Evaluation of Hydrophobicity for Fatty Acids Using Reversed-phase Thin Layer Chromatography

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Abstract: Hydrophobicity of ingredients is important for designing food, pharmaceuticals, and cosmetics. Here, we evaluated the hydrophobicity of fatty acids and showed the effect of the alkyl length and the unsaturation degree using reversed-phase thin layer chromatography (RP-TLC). A linear relation was obtained between the methanol concentration in a mobile phase and the \( R_m \) value. The linear regression analysis was achieved and the hydrophobicity value \( R_m \) was obtained using the robust regression (MM-estimator). The hydrophobicity of fatty acids depends on the structure of alkyl chain as follows: a longer alkyl chain in fatty acid increased the hydrophobicity. Additionally, the hydrophobicity increased as the number of unsaturated parts increased. Fatty acid with branched structures were less hydrophobic than that with straight chained structures.

Key words: hydrophobicity, fatty acid, reversed-phase thin layer chromatography

1 Introduction

Fatty acids are amphiphilic materials containing a hydrophobic alkyl chain and a hydrophilic carboxyl group in a molecule. Fatty acids and their derivatives are superior emulsifiers because they form viscoelastic interfacial film and inhibit the coalescence of liquid droplets\(^1\)\(^{-}\)\(^3\). Recently, researches have been conducted regarding physiological activities of some fatty acids such as bactericidal properties, immune activity, and inflammatory reaction\(^4\)\(^{-}\)\(^12\). Furthermore, fatty acids have been used as ingredients for food, pharmaceuticals, and cosmetics\(^13\). When fatty acids exhibit surface and physiological activities, a suitable balance of their hydrophilicity and hydrophobicity is required. One of the physicochemical parameters, which reflect this character, is the water–octanol partition coefficient (log \( P_{ow} \))\(^14\)\(^{-}\)\(^16\). Hansch \textit{et al.} proposed a quantitative structure-activity relationship using log \( P_{ow} \), which is referred if chemical substances are regulated by law or international standards\(^17\)\(^{-}\)\(^18\).

Generally, value of log \( P_{ow} \) is evaluated by the shake-flask method in which the amount of the substance in aqueous phase is evaluated after shaking the binary system containing oil and water phases\(^14\)\(^,\)\(^16\). Although partition coefficients of various substances can be evaluated directly in this method, the precise evaluation is difficult if the solubility of the substance is very low. Therefore, some alternative methods have been developed, such as computer simulation and high-performance liquid chromatography (HPLC) methods\(^18\)\(^{-}\)\(^22\). In particular, the method using reversed-phase thin layer chromatography (RP-TLC) is facile and cost effective. Some researchers have evaluated the hydrophobicity for many compounds\(^15\)\(^,\)\(^21\). In the case of RP-TLC, the silica gel particles are modified with a hydrophobic agent such as octadecyl silane. The moving distance of the compounds spotted on TLC plate is determined by the affinity of the substances between the mobile phase and the stationary phase (Fig. 1). This relationship enables the evaluation of the hydrophobicity of the substances by changing the concentration of the organic solvent in the mobile phase systematically. Pyka \textit{et al.} evaluated the hydrophobicity of saturated fatty acids from acetic acid (C2:0 fatty acid) to stearic acid (C18:0 fatty acid) using reversed-phase high-performance TLC\(^26\).

Here, we evaluate the hydrophobicity of fatty acids with alkyl chain length of C8 to C18 involving saturated, unsaturated and branched fatty acid using RP-TLC method to evaluate the effect of alkyl structure on the sample’s hydrophobicity (Fig. 2). In this evaluation, we improved the
composition of the mobile phase and fitting method. We selected aqueous methanol solution as the mobile phase because the organic solvent, whose dipole moment and hydrogen bonding ability is close to those of water, is suitable. In addition, linear regression was achieved by MM-estimator, which is a robust regression method to obtain reliable coefficients by weighing outliers.

