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Analysis of hydroxy triacylglycerol as a lactone precursor in milk fat using liquid chromatography electrospray ionization tandem mass spectrometry

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ABSTRACT

Heating milk fat leads to lactone formation. Hydroxy fatty acids, esterified in triacylglycerol (TAG), are likely precursors of lactones in milk fat, but respective hydroxy TAG isomers have not been directly detected for several decades. In this study, we separated hydroxy TAG isomers—1,2-dipalmitoyl-3-(5-hydroxy decanoyl)-*rac*-glycerol (PP(C10-5OH)-TAG), 1,2-dipalmitoyl-3-(5-hydroxy dodecanoyl)-*rac*-glycerol (PP(C12-5OH)-TAG), 1,2-dipalmitoyl-3-(5-hydroxy tetradecanoyl)-*rac*-glycerol (PP(C14-5OH)-TAG), and 1,2-dipalmitoyl-3-(4-hydroxy dodecanoyl)-*rac*-glycerol—by using liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) with an octacocyl silylation column. This method revealed the presence of PP(C10-5OH)-TAG, PP(C12-5OH)-TAG, and PP(C14-5OH)-TAG in butter oil, whereas no hydroxy TAG isomers were detected in heat-treated butter oil. Furthermore, a heating test of hydroxy TAG standards showed a decrease in hydroxy TAG levels and an increase in the corresponding lactone levels. These changes were stimulated by adding a small amount of water. This is the first reported analysis of respective hydroxy TAG isomers in milk fat using LC-ESI-MS/MS.

1. Introduction

Milk fat is a valuable fat in food manufacturing because of its attractive flavor. More than 230 volatile compounds have been identified as natural constituents in milk fat (Mallia, Escher & Schlichtherle-Cerny, 2008). Among them, lactone substantially contributes to the flavor of milk. Therefore, many researchers have studied lactones in milk fat. Keeny and Patton (1956) first identified δ-decalactone as a coconut-like flavor of milk fat, and Tharp and Patton (1960) isolated δ-decalactone and δ-dodecalactone from the steam distillate of butter. Boldingh and Taylor (1962) detected a series of δ-lactones and smaller amounts of γ-lactones in milk fat as desirable contributors to the flavor of butter and detected δ-octalactone, δ-decalactone, δ-dodecalactone, and δ-tetradecalactone. The aroma thresholds of the individual lactones were established by Siek, Albin, Sather and Lindsay (1971). They reported taste thresholds for δ-octalactone, δ-decalactone, δ-dodecalactone, and δ-tetradecalactone of 3.0, 1.4, 95.0, and 500 ppm in oil, respectively; the thresholds in water were 5 to 950 times lower than those in oil. Altogether, short chain lactones indicate lower thresholds than long chain lactones based on the difference in the individual volatilities. Incidentally, Schlutt, Moran, Schieberle and Hofmann (2007) found that semi-volatile lactones, such as δ-tetradecalactone, could enhance the typical creamy flavor of butter. These points indicate that it is important to analyze a series of lactones to estimate the flavor of milk fat. Several methods have been provided to analyze the key volatile flavors in milk fat and dairy products using dynamic headspace sampling or solid phase microextraction (Izco & Torre, 2000; Mallia et al., 2008; Povolo & Contarini, 2003). These methods can sensitively detect the volatile flavor. However, the recovery of semi-volatile compounds, such as δ-tetradecalactone and δ-hexadecalactone, is very low and their quantification is difficult to perform. In contrast, solvent extraction is a rapid and simple established method (Alewijn, Sliwinski & Wouters, 2003; Vandeweghe & Reineccius, 1990; Wong & Parks, 1968). Our laboratory recently developed a new analytical method that provides a good recovery for lactones using gas chromatography-mass spectrometry (GC–MS) after solvent extraction (Obi et al., 2018).

