J. Dairy Sci. 98:8473–8485
http://dx.doi.org/10.3168/jds.2015-9889

Identification and quantification of triacylglycerols containing n-3 long-chain polyunsaturated fatty acids in bovine milk

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ABSTRACT

The n-3 long-chain polyunsaturated fatty acids (LC-PUFA) are low-abundance components in milk fat, but have great potential in promoting human health. A comprehensive survey on triacylglycerol (TAG) molecular species in milk that contain at least one type of n-3 LC-PUFA, namely eicosapentaenoic acid, docosahexaenoic acid, and docosapentaenoic acid, was conducted in this work using HPLC-linear trap quadrupole-Orbitrap and HPLC-triple quadrupole mass spectrometry techniques. A total of 51 TAG species that contain n-3 LC-PUFA have been identified in bovine milk and their structures assigned. The TAG species containing docosahexaenoic acid were found in much smaller number and at much lower abundance compared with the other 2 types of TAG. An HPLC-triple quadrupole mass spectrometry-based method was developed, which provides relative quantification of all these TAG species in a run of 36 min. Application of this method to the quantification of n-3 LC-PUFA-incorporated TAG in 32 individual animal milk samples allowed us to determine variation between animals, identify strong metabolic relationships between TAG species, and reveal negative effect of a grape marc supplement on the accumulation of eicosapentaenoic acid in milk.
Key words: milk, n-3 long-chain polyunsaturated fatty acids, liquid chromatography-mass spectrometry

INTRODUCTION

Although not all health benefit claims of n-3 FA have been substantiated, strong evidence exists that n-3 log-chain (≥C20) polyunsaturated fatty acids (**n-3 LC-PUFA**), especially eicosapentaenoic acid (**EPA**; C20:5n-3) and docosahexaenoic acid (**DHA**; C22:6n-3), have multiple beneficial functions for human health and development, such as prevention of cardiovascular disease, enhancing brain development in infants, and anti-inflammatory activity (Mori, 2006; Ruxton et al., 2007; Abeywardena and Patten, 2011). The n-3 LC-PUFA are typically found in fish oils, which may not meet the rising demand for nutraceutical n-3 LC-PUFA products, thus an additional source of n-3 LC-PUFA is required (Nichols et al., 2010). Recently, algae have been cultivated to preferentially produce DHA and their oil has been extracted to make supplements (Sijtsma and de Swaaf, 2004). In addition, good progress has been made in metabolic engineering of n-3 LC-PUFA in oilseed crops by several groups in recently years (Wu et al., 2005; Cheng et al., 2010; Petrie et al., 2012). Breast milk is an important source of DHA and essential FA in early human life, but ruminant milk is known to be a poor source of n-3 LC-PUFA (Gastaldi et al., 2011). As milk is a staple drink and food ingredient, increasing its n-3 LC-PUFA content could increase the intake of such beneficial FA for a large number of people. Recently it has been shown that the concentrations of n-3 LC-PUFA in cow milk can be substantially increased by supplementing the cow diet with feed containing high quantities of n-3 FA (Moghadasian, 2008; Nelson and Martini, 2009; Moate et al., 2013). One study also found that the concentration of n-3 FA, including EPA and docosapentaenoic acid (**DPA**; C22:5n-3), was higher in organic milk as compared with conventional milk (Benbrook et al., 2013). A recent study into the heritability of lipid classes in milk (SFA, MUFA, and PUFA) suggests that potential to select for a healthy milk composition exists (Penasa et al., 2015). Underpinning any genetic or management strategy to change the composition of milk lipids is the ability to accurately measure the lipid species.

The global FA composition of any food sample can be readily measured by transesterification and GC analysis (Slover and Lanza, 1979). Consequently, numerous

Received June 1, 2015.
Accepted August 9, 2015.
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data can be found regarding the FA profile of bovine milk fat (Moate et al., 2007, 2014; Mansson, 2008; Frelich et al., 2009; Gastaldi et al., 2011); the major FA species are consistent, which include C4, C6, C8, C10, C12, C14, C14:1, C16, C16:1, C18, C18:1, C18:2, and C18:3. However, the concentration of n-3 LC-PUFA was not given in most reports, presumably due to the low abundance of these FA, which were below the limit of quantification in those studies.