2 Experimental Procedures

2.1 Materials

Figure 3 shows the chemical structures of the fatty acids used in this study. Caprylic acid (C8:0 fatty acid, ≥ 98%), capric acid (C10:0 fatty acid, ≥ 99%), lauric acid (C12:0 fatty acid, ≥ 99%), palmitic acid (C16:0 fatty acid, ≥ 99%), palmitoleic acid (C16:1 fatty acid, ≥ 99%), stearic acid (C18:0 fatty acid, ≥ 99%), oleic acid (C18:1 fatty acid, ≥ 99%), and chloroform (guaranteed reagent) were purchased from FUJIFILM Wako Pure Chemical Corporation (Miyazaki, Japan). Myristic acid (C14:0 fatty acid, ≥ 99%) was obtained from Sigma-Aldrich Co. LLC (St. Louis, USA). Linoleic acid (C18:2 fatty acid, ≥ 99%) and iso-stearic acid (iso-C18:0 fatty acid, ≥ 90%) were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Methanol (c.p. reagent) and ethanol (guaranteed reagent) were obtained from Kanto Chemical Co. Inc. (Kanagawa, Japan). 12-Molybdophosphoric acid (n-hydrate) was purchased from Kishida Chemical Co. Ltd. (Osaka, Japan).

2.2 Evaluation of Rf value by RP-TLC

Fatty acid chloroform solution (1 μL) was spotted with a microcapillary (Funakoshi Co., Tokyo, Japan) on the RP-TLC plate (size = 50 mm × 200 mm, TLC Silica gel 60RP-18F254 20 Aluminum sheets 20 × 20 cm Batch HX60378659, Merck Millipore, Darmstadt, Germany). The plate was set vertically in a two-layer chromatographic chamber (AS ONE Co., Osaka, Japan) in a cool incubator (AS ONE Co., Osaka, Japan) at 20 ± 0.3°C. A filter paper containing 30 mL of the mobile phase was placed on a wall of the chamber. In order to saturate by the atmosphere of the mobile phase, the chamber was left for 2 hours before developments. The RP-TLC plate was developed with the mobile phase when the concentration of methanol in the mobile phase was 55.0, 60.0, 65.0, 70.0, 72.5, 75.0, 77.5, 80.0, 82.5, 85.0, 87.5, 90.0, 95.0, and 100 v/v%. The plate was dried and dipped in an aqueous solution of phosphomolybdic acid containing 5 wt/vol% ethanol. The plate was then heated at 200°C to visualize the spots.
2.3 Analytical method

The $R_f$ value is defined by the equation (1) containing the migration distance of the mobile phase ($b$) and fatty acid on the RP-TLC plate ($a$) (Fig. 1):

$$R_f = \frac{a}{b}$$  \hspace{1cm} (1)

The $R_m$ value was then calculated by the equation (2):

$$R_m = \log\left(\frac{1}{R_f} - 1\right).$$  \hspace{1cm} (2)

A linear relationship was obtained between the $R_m$ value and the methanol concentration $\phi$ in the mobile phase. An intercept $R_{mw}$ was calculated from the linear regression analysis (MM estimation):

$$R_m = S\phi + R_{mw},$$  \hspace{1cm} (3)

where $S$ is the slope of linear regression depending on the kind of organic solvent in the mobile phase. Statistical software $R$ was used for MM estimation. The package robustbase was included in the CRAN package.

3 Results and Discussion

Figure 3 shows the image of RP-TLC plates when a C12:0 fatty acid was developed with the mobile phases containing methanol. C12:0 fatty acid formed a circular spot on the RP-TLC plate after the visualization with phosphomolybdic acid. The position of the spot increased with the methanol concentration in mobile phase. Figure 4 shows the values of $R_f$ and $R_m$ for the C12:0 fatty acid. The left and the right vertical axes are for $R_f$ and $R_m$ values, respectively. If the affinity to the stationary phase is stronger, the $R_f$ and $R_m$ values become smaller and larger, respectively. The $R_f$ value increases with the increase of the methanol concentration in the mobile phase. For example, the $R_f$ value was $0.02 \pm 0.00$ and $0.47 \pm 0.02$ when $\phi = 65.0$ and $100 \text{ vol}\%$, respectively. In addition, the spots of C12:0 fatty acid did not move when the methanol concentration was lower than $60.0 \text{ vol}\%$. The $R_m$ value was $1.71 \pm 0.10$ and $0.36 \pm 0.05$ when $\phi = 65.0$ and $90.0 \text{ vol}\%$. The $R_m$ value decreased as the methanol concentration increased, which showed their linear relation, and the $R_m$ value became $0.05 \pm 0.03$ when the mobile phase was methanol. The intercept (the $R_{mw}$ value) of the regression line was $4.27 \pm 0.22$, which was calculated from the graph of methanol concentrations vs. the $R_m$ values. This $R_{mw}$ value shows the hydro-
Fig. 5 Relationship between the hydrophobicity values for the fatty acid at methanol fraction of 55 to 90vol%. The symbol □ is for the \( R_{mw} \) value (the hydrophobicity value evaluated by TLC experiment); the symbol ○ is for the \( \log k_w \) value (the hydrophobicity value obtained by HPLC (stationary phase: octadecyl silica, mobile phase: 50 wt% acetonitrile aqueous solution)\(^{20}\)). The □ is for the \( \log P \) value (calculated value of hydrophobicity by computer)\(^{22}\).