The content of free lactone increases when milk fat is heated (Boldingh & Taylor, 1962; Mattick, Patton, & Keeney 1959; Parliment, Nawer & Fagerson, 1966). The amount of released lactones when milk fat is heated in the presence of water is defined as the lactone potential. The lactone potential in milk fat is affected by environmental factors, such as the season, feeding regime, and stage of lactation (Dimick &

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Harner, 1968; Urbach & Stark, 1978; Urbach, 1990; Villeneuve et al., 2013). For instance, Villeneuve et al. (2013) found that milk from cows fed hay was characterized by higher contents of lactones than milk from cows on pasture. Besides, more significant effects of forage types were observed on the contents of γ-lactones than δ-lactones in milk. Many researchers have investigated the origin of lactone in milk and suggested that γ- and δ-lactones are formed from 4- and 5-hydroxy fatty acid, respectively. Furthermore, hydroxy fatty acids are likely esterified in triacylglycerol (TAG) (Boldingh & Taylor, 1962; Jurriens & Oele, 1965; Kinsella, Patton & Dimick, 1967; Parliment et al., 1966; Wyatt, Pereira & Day, 1967). The esterified hydroxy fatty acids are hydrolyzed from TAG to free fatty acids by heating; then, the free hydroxy fatty acids are spontaneously lactonized. Alewijn, Sliwinski, and Wouters (2005) observed the formation of lactones during the ripening of Gouda cheese. Alewijn, Smit, Sliwinski and Wouters (2007) proposed direct lactonization of hydroxy fatty acids esterified in TAG without hydrolysis because the addition of an aqueous acid and alkaline solution did not affect lactone formation. They also suggested that water plays a catalytic role in the proposed reaction. Hydroxy TAG has been indirectly confirmed as a lactone precursor by column chromatography, thin-layer chromatography (TLC), and GC. For example, Jurriens and Oele (1965) separated a milk fat into several fractions by column chromatography and TLC, and they analyzed the amount of released lactone using GC after saponification of respective fractions. They observed that the fraction consisted mainly of diacylglycerol (DAG) lactones that were released by heating. Kinsella et al. (1967) treated a hydroxy TAG fraction with pancreatic lipase and suggested that the hydroxy fatty acids were esterified to the *sn*-1 or 3 position of TAG. These TAGs contained palmitic acid, myristic acid, oleic acid, and stearic acid. Dolendo, Means, Tobias and Perkins (1969) synthesized 1-(5-hydroxy dodecanoyl)-2,3-dipalmitoyl glycerol and observed δ-dodecalactone after performing a heating test on synthesized TAG. The obtained chemical property was consistent with that of milk fat. Previous studies provided indirect evidence of the presence of hydroxy TAG. However, direct identification of hydroxy TAG has not been achieved because the concentration of the lactone precursor in milk fat is very low, and it is co-eluted with DAG and other compounds with similar structures (Parliment et al., 1966). Liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) is a highly selective method, but pure TAG standards are needed to quantify TAG because the responses of individual TAGs on the chromatogram are different. It is difficult to obtain the necessary TAG standards because of their cost and limited number of suppliers. Furthermore, it is unclear whether the LC-ESI-MS/MS method can separate and detect several kinds of hydroxy TAG isomers that exist in milk fat.

The aim of the present study was to develop an analytical method to detect hydroxy TAG isomers using LC-ESI-MS/MS. For this purpose, four hydroxy TAG standards consisting of two palmitic acids and one 4-hydroxy fatty acid or 5-hydroxy fatty acid were synthesized, allowing for a detailed investigation into the separation character and chemical properties of these isomers. Furthermore, we identified the hydroxy TAG isomers in butter oil, heat-treated butter oil, and reduced butter oil using $NaBH_4$.

2. Materials and methods

2.1. Chemicals and materials

The hydroxy TAG standards used in this study—1,2-dipalmitoyl-3-(5-hydroxy decanoyl)-*rac*-glycerol (PP(C10-5OH)-TAG), 1,2-dipalmitoyl-3-(5-hydroxy dodecanoyl)-*rac*-glycerol (PP(C12-5OH)-TAG), 1,2-dipalmitoyl-3-(5-hydroxy tetradecanoyl)-*rac*-glycerol (PP(C14-5OH)-TAG), and 1,2-dipalmitoyl-3-(4-hydroxy dodecanoyl)-*rac*-glycerol (PP(C12-4OH)-TAG)—were synthesized according to the methods reported by Dolendo et al. (1969), Lísa and Holčapek (2013), and Obi et al. (2018) with > 99% purity. All other regents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Butter oil was manufactured in-house (Tsukishima Foods Industry Co., Ltd., Tokyo, Japan).

