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Liquid chromatography–tandem mass spectrometry method for the determination of vitamin K homologues in human milk after overnight cold saponification

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ABSTRACT

Human milk is the only source of vitamin K for exclusively breastfed neonates. This vitamin is crucial both for blood coagulation (vitamin K_1) and for the normal neurological and skeletal development of the foetus and newborn (vitamin K_2). Since vitamin K is ubiquitous in foods, deficiency is not common in adults, but plasma levels and hepatic storage are very low at birth. In light of the importance of this valuable micronutrient, a non-invasive method for verifying that exclusively breastfed infants are receiving an adequate supply of the vitamin is clearly a topic of great significance. In spite of this, the determination of the several vitamin K homologues in human milk has still not been completely elucidated. This paper presents an HPLC–MS/MS method for the simultaneous determination of phylloquinone, menaquinone-4 (MK-4), and menaquinone-7 (MK-7) in human milk after a simple and effective isolation procedure. Overnight cold saponification and extraction of the analytes with hexane provided yields above 75%. This procedure, combined with high performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS), made it possible to achieve limits of detection (LODs) below 0.8 ng/mL. After a complete validation study, the method was applied to measure vitamin K congeners in several human milk samples; results showed vitamin K_1 concentrations comparable with those reported in the literature. In addition, this is the first study performed for the determination of MK-4 and MK-7 in the maternal milk of Italian women.

1. Introduction

Many developed countries, adopting the recommendations of the World Health Organization, have undertaken information campaigns to promote breastfeeding as the exclusive method of feeding infants for the first six months of life (see e.g. World Health Organization, "10 facts on Breastfeeding", http://www.who.int/features/factfiles/breastfeeding/en/ World Health Organization, 2015). Breast milk is the best nourishment for infants because it is rich in antibodies and provides an adequate intake of almost all nutrients, with the exception of vitamin K.

At birth, the plasma levels of this vitamin are low due to the limited placental transfer and hepatic storage capacity of the foetus (Kries et al., 1988). Maternal consumption of vitamin K antagonists and/or inadequate nutrition during pregnancy are other factors which may contribute to a serious neonatal K hypovitaminosis (Kries et al., 1988). The most severe consequence is a hematologic disorder of the newborn known as vitamin K deficiency bleeding (Kries et al., 1988; Shearer, 2009). Besides negative implications on blood coagulation, a deficiency of vitamin

Abbreviations: CID, collision induced dissociation;; CV, coefficient of variation; FDA, Food and Drug Administration; IS, internal standard; K_1, phylloquinone; K_1-d_7, phylloquinone-d7; LC-F, liquid chromatography with fluorescence detection; HPLC–MS/MS, high performance liquid chromatography tandem mass spectrometry; LLE, liquid–liquid extraction; LLOQ, lower limit of quantitation; LOD, limit of detection; MGP, matrix Gla protein; MK-n, menaquinones; MK-4-d_7, menaquinones-4-d7; MRM, multiple reaction monitoring; NARP, non-aqueous reversed phase; NC, needle current; PIVKA, proteins induced by Vitamin K absence or antagonism; QC, quality control; R^2, coefficient of determination; VKDP, vitamin K-dependent proteins.

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K also has negative repercussions on bone mineralization and neurological development of the infant (Shearer, 2009).

Two molecular forms of vitamin K are known, namely phylloquinone (vitamin K1) and menaquinones (MK-*n*, where *n* is the number of prenyl units of the lateral chain, ranging from 4 to 13; see Fig. 1), which are provided by a common naphthoquinone nucleus and by a different chain in C3 (Shearer and Newman, 2014). Phylloquinone has a phytyl side chain, occurs in leafy-green vegetables and is the major dietary form of this vitamin. MKs-*n* are bacterial catabolites collectively known as vitamin K_2 that occur in fermented foods and in the intestines (Conly and Stein, 1992).

The biological role of vitamin K is that of cofactor for an enzyme involved in the carboxylation of vitamin K-dependent proteins (VKDPs) (Shearer and Newman, 2014). VKDPs are involved in the physiology of bone, soft tissues, kidneys and brain and thus a deficient intake of vitamin K reduces biological functionality with negative implications for coagulation, bone mineralization and the health of the involved soft tissues (Shearer, 1993; Thijssen et al., 2002). On the other hand, there are several studies that correlate the high concentration of MK-4 with the levels of sphingomyelin and sulfatides in the brain, suggesting its role in the biosynthesis of this important class of lipids (Denisova and Booth, 2005; Ferland, 2012). For all these reasons, vitamin K is crucial for proper neurological development during the foetal and neonatal period (Shearer, 2009).

