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Comparison and validation of 2 analytical methods for the determination of free fatty acids in dairy products by gas chromatography with flame ionization detection

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ABSTRACT

Accurate quantification of free fatty acids (FFA) in dairy products is important for quality control, nutritional, antimicrobial, authenticity, legislative, and flavor purposes. In this study, the performance of 2 widely used gas chromatographic flame ionization detection methods for determination of FFA in dairy products differing in lipid content and degree of lipolysis were evaluated. We used a direct on-column approach where the isolated FFA extract was injected directly and a derivatization approach where the FFA were esterified in the injector to methyl esters using tetramethylammonium hydroxide as a catalyst. A comprehensive validation was undertaken to establish method linearity, limits of detection, limits of quantification, accuracy, and precision. Linear calibrations of 3 to 700 mg/L ($R^2 > 0.999$) and 20 to 700 mg/L ($R^2 > 0.997$), and limits of detection and limits of quantification of 0.7 and 3 mg/L and 5 and 20 mg/L were obtained for the direct injection on-column and the derivatization method, respectively. Intraday precision of 1.5 to 7.2% was obtained for both methods. The direct injection on-column method had the lower levels of limits of detection and quantification, because FFA are directly injected onto the GC as opposed to the split injection used in the derivatization method. However, the direct injection on-column method experienced accumulative column phase deterioration and irreversible FFA absorption because of the acidic nature of the injection extract, which adversely affected method robustness and the quantification of some longer chain FFA. The derivatization method experienced issues with quantification of butyric acid at low concentrations because of coelution with the injection solvent peak, loss of polyunsaturated FFA due to degradation by tetramethylammonium hydroxide, and the periodic emergence of by-product peaks of the tetramethylammonium hydroxide reaction that interfered with the quantification of some short-chain FFA. The derivatization method is more robust, and because the derivatization step can be automated, it is more suitable for routine analysis of FFA in dairy products. However, considerable scope exists to develop an alternative gas chromatography with flame ionization detection method to quantify FFA in dairy products without any limitations that is robust and accurate.

Key words: free fatty acid, dairy, gas chromatography, methyl ester

INTRODUCTION

Free fatty acids (**FFA**) are an important class of compounds in food and dairy products from a flavor, nutritional, and antibacterial perspective. They have a large effect on organoleptic quality because of their low odor thresholds, especially the short-chain fatty acids, which provide the characteristic odors for many dairy products but are also responsible for rancidity defects. In the past the main requirement for the quantification of FFA was for quality control of milk and dairy products. Even though other chromatographic methods exist to quantify FFA, the most popular method of analysis involves gas chromatography with flame ionization detection (**GC-FID**) because of its precision and reliability and relative low cost (Christie, 1993; Delmonte et al., 2009). In the case of FFA they can be analyzed after conversion to methyl esters (Metcalffe and Wang, 1981; Needs et al., 1983; Martínez-Castro et al., 1986) or directly after extraction from the product (Woo and Lindsay, 1982; Deeth et al., 1983; De Jong and Badings, 1990). The isolation of FFA by aminopropyl solid-phase extraction (**SPE**) columns followed by GC-FID analysis is a widely used approach to quantify FFA (De Jong et al., 1994; Hickey et al., 2006; Hickey et al., 2007; Kilcawley et al., 2012; Calzada et al., 2014). In this case all of the FFA are isolated with a reportedly high degree of purity (Kaluzny et al., 1985), and no further treatment is required before GC analysis, thus mak-

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ing it a convenient alternative to derivatized methods. For derivatization reactions, tetramethylammonium hydroxide (**TMAH**) is a commonly used derivatizing agent to convert FFA to FAME before GC-FID analysis (Martínez-Castro et al., 1986; Martin-Hernández et al., 1988; Juárez et al., 1992; Chavarri et al., 1997; Gomes Reis et al., 2011), because of its ability to simultaneously create methyl esters of glycerides and form salts of FFA (which are then converted to methyl esters in a heated injector) in separate phases. This makes it possible to analyze both components of the lipid extract without the need for prior separation.

