J. Dairy Sci. 97:6719–6728
http://dx.doi.org/10.3168/jds.2014-8128

Total milk fat extraction and quantification of polar and neutral lipids of cow, goat, and ewe milk by using a pressurized liquid system and chromatographic techniques

M. P. Castro-Gómez,* L. M. Rodriguez-Alcalá,* M. V. Calvo,* J. Romero,† J. A. Mendiola,* E. Ibañez,* and J. Fontecha*[1]
*Bioactivity and Food Analysis Department, Instituto de Investigación en Ciencias de la Alimentación, Consejo Superior de Investigaciones Científicas (CIAL-CSIC), C/Nicolás Cabrera 9, 28049 Madrid, Spain
†Laboratorio Interprofesional Lácteo de Castilla la Mancha, Avda. Portugal 42, 45600 Talavera de la Reina, Toledo, Spain

ABSTRACT

Although milk polar lipids such as phospholipids and sphingolipids located in the milk fat globule membrane constitute 0.1 to 1% of the total milk fat, those lipid fractions are gaining increasing interest because of their potential beneficial effects on human health and technological properties. In this context, the accurate quantification of the milk polar lipids is crucial for comparison of different milk species, products, or dairy treatments. Although the official International Organization for Standardization-International Dairy Federation method for milk lipid extraction gives satisfactory results for neutral lipids, it has important disadvantages in terms of polar lipid losses. Other methods using mixtures of solvents such as chloroform:methanol are highly efficient for extracting polar lipids but are also associated with low sample throughput, long time, and large solvent consumption. As an alternative, we have optimized the milk fat extraction yield by using a pressurized liquid extraction (PLE) method at different temperatures and times in comparison with those traditional lipid extraction procedures using 2:1 chloroform:methanol as a mixture of solvents. Comparison of classical extraction methods with the developed PLE procedure were carried out using raw whole milk from different species (cows, ewes, and goats) and considering fat yield, fatty acid methyl ester composition, triacylglyceride species, cholesterol content, and lipid class compositions, with special attention to polar lipids such as phospholipids and sphingolipids. The developed PLE procedure was validated for milk fat extraction and the results show that this method performs a complete or close to complete extraction of all lipid classes and in less time than the official and Folch methods. In conclusion, the PLE method optimized in this study could be an alternative to carry out milk fat extraction as a routine method.

Received March 12, 2014.
Accepted July 24, 2014.
[1]Corresponding author: j.fontecha@csic.es

Key words: pressurized liquid extraction, milk lipid, fatty acid, phospholipid

INTRODUCTION

Milk lipid analysis is an important area of research and the field has experienced a new renaissance in the last decades. Although some concern exists about the high amount of saturated fat present in whole milk, the latest advances indicate the presence of bioactive FA, such as short-chain FA and CLA, and other minor components, such as polar lipids (phospholipids and sphingolipids), which may have favorable effects on human blood lipids and other cardiometabolic risk factors (Hilmarsson et al., 2006; Heinze and Actis, 2012; Küllenberg et al., 2012). Polar lipids in milk are the main constituents of the milk fat globule membrane, mainly constituted of phosphatidylcholine (**PC**), phosphatidylethanolamine (**PE**), phosphatidylinositol (**PI**), phosphatidylserine (**PS**) and sphingomyelin (**SM**; Singh, 2006). The interest in these molecules is high due to the potential positive effects on human health of dietary phospholipids (Küllenberg et al., 2012).

For an analysis of the total milk lipid composition, it is necessary to select the appropriate method of lipid extraction for preventing either the loss of some of these components or their chemical changes. The standard milk fat extraction methods, such as the Röse-Gottlieb (ISO, 2001), using a mixture of diethyl ether and *n*-pentane, as well as the method based on extraction with a mixture of hexane:isopropanol proposed by Hara and Radin (1978), give satisfactory results for neutral lipid extraction but they present important disadvantages due to losses of some phospholipids and sphingolipids (Feng et al., 2004; Avalli and Contarini, 2005). In addition, they are often performed manually, involving exhaustive and time-consuming steps and hazardous solvents at the large amounts required to remove the fat from the sample matrix. Moreover, these methods either are incompatible with the extraction of lipids with a wide range of hydrophobicity as phospholipids

or result in lower recoveries (Avalli and Contarini, 2005; Gallier et al., 2010).

One of the most commonly used methods for extracting and purifying lipids is the Folch procedure (Folch et al., 1957). Even though this method is highly efficient for extracting polar lipids, it is also associated with low sample throughput, long time, and large solvent consumption.