The concentrations of the several homologues of vitamin K in human milk are especially influenced by diet (Greer, 1999; Wijnhoven et al., 2009). K_1 is the most abundant form of this vitamin found in breast milk (0.6–10 ng/mL), principally from the consumption of green leafy vegetables, broccoli and seasoning with oils and vegetable margarine. Foods of animal origin (meat, fish, milk, cheese, eggs) contain K_1 and MK-4, with relatively high concentrations of MK-4 in butter and egg yolk (Schurgers and Vermeer, 2000), but data on MK-4 levels are very few; one report details a concentration of about 0.5 ng/mL in pooled human milk (Indyk and Woollard, 1997). The long-chain MK-*n* are present in ricotta and some other cheeses, and they are probably derived from a process of bacterial fermentation (Schurgers and Vermeer, 2000); maybe for this reason their presence has been highlighted in the milk of Japanese women (Kamao et al., 2007), whose diet is based on the consumption of natto, which is fermented soybean rich in MK-*n* (MK-7 in particular). Since the topic related to the distribution of MK-*n* in human milk still remains controversial, it deserves to be investigated in more detail above all for milk from non-Asian women.

Most studies have been aimed at measuring K_1 by LC coupled to fluorescence detection (LC-F) after a post-column reduction (Haroon et al., 1982; Indyk and Woollard, 2000; Lambert et al., 1992). Two LC-F methods have included the measurement of MK-4 (Thijssen et al., 2002; Indyk and Woollard, 1997), another one has also determined MK-5, MK-7 and MK-10 (Shino, 1988), while only one method has utilized LC–MS/MS for the simultaneous determination of K_1, MK-4 and MK-7 (Kamao et al., 2007). However, these last two methods (Shino, 1988; Kamao et al., 2007) have a sensitivity suitable for measuring the high concentrations of MK-*n* occurring in the milk of Japanese women who

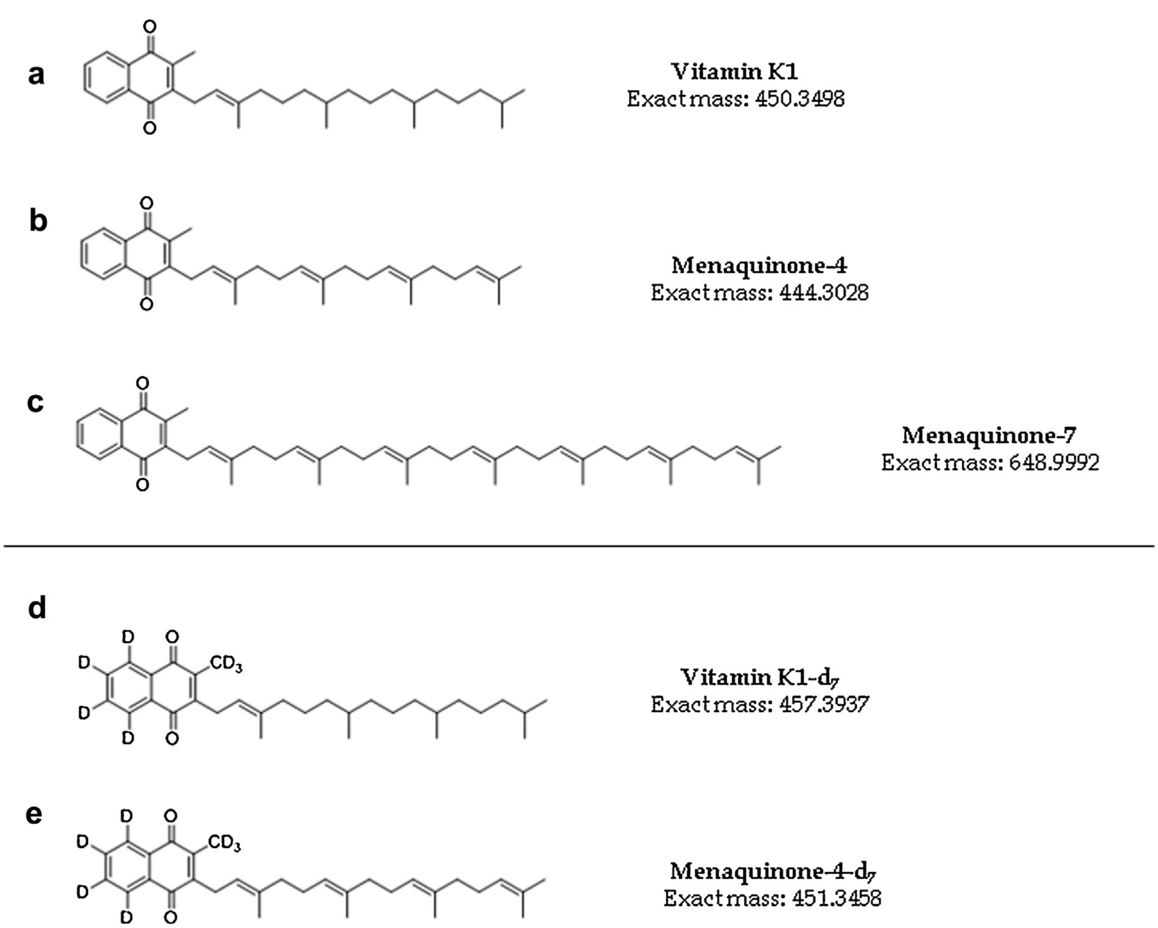

Fig. 1. Chemical structures of (a) Vitamin K_1, (b) menaquinone-4, (c) menaquinone-7, (d) phylloquinone-d7, and (e) menaquinone-4-d7 with their respective exact mass.