The unsaturated moiety in an alkyl chain lowers the hydrophobicity and a partition property of the substances when the mobile phase is water.

Figure 5 shows the hydrophobicity of various fatty acids. For example, the \( R_{mw} \) value was 2.44 ± 0.31 and 6.48 ± 1.35 for the C8:0 and C16:0 fatty acids, respectively. This suggests that the fatty acid’s hydrophobicity is stronger if the alkyl chain is longer. Furthermore, considering the effect of unsaturation degree, the spot of C18:0 fatty acid moved only for a short distance when the mobile phase contained less methanol because it has strong affinity with the stationary phase owing to its long alkyl chain. Conversely, the \( R_{mw} \) value for the C18:1 and C18:2 fatty acids were 6.81 ± 0.90 and 6.78 ± 0.48, respectively. This suggests that the unsaturated moiety in an alkyl chain lowers the hydrophobicity. In addition, the \( R_{mw} \) value for iso-C18:0 fatty acid, which has a branched alkyl chain was 6.07 ± 0.73, which is lower hydrophobicity than that in C18:0 fatty acid. The solubility of the C8:0 and C18:0 fatty acids in 100 g of water is 0.068 g and 0.00029 g, respectively\(^{20}\). The trend on the \( R_{mw} \) values is consistent with that of the solubility data.

Finally, we compared the \( R_{mw} \) values with other parameters related with hydrophobicity. Figure 5 shows the experimental value of \( \log k_w \), which was evaluated by HPLC method and the average value of \( \log P \), which was calculated by the ALOGPS program\(^{22,20}\). To quantitatively evaluate the reliability of the \( R_{mw} \) value, we fitted the values to the Collander equation defined as\(^{4}\):

\[
\log P_1 = a \log P_2 + b,
\]

where \( \log P_1 \) and \( \log P_2 \) are hydrophobicity parameters, \( a \) is the tendency of hydrophobicity, and \( b \) is the correction value for the corresponding hydrophobicity parameter. When the value of the slope \( a \) was closer to 1.0, the tendency of hydrophobicity \( (a) \) became similar as in previous study. Therefore, two additional equations were obtained:

\[
R_{mw} = 0.98 \log k_w - 0.46, \quad R^2 = 0.984
\]

\[
R_{mw} = 1.18 C \log P - 1.10, \quad R^2 = 0.986
\]

In addition, Kendall’s coefficient of concordance was evaluated to check the correlation between three parameters of hydrophobicity. The degree of coincidence was 0.71 (P value = 0.0059): These parameters correlate each other. These results show that the tendency to hydrophobicity (based on \( R_{mw} \)) roughly agrees with the results of the previous experiments or calculations.

4 Conclusion

In this study, we evaluated the hydrophobicity of fatty acids using RP-TLC. A linear relationship was obtained between the methanol concentration in a mobile phase and the \( R_{mw} \) value. The data were fitted with a linear regression line using the MM-estimator method to obtain the hydrophobicity value \( R_{mw} \). The \( R_{mw} \) values were 2.44 ± 0.31 and 6.48 ± 1.35 for C8:0 fatty acid and C16:0 fatty acid, respectively. Therefore, the hydrophobicity is higher when the alkyl chain is longer in the molecule. The \( R_{mw} \) values for the C18:1 and C18:2 fatty acids with unsaturated sites were 6.81 ± 0.90 and 6.78 ± 0.48, respectively, suggesting that the hydrophobicity reduces as the number of the unsaturated sites increases. In addition, the \( R_{mw} \) value of the iso-C18:0 fatty acid, which has a branched structure was 6.07 ± 0.73. Such fatty acids with branched alkyl chain are less hydrophobic than those molecules with straight alkyl chains. These trends are similar with the findings obtained by other methods.

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