All these classical extraction schemes for fat extraction have meanwhile been outperformed by pressurized liquid extraction (**PLE**). Pressurized liquid extraction has developed into the most powerful extraction approach in routine analysis of lipids/FA in biological matrices as well as foods (Schäfer, 1998; Herrero et al., 2005; Señoráns and Luna, 2012). By means of a proper combination of temperature, pressure, time, and number of cycles of extraction, a reduction both in solvent consumption and in the extraction time per sample could be achieved, using the same mixture of solvents as in the traditional methods and offering as an additional advantage the possibility of process automatization (Conte et al., 1997; Macnaughton et al., 1997; Jansen et al., 2006). The aim of this study was to compare the classical extraction methods with a PLE procedure and to validate the procedure for milk fat extraction. Fat yield, FAME composition, triacylglyceride species, cholesterol (**CHOL**) content, and lipid class compositions, with special attention to polar lipids such as phospholipids and sphingolipids, were determined in raw whole cow, ewe, and goat milk.

MATERIALS AND METHODS

Samples

Raw whole milk from 3 different ruminant species (cows, ewes, and goats) was obtained from different farms of Castilla-La Mancha, Spain (10 samples for each species), and analyzed for composition in milk fat and protein by the Interprofessional Dairy Laboratory of Castilla-La Mancha (LILCAM, Castilla-La Mancha, Spain). One hundred milliliters of drawn milk was rapidly frozen and shipped to our laboratory in isothermal containers and then freeze-dried and stored at −35°C until use. A commercial powder skim milk with maximum 1% fat content [Corporación Alimentaria Peñasanta S.A. (CAPSA), Granda-Siero, Asturias, Spain] was used to optimize the lipid extraction conditions by the PLE method.

Reagents

All solvents (dichloromethane, chloroform, hexane, methanol, isooctane, and isopropanol) were HPLC grade and purchased from Avantor Performance Materials Poland S.A. (Gliwice, Poland; Labscan brand). Sodium carbonate and sea sand were obtained from Panreac Química S.A. (Barcelona, Spain). Formic acid (98%), trifluoroacetic acid, triethylamine (99.5%), trinonanoin, tritridecanoin, pelargonic acid (C9), tridecanoic acid (C13), myristic acid (C14), palmitic acid (C16), estearic acid (C18), arachidonic acid (20:4), eicosapentaenoic acid (20:5), docosahexapentaenoic acid (22:6), monostearin, diolein, PI, PS, PE, SM, PC, and *N*-oleoylethanolamine were purchased from Sigma (Bellefonte, PA). Reference butterfat BCR-164 and BCR-519 (EU Commission, Brussels, Belgium) were purchased from Fedelco Inc. (Madrid, Spain).

Fat Extraction

First, total milk fat amount was determined in the Interprofessional Dairy Laboratory (LILCAM) by either the Röse-Gottlieb method based on solvent extraction according to the official reference procedure (ISO, 2001) and by using an infrared spectrophotometer (MilkoScan; Foss Electric España S.A., Barcelona, Spain) as fat total content determination method.

Milk fat was extracted in our laboratory from each of the 30 stored freeze-dried milk samples (cow, ewe, and goat milk; n = 10) using the following 2 methods:

1) Folch method according to Iverson et al. (2001), modified as follows: from a well-mixed freeze-dried milk sample, a 2-g aliquot was placed in 50-mL centrifuge tubes with 1 mg of previously added trinonanoin as internal standard. Fifteen milliliters of a dichloromethane-methanol solution (2:1, vol/vol) was then added to each tube. The mixture was shaken mechanically for 30 min and centrifuged at 6,600 × *g* for 5 min at 4°C. As much of the upper organic solvent fraction as possible was carefully removed with a pipette. The sediment was washed with 12 mL of a dichloromethane-methanol solution (2:1, vol/vol) and, after shaking for 1 min, the sample was, again centrifuged at 6,600 × *g* for 5 min at 4°C. The removed organic solvent was combined with that previously collected and 3 mL of a 0.9% solution of sodium chloride was added and mixed mechanically for 1 min before the tubes were stored overnight at 4°C. Afterward, they were again centrifuged at 6,600 × *g* for 5 min at 4°C and the bottom dichloromethane layer was collected and filtered through a Whatman 1-phase separator filter paper (Whatman, Maidstone, UK) containing approximately 3 g of anhydrous sodium sulfate. Finally, the extract