognize not only FAME but also CLA-Men.

The areas of each peak group obtained by GC with a DB-23 column were summed up. The summed value was then multiplied by a correction factor to estimate the degree of esterification. The degree of esterification (47.1 ± 0.4%, relative standard deviation (RSD) = 0.8%, n = 5) corresponded well with that obtained by alkali titration (46.0 ± 1.1%, RSD = 2.4%, n = 5) when a dose of 200 U/g of lipase was used. In addition, when a dose of 3000 U/g of lipase was used, the reaction reached equilibrium; the results at equilibrium based on the GC analysis and alkali titration were also quite consistent (78.5 ± 0.9%, RSD = 0.8%).
1.1% by GC, \( n = 5 \); 77.3 \( \pm \) 2.1%, RSD = 2.7% by titration, \( n = 5 \). As described above, consistent results could be obtained, indicating that only FFA is converted to FAME, whereas \( \pi \)-menthol and CLA-Men are little changed. Therefore, this method can be applied to estimate the degree of esterification in the synthesis of CLA-Men.

The order of elution of FAs in FAME was C16:0, C18:0, C18:1, 9-cis,11-trans-CLA, 10-trans,12-cis-CLA, and other CLAs, as shown in the FAME group in Fig. 1A. The FA composition of FAME was as follows: 12% 9-cis,11-trans-CLA, 62% 10-trans,12-cis-CLA, 9.1% other CLAs, and 17% other FAs (fractionation efficiency: 10-trans,12-cis/ (9-cis,11-trans + 10-trans,12-cis) \( \times 100 = 84\% \)). C18:0 was esterified only slightly because of the low substrate specificity, and these results were almost identical to those obtained by the conventional method\(^*\). The order of elution of FAs in the CLA-Men group was also the same as that in the FAME group. Furthermore, the FA composition of CLA-Men could be quantified as follows: 67% 9-cis,11-trans-CLA, 8.9% 10-trans,12-cis-CLA, 14% other CLAs, and 23% other FAs (fractionation efficiency: 9-cis,11-trans/(9-cis,11-trans + 10-trans,12-cis) \( \times 100 = 88\% \)).

As already mentioned, the order of elution of FAs for FAME and CLA-Men was the same. This may be attributed to the fact that a DB-23 column recognizes the acyl residue but not the alcohol residue in this case. A DB-23 column has a cyano group in its stationary phase and \( \pi \) electrons in the functional group. The \( \pi \) electrons in the FA residues of FAME or CLA-Men interact well with those in the stationary phase and significantly contribute to the rise in separation efficiency. The alcohol residue does not contain double bonds and has the role of accommodating the difference in molecular weights between FAME and CLA-Men. The difference in the molecular weight causes the difference in the vapor pressures of the two esters. An efficient separation can be realized by these two factors. Based on these results, the separation of FFA and ester fractions can be achieved, i.e., the degree of esterification and the composition of the FA isomers can be simultaneously determined by GC.

To generalize the above results, the analysis of a CLA ester containing a different alcohol residue is now discussed. In this study, CLA-Lau was used as the model compound. Figure 1B shows the chromatogram obtained after methylation of FFA in the reaction mixture for the lipase-catalyzed hydrolysis of CLA-Lau. Lauryl alcohol was first eluted, followed by the FAME and CLA-Lau groups. The order of elution of the peak groups also follows the vapor pressure of the compounds. However, the column temperature must be maintained higher than that of the analysis of CLA-Men, and a long analysis period was required. The degree of hydrolysis and the FA composition of CLA-Lau could also be determined; the degree of hydrolysis at equilibrium was 12.3 \( \pm \) 0.3%, RSD = 2.4%, \( n = 5 \); the FA composition of CLA-Lau was 41% 9-cis,11-trans-CLA, 42% 10-trans,12-cis-CLA, 4.6% other CLAs, and 12% other FAs. These results agreed well with the results obtained by the conventional method. Even for the analysis of CLA-Lau, the difference in the vapor pressure and interaction of the \( \pi \) electrons between the acyl residue and the stationary phase both play an important role in the GC separation.