2.2. Analysis of hydroxy TAG by LC-ESI-MS/MS

The sample solution was analyzed using an LC-ESI-MS/MS system composed of an octacosyl silylation (C28) column (Sunrise C28, 4.6 mm internal diameter (i.d.) × 250 mm, 5 μm, ChromaNik Technologies Inc., Osaka, Japan), an LC system (Alliance e2695, Waters Corporation, Milford, USA), and an ESI/MS system (Quattro micro API, Waters Corporation). The column temperature and flow rate were 35 °C and 1.0 mL/min, respectively. The initial composition of the mobile phase was methanol/acetone (70/30, v/v); the ratio of acetone was then linearly increased to methanol/acetone (0/100, v/v) over a period of 30 min, and then held constant until all components were eluted. The ion source parameters were as follows: polarity, ESI positive; source temperature, 120 °C; desolvation temperature, 450 °C; cone gas flow, 50 L/h; desolvation gas flow, 800 L/h; and data acquisition mode, multiple reaction monitoring (MRM). The precursor-to-product ion MRM transitions were *m/z* 756 $[M+NH_4]^+$ > 551 $[PP]^+$ for PP(C10-5OH)-TAG, *m/z* 784 $[M+NH_4]^+$ > 551 $[PP]^+$ for PP(C12-4OH)-TAG and PP(C12-5OH)-TAG, *m/z* 812 $[M+NH_4]^+$ > 551 $[PP]^+$ for PP(C14-5OH)-TAG, and *m/z* 317 $[M+H]^+$ > 299 $[M-OH]^+$ for monopentadecanoin (C15-MAG). The analyses were performed three times.

2.3. Calibration curves for respective hydroxy TAG standards

To obtain calibration curves for the quantification of hydroxy TAG isomers, PP(C10-5OH)-TAG, PP(C12-5OH)-TAG, PP(C14-5OH)-TAG, and PP(C12-4OH)-TAG were adjusted to 1, 2, 5, 10, 25, 50, 100, and 200 μg/mL using 200 μg/mL C15-MAG as the internal standard in acetone. A 10-μL aliquot of each standard solution was injected into the LC-ESI-MS/MS system. Calibration curves were prepared for each hydroxy TAG isomer by plotting the hydroxy TAG concentration on the *x*-axis and the ratio of the chromatogram peak areas (hydroxy TAG/C15-MAG) on the *y*-axis. The limits of detection (LOD) and quantification (LOQ) of this method were calculated from the signal/noise ratio (S/N). The LOD and LOQ were defined as S/N = 3 and 10, respectively.

2.4. Determination of hydroxy TAG isomers in milk fat

To obtain the butter oil from which hydroxy TAG was removed, butter oil was heated at 160 °C for 2 h in the presence of 1% (w/w) water and then dried over anhydrous sodium sulfate; it was used as the heat-treated butter oil. To obtain butter oil with a high amount of hydroxy TAG, butter oil was reduced with $NaBH_4$ in ethanol at room temperature for 2 h; then, the mixture was diluted with diethyl ether and saturated saline. The organic layer was separated, dried over anhydrous sodium sulfate, and concentrated by evaporation. The obtained oil was used as the $NaBH_4$-reduced butter.

The butter oil samples (500 mg) were transferred to a 10-mL volumetric flask and mixed with 1 mL of a 5-mg/mL solution of C15-MAG in diisopropyl ether as an internal standard, and the volumetric flask was then filled with hexane. The hydroxy TAG fraction in the samples was separated using a Sep-Pak Silica cartridge (1 g, Waters Corporation, Milford, USA). Briefly, 2 mL of the sample solution was loaded into a Sep-Pack Silica cartridge that had been pre-equilibrated using a solvent mixture of hexane and ethyl acetate (20:1, v/v). TAG was then removed by eluting with 15 mL of the same solvent mixture. Finally, hydroxy TAG was collected using 15 mL of ethyl acetate. After solvent evaporation, the residue was dissolved in 0.5 mL of acetone for subsequent LC-ESI-MS/MS analysis.

The individual hydroxy TAG isomers were identified using the retention times and fragmentation patterns of the pure reference standards. The hydroxy TAG content (mg/kg oil) was calculated using the