Martínez-Castro et al. (1986) and De Jong and Badings (1990) have described some limitations in relation to both of these methods that need further investigation. Underivatized FFA have a strong interaction with column phases, which can lead to irreversible adsorption, a phenomenon referred to as "memory effect" that can result in overestimation of FFA content. Also, the direct injection method involves the isolation of FFA in 2% formic acid in diethyl ether, which is very acidic and has an adverse effect on column lifespan (De Jong and Badings, 1990). This can be very costly but also adversely affect the responses of analytes. Such drawbacks may also affect retention times, limits of detection (**LOD**), limits of quantification (**LOQ**), and linearity values. The use of TMAH as an esterification reagent for FFA also has limitations (Martínez-Castro et al., 1986); the glyceride component of extracted lipids was shown to interfere with FFA determination. This led Martínez-Castro et al. (1986) to modify the extraction steps to include solvent washing of the separate layers, to remove interfering compounds before analysis. This issue of glyceride interference was further highlighted by Chavarri et al. (1997), who reported a significant disagreement between the results obtained between FFA isolation using aminopropyl SPE columns and direct injection, and the derivatization method where FFA are converted into methyl esters using TMAH. They recommended isolating the FFA from the lipid extract before treatment with TMAH when analyzing samples with a large triglyceride-to-FFA ratio, which is the case with most dairy samples.

Oddly, very few studies have been published relating to the effectiveness, LOD, LOQ, linearity, and detection range of these routinely used methods in relation to dairy products, despite the fact that both have been in use for more than a couple of decades. Also, the practical application of these methods to quantify FFA in a range of different dairy sample matrices has not been fully explored. In addition, both of these methods are relatively laborious and time consuming and require a large of amount of solvents and reagents; thus, the incorporation of a degree of automation into the methodology would likely be of significant benefit. Therefore, this study investigated the performance of both the FAME method (using TMAH for FFA derivatization) and the direct injection method after SPE of FFA on a wide range of dairy products. A modification of the FAME method was employed based on the findings of Chavarri et al. (1997), where the FFA was initially isolated from the sample extract before conversion to methyl esters using TMAH. Automation was incorporated in standard preparation and FFA derivatization.

MATERIALS AND METHODS

Materials

Hexane, heptane, diethyl ether, formic acid, 25% tetramethylammonium hydroxide in methanol (TMAH), butyric acid ($C_{4:0}$), valeric acid ($C_{5:0}$), caproic acid ($C_{6:0}$), caprylic acid ($C_{8:0}$), capric acid ($C_{10:0}$), undecylic acid ($C_{11:0}$), lauric acid ($C_{12:0}$), myristic acid ($C_{14:0}$), palmitic acid ($C_{16:0}$), margaric acid ($C_{17:0}$), stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$), and linolenic acid ($C_{18:3}$) were purchased from Sigma-Aldrich (Dublin, Ireland). Certified FFA standard mix containing $C_{4:0}$ to $C_{22:0}$ free acids (GLC Reference standard 74 "Free acid") and FAME standard mix containing $C_{4:0}$ to $C_{22:0}$ methyl esters (GLC Reference Standard 74) were purchased from Nu-Chek Prep Inc. (Waterville, MN). Aminopropyl cartridges (500 mg) were obtained from Agilent Technologies Ireland Ltd. (Little Island, Cork, Ireland).

Samples

Milk, whole milk powder, infant formula powder, yogurt, ice cream, Cheddar cheeses, blue cheese, processed cheese, Brie, enzyme modified cheeses (**EMC**), and butter were purchased from local commercial suppliers or local retail outlets.

Infant formula, milk powder, and EMC powder samples were stored under nitrogen in sealed containers at room temperature in darkness. Milk, yogurt, butter, EMC paste, and ice cream samples were transferred into sterile containers, which were frozen at −18°C until required. All cheese samples were vacuum packed and frozen at −18°C.

Instrumentation

The FFA and FAME analyses were carried out on a Varian CP3800 gas chromatograph (JVA Analytical Ltd., Dublin, Ireland) equipped with a CP8400 au-