Currently, information on the molecular species and the relative abundance of triacylglycerol (**TAG**) that contain n-3 LC-PUFA in milk fat is also scarce, although detailed profiling of the relatively abundant TAG species has been reported extensively (Gresti et al., 1993; Mottram and Evershed, 2001; Månsson, 2008; Gastaldi et al., 2011; Haddad et al., 2011; Zhou et al., 2014a). To investigate the accumulation pattern of n-3 LC-PUFA in milk fat in relation to various genetic and environmental factors, a systematic characterization of the TAG species that contain n-3 LC-PUFA as well as a robust method for their quantification is required.

Grape marc (skins, seeds, and stems that remain after grapes are pressed for juice-making), is currently a by-product used as a feed supplement by the dairy and beef industries. A recent study showed that grape marc could reduce methane emissions when fed to dairy cow, indicating that this by-product could play a role in methane abatement (Moate et al., 2014). The influence of grape marc diet on the concentration of major FA of milk fat has been investigated in the same study (Moate et al., 2014). However, whether low-abundance n-3 LC-PUFA is affected by grape marc diet remained unknown. The objectives of our study were (1) comprehensive identification of molecular species of TAG that contain EPA, DHA, or DPA; (2) developing a HPLC-triple quadrupole MS-based method for simultaneous quantification of the identified n-3 LC-PUFA-incorporated TAG species; (3) investigating the variance in abundance of n-3 LC-PUFA-containing TAG species between cows; and (4) determining of the effect of grape marc diet on the accumulation of n-3 LC-PUFA in milk.

MATERIALS AND METHODS

Cows, Diets, and Milk Samples

Thirty-two lactating, multiparous Holstein-Friesian cows (500 ± 51.9 kg of BW, 22.1 ± 5.1 DIM, 3.4 ± 0.50 yr of age; average ± SD) were assigned to 3 groups balanced for BW, DIM, and age. Each group was then randomly allocated to 1 of 3 dietary treatments: (1) a control diet (**CON**) in which cows were individually offered 5.0 kg of DM of cracked corn, 0.2 kg of DM of minerals, and 15.0 kg of DM of freshly cut pasture (predominantly ryegrass); (2) white-grape marc diet (**WGM**) in which cows were individually offered 5.0 kg of DM of cracked corn, 0.2 kg of DM of minerals, 5.0 kg of DM of white-grape marc, and 10.0 kg of DM of freshly cut pasture; and (3) a red-grape marc diet (**RGM**) in which cows were individually offered 5.0 kg of DM of cracked corn, 0.2 kg of DM of minerals, 5.0 kg of DM of red-grape marc, and 10.0 kg of DM of freshly cut pasture. The CON treatment had 12 cows assigned to it, whereas the WGM and RGM treatments each had 10 cows. These diets were fed to the cows for 28 d and samples of milk were collected on the last day of the experiment and kept at −80°C before analysis.

Chemicals

The n-3 LC-PUFA methyl ester standards, TAG tri-C22:1 (used as internal standard; **IS**), and ammonium formate (≥99% purity, used as a mobile phase additive) were purchased from Sigma Aldrich (St. Louis, MO). Solvents used for lipid extraction and mobile phase preparation were of chromatographic grade and were from Merck (methanol; Merck, Kenilworth, NJ) and Sigma Aldrich (chloroform, isopropanol, and acetonitrile containing 0.1% formic acid). Sulfuric acid used in lipid transesterification was of analytical grade (Ajax Finechem, Seven Hills, Australia).

Method for Lipid Extraction from Milk

Milk lipid was extracted as described in our previous report (Liu et al., 2015). Briefly, 0.2 mL of full-cream milk, 50 μL of IS (TAG tri-C22:1, 0.1 mg/mL in chloroform and methanol at a 2:1 ratio), and 0.8 mL of Milli-Q water (Merck Millipore, Billerica, MA) was added; the diluted milk was mixed with 4 mL of chloroform and methanol (2:1, vol/vol) and shaken in a vortex mixer thoroughly for 10 min at room temperature. The mixture was then centrifuged for 10 min at 1,900 × g at room temperature to facilitate phase separation. After transferring the organic phase to a new tube, the aqueous phase was extracted again with 2 mL of chloroform and methanol (2:1, vol/vol). The combined organic phase (ca. 4.5 mL), which contained the lipid fraction, was dried under a stream of nitrogen and reconstituted at a 3:1 ratio in isopropanol and chloroform (2:1, vol/vol) before analysis by HPLC-MS.

Method for Identification and Quantification of n-3 LC-PUFA-Containing TAG

Two types of HPLC-MS configurations were employed in our study: HPLC-linear trap quadrupole