We have discussed the analyses of the CLA esters. The application of the GC method was also investigated in the analyses of other FA esters. The syntheses of oleic acid alkyl esters (alkyl chain length: 3 to 10) from FFA and alcohol were used as models, where oleic acid contained small amounts of other FFAs as impurities. A good separation could be achieved in some cases (Table 1). When an alcohol having five or more carbons was used, the impurities in oleic acid could also be observed. The order of elution was alcohol, the FAME group, and the group of corresponding alkyl esters. The separation was more favorable when using a longer-chain alcohol because the differences in vapor pressures between each component increased. However, when a short-chain alcohol (1-propanol, 2-propanol, or 1-butanol) was used, the peak groups of FAME and the alkyl esters approached each other. As a result, the separation was poor, and good separation of impurities could not be achieved. These results show that the GC analytical method can be effectively used during the synthesis of FA alkyl esters when the alkyl chain length of the alcohol

Table 1 Retention times for the various alkyl esters in GC analysis.

<table>
<thead>
<tr>
<th>FA</th>
<th>Alcohol</th>
<th>RT&lt;sup&gt;a&lt;/sup&gt; of alcohol (min)</th>
<th>RT&lt;sup&gt;a&lt;/sup&gt; of OAME&lt;sup&gt;b&lt;/sup&gt; (min)</th>
<th>RT&lt;sup&gt;c&lt;/sup&gt; of OAEE&lt;sup&gt;d&lt;/sup&gt; (min)</th>
<th>GC condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>C18:1</td>
<td>1-Propanol</td>
<td>2.74</td>
<td>13.4</td>
<td>14.5</td>
<td>d</td>
</tr>
<tr>
<td>C18:1</td>
<td>2-Propanol</td>
<td>2.62</td>
<td>13.4</td>
<td>13.7</td>
<td>d</td>
</tr>
<tr>
<td>C18:1</td>
<td>1-Butanol</td>
<td>2.42</td>
<td>9.70</td>
<td>10.7</td>
<td>e</td>
</tr>
<tr>
<td>C18:1</td>
<td>1-Pentanol</td>
<td>2.92</td>
<td>9.70</td>
<td>11.2</td>
<td>e</td>
</tr>
<tr>
<td>C18:1</td>
<td>1-Hexanol</td>
<td>2.62</td>
<td>9.57</td>
<td>12.3</td>
<td>f</td>
</tr>
<tr>
<td>C18:1</td>
<td>Cyclohexanol</td>
<td>2.96</td>
<td>9.57</td>
<td>14.1</td>
<td>f</td>
</tr>
<tr>
<td>C18:1</td>
<td>1-Octanol</td>
<td>2.45</td>
<td>5.77</td>
<td>9.18</td>
<td>g</td>
</tr>
<tr>
<td>C18:1</td>
<td>1-Decanol</td>
<td>2.89</td>
<td>5.53</td>
<td>10.7</td>
<td>h</td>
</tr>
</tbody>
</table>

<sup>a</sup> Retention time
<sup>b</sup> Oleic acid methyl ester.
<sup>c</sup> Oleic acid alkyl ester.
<sup>d</sup> 40 to 55°C at 5°C/min, 55 to 200°C at 20°C/min, 200 to 230°C at 5°C/min.
<sup>e</sup> 70 to 100°C at 10°C/min, 100 to 240°C at 25°C/min, then stored at 240°C for 5 min.
<sup>f</sup> 100 to 140°C at 10°C/min, 140 to 230°C at 20°C/min, then stored at 230°C for 10 min.
<sup>g</sup> 150 to 175°C at 10°C/min, 175 to 240°C at 30°C/min, then stored at 240°C for 6 min.
<sup>h</sup> 150 to 175°C at 10°C/min, 175 to 240°C at 30°C/min, then stored at 240°C for 9 min.
residue is greater than five.

In conclusion, this GC analytical method can contribute to the development of a high-performance analysis for the synthesis or hydrolysis of esters. Because the limiting temperature of the GC column is presently 250°C, the FA/alcohol combination is limited. This GC method will become more general when better heat-resistant columns become available.

References