Comparison of Extraction Methods for Fatty Acid and Conjugated Linoleic Acid Quantification in Milk

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Abstract. The purpose of this study was to compare acid and base catalyst for the extraction of Fatty acids (FA) and conjugated linoleic acid from milk product. Lipid extraction from milk by using acid catalyst were carried out in H₂SO₄/methanol solvents. Methylation was performed for 2 h at 80°C, and FA methyl esters were recovered for chromatographic analysis by the addition of isoctane. On the other hand, the extraction using base catalysts was conducted by AOAC 989.05 (2012) official method, followed by AOAC 969.33 (2012) for methylation. Extraction process by acid catalyst shown better performance compared to base catalyst due to it could be produced higher recoveries. The advantage of extraction method by using acid-catalyst allowed the measurement of high number of samples. Moreover, it can be reduced the sample manipulation and consequently reduce sample loss and contamination. In conclusion, the acid-catalyst method was a simple and rapid method, low cost, and achieves good results for Fatty acids (FA) and conjugated linoleic acid extraction from milk product.

1. Introduction

Conjugated Linoleic Acid (CLA) is an essential fatty acid which is important for human health. CLA can be found naturally in food, predominantly in dairy products and meat, which are originated from ruminants. In this case, linoleic acid contained conjugated double bond in different position and geometric isomer. [1]. Some CLA isomers shown several biological activities in experimental animals, such as anti-carcinogenic, and has potential benefit for human health. CLA is considered to have important role for various human physiological functions such as anti-diabetic, anticancer, as well as to improve the mineralization of bone and modulate the immune system [2]. So that an analytical technique that is accurate, reliable, and quick to assess CLA concentration in milk is very important to find.

The determination of fatty acids in food product can be analyzed by Gas Chromatography (GC), which usually requires fat extraction process, followed by the methylation process, then conversion of the FAs into methyl esters (FAMEs), and finally determination through Gas Chromatography. The first proposed method was using a mixture of chloroform-methanol to isolate the total fat content in animal tissue [3]. However, FAs is polar compound with low volatility that tends to adhere on the walls of GC columns or other surfaces and result in problem of chromatographic separation. In order to avoid this...
problem, the FAs should be modified. Methylation process is necessary to convert the polar, non-volatile long-chain FAs into methyl ester derivatives, that are less polar, relatively volatile, and thermally stable [4]. In the last few years, AOAC 989.05 (2012) and AOAC 969.33 (2012) methods have been adopted as official methods for lipid extraction and preparation of FAMEs. However, it is recognized that conventional methods are impractical since they are time consuming, require large samples, and consume large amount of solvents. Flow charts in extraction methods for lipid complex are responsible for partial loss of the lipid phase and can cause contamination [5]. For this reason, fast, low cost, simple, and accurate methods for determining FA concentration in milk product are necessary.

Sulphuric acid has been used as acid catalyst in the formation of isopropyl esters in milk lipid. Although it has not been thoroughly investigated, the utilization of H$_2$SO$_4$ in methanol has shown better results [6]. Besides that, base methylation is commonly used method for adding methyl groups to milk lipid. However, this method requires strict anhydrous conditions so that it is difficult to apply raw milk samples directly [7].

The purpose of this research was to compare acid and base catalyst performance on lipid extraction for FAs and CLA quantification in milk product.

2. Research Methodology

Reagents and solvents were analytical grade for Gas Chromatography (GC) analysis. Ethanol, diethyl ether, ammonia solution, sodium sulphate anhydrous, sodium chloride, sulphuric acid, methanol, petroleum benzene, boron tri fluoride, isooctane were purchased through Merck.

The CLA and FAME standards were purchased from Sigma-Aldrich, while the milk samples were taken at the Pemijahan Village dairy farm in Bogor Regency, from dairy cattle Friesian Holstein (FH) breeds.

The separation and quantification of CLA was performed using the GC G4350B with serial number CN17392056 (Agilent Technologies, Palo Alto, CA, USA), equipped with a flame ionization detector (FID) and HP-5 capillary column (30 m length, 0.320 mm diameter, 0.25 µm film thickness).

The chromatographic conditions were as follows: the initial oven temperature was 35°C for 2 minutes and then increased to 100°C with an increase of 30°C per minutes. The oven temperature was increased to 195°C with an increase of 10°C per minute, conducted for 5 min. The temperature was raised to 205°C with a rise of 7 °C every min, and conducted for 9 min. Then the temperature was increased again to reach 240°C with an increase of 3°C per min, and conducted for 7 min. The injection ports and detector temperatures were set to 250 and 250°C, respectively. Helium was used as a carrier gas at a flow rate of 1.3 mL/minute. The split ratio of injection solution was 10:1, and 1 µL. Data were recorded by GC ChemStation Software integrator version B.03.02 (Agilent Technologies).

Milk samples were homogenized by shaking for 10 min in a water bath at 38°C. Extraction of this method was performed as outlined by AOAC 989.05 (AOAC, 2012). The FAMEs were prepared by base-catalysed using NaOH in methanol as described in AOAC 969.33 years 2012.

Milk samples were homogenized and 10 mL of milk was placed in a tube sealed with a Teflon-lined cap, then 2 mL of a 25 mL/ L H$_2$SO$_4$ solution in methanol was added. The tube was shaken for 30 s and stored in darkness at -20°C overnight (12–16 h) for lipid extraction. After that, methylation was carried out in a water bath for 2 h at 60°C. Then, 2 mL of a saturated NaCl solution and 1 mL of isooctane were added, the mixture was shaken for 30 s and centrifuged for 10 min at 2000 rpm. The aqueous layer was removed, and a small amount of anhydrous sodium sulphate was added to eliminate any water residue.

Differences in the levels of fatty acids in milk were analysed by extraction methods with acid catalysts and alkaline catalysts were statistically analysed by t-Student Test. The difference was considered significant when P <0.05. Data analysis was performed using SPSS, version 13.0 for Windows.

3. Result and Discussion
Fatty acid is an organic acid compound consisting of a hydrocarbon chain with carboxyl group (COOH) in head side and methyl group (CH$_3$) in another side. The component of fatty acid has a large effect on milk quality such as physical and organoleptic characteristics as the result of acid free short-chain fat and unsaturated fatty acid oxidation [8]. In this research, there were 15 types of fatty acids that were extracted by using acid and base catalyst, including Conjugated Linoleic Acid (CLA). All samples were identified and the result were displayed on table 1. According to the result, fatty acid yield that was obtained by using acid catalyst significantly higher than alkaline catalysts. However, no significant interactions different were obtained from milk samples analysed by both methods for cases C17: 1, C18: 2n6c, and CLA. The difference of fatty acid levels in milk was more obvious (2-fold in some cases) for the length chain FA than for the short one (except for C6: 1, C8: 0, and C16: 1). Especially for C18: 2n6t fatty acid concentrations, the acquisition with an acid catalyst was almost ten times greater when compared to base catalyst.

Table 1. The amount of FAME (mg/L) in fresh cow's milk performed by acid and base catalyst

| Fatty Acid | Acid Catalyst | Alkaline Catalyst | P   |
|-----------|---------------|-------------------|-----|
| C6:0      | 547.8±39.5    | 204.1±22.7        | **  |
| C8:0      | 234.5±5.1     | 111.4±14.2        | **  |
| C10:0     | 429.7±30.4    | 235.6±30.6        | *   |
| C12:0     | 890.5±64.1    | 512.4±49.3        | *   |
| C14:0     | 146.6±8.1     | 88.4±5.3          | *   |
| C14:0     | 2213.4±164.9  | 1423.4±65.8       | *   |
| C15:1     | 265.5±11.2    | 194.6±14.1        | *   |
| C16:1     | 335.9±28.9    | 130.6±13.5        | **  |
| C16:0     | 6517.3±453.1  | 4690.5±251.7      | *   |
| C17:1     | 141.5±7.1     | 130.1±25.5        | ns  |
| C18:1n9t  | 705.8±50.5    | 585.1±33.5        | *   |
| C18:1n9c  | 3701.1±164.2  | 3029.4±156.3      | *   |
| C18:2n6t  | 1310.5±81.9   | 723.3±16.2        | *** |
| C18:2n6c  | 2342.1±114.7  | 2094.7±114.1      | ns  |
| CLA       | 637.8±114.3   | 528.3±44.3        | ns  |

Note: values listed is average ± standard deviation (n = 5). ns= not significant (P ≥ 0.05), *P ≤ 0.05 ** P ≤ 0.01, *** P ≤ 0.001.

These outcomes were consistent with the fact that transesterification using alkaline catalysts have two main disadvantages, such as free fatty acids and sphingolipids partially unreacted, and the ester undergoes a saponification reaction [9]. In the presence of methoxide, water produces free hydroxide ions. With the presence of these ions, the esters and FAME product can be hydrolysed [10]. For this reason, anhydrous conditions are very necessary [7]. These revealed that alkaline catalysts produced the low yield of fatty acid product compared to acid catalysts.

Other modification methods for the analysis of fatty acids have been carried out and there were no significant differences between these compared to base catalysts method, but this method shows a prominent advantage in terms of time, chemicals, and labor [10]. Furthermore, other acid catalysts were executed for other matrices in order to achieve the preferred results than those obtained with basic catalyst. This fact was caused by the prevention of loss of fatty acids by handling complex steps and high number of samples and solvents [11].

4. Conclusion
The acid catalysts method is able to extract CLA and some fatty acids with higher yield compared to base catalyst. In addition, this method reduces the risk of fatty acid losses and contamination through a
reduction in sample handling and this is very relevant for analysing large number of samples at the same time.

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