Determination of vitamin B_{12} in dairy products by ultra performance liquid chromatography-tandem mass spectrometry

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Abstract

Vitamin B_{12} is a water-soluble molecule composed of a tetrapyrrolic complex with a cobalt atom at its centre. It is an essential regulatory element, synthesized only by bacteria; for this reason it is present only in food of animal origin and the daily requirement for humans is about 1 to 2 μg. Since milk and dairy products provide a significant dietary cobalamin intake, an ultra performance liquid chromatography-tandem mass spectrometry method was applied to samples collected at different stages along the process of cheese making in order to evaluate the distribution of this molecule. In particular, samples of milk, rennet, whey, ricotta cheese, curd, mozzarella cheese and caciotta cheese were analysed. Results showed a level of vitamin B_{12} about 10 times higher in whey and ricotta cheese with respect to the milk they are derived from. These data would confirm the tendency of cobalamine to concentrate in the proteic fractions along the cheese production process.

Introduction

Vitamin B_{12}, or cobalamine, is a water-soluble molecule composed of a tetrapyrrolic complex with a cobalt atom at its centre. It is an essential regulatory element, very important to human physiology since it is involved in the red blood cells formation and nervous system functioning, and its deficiency may lead to anaemia and nerve degenerations. Differently from other vitamins belonging to the B class, vitamin B_{12} is synthesized only by bacteria; for this reason it is present only in food bacterially fermented or obtained from animals that got this cobalamin from their gastrointestinal microflora or diet (Girard *et al*., 2009).

Food of animal origin is the main source of this vitamin for humans and daily requirement is about 1 to 2 μg per day (Chassigne and Lobinski, 1998). In foodstuff, vitamin B_{12} is present in its coenzyme forms: hydroxylcobalamin, 5'-deoxyadenosylcobalamin, methylcobalamin, and cyanocobalamin, which occurs only in small amounts naturally (Kelly *et al*., 2005). Cyanocobalamin is more stable than the other forms which are light sensitive (Kumar *et al*., 2010). Cobalamins are naturally protected from degradation by protein-binding; in bovine milk, naturally occurring vitamin B_{12} is associated with the protein fraction, with high affinity to whey protein (Campos-Giménez *et al*., 2008).

Since milk and dairy products provide a significant dietary cobalamin intake, an ultra performance liquid chromatography-tandem mass spectrometry method (Zironi *et al*., 2013) was applied to samples collected at several stages along the process of cheese making in order to evaluate the distribution of this molecule in different dairy products.

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Materials and Methods

Sample preparation

Samples of milk, rennet, whey, ricotta cheese, curd, mozzarella cheese and caciotta cheese were collected twice along the production processes, which were repeated four times on different days. Routine control on serum reported a pH of 4.9 and a protein content of 1.14%.

Mozzarella was produced using 50 L of unpasteurized milk and natural whey culture as starter. Briefly, the raw milk was heated to 37-38°C, then the whey and rennet were added and left to ripen at 37-38°C for about 4 h. Afterwards, the curd was extracted from the whey, stretched with hot water (90°C) for about 3 min and then molded into the traditional round shape. Each mozzarella weighed about 250 g (Serraino *et al*., 2013).

The extraction procedure was conducted on 5 g of the various matrices adding sodium acetate buffer and potassium cyanide; samples were submitted to heat treatment that, in presence of cyanide, converts all the less stable cobalamins in cyanocobalamin (Zironi *et al*., 2013). For solid matrices it has been necessary to introduce a centrifugation step before solid phase extraction (SPE) clean-up, in order to obtain a better separation between the liquid phase and the solid residue. Samples were then purified by a single SPE step and analysed using reverse phase liquid chromatography coupled with tandem mass spectrometry in positive electrospray ionization (ESI+). Methotrexate was used as internal standard for quantification (Lu *et al*., 2008).

Analytical conditions

The separation was performed by an acquity ultra-performance liquid chromatographic system consisting of a binary pump, solvent degasser, autosampler and column heater fitted with a Waters HSS T3 column (1.7 μm, 2.1×50 mm) equipped with a guard column with the same packing (Waters Corporation, Milford, MA, USA). Flow rate was 0.3 mL/min and the column temperature was kept at 45°C. Separation was carried out in programmed conditions with mobile phase consisting of water (A) and acetonitrile containing 0.1% formic acid (B), for a total run time of 5 min. The gradient was T_0 min: 90% A, 10% B; T_2 min: 20% A, 80% B; T_3 min to the end: 90% A, 10% B.

The mass spectrometer was a Quattro Premiere XE, a triple quadrupole instrument equipped with an ESCI™ Multi-Mode Ionization Source (Waters Corporation).

The whole analysis was conducted in ESI+ mode using multiple reaction monitoring (MRM). The monitored transitions were *m/z* 678.36→147.10 and 678.36→359.30 for vitamin B_{12}, and *m/z* 455.22→175.13 and 455.22→308.22 for methotrexate (IS).

Results and Discussion

The complexity of vitamin B_{12} quantification in food matrices has been investigated by several Authors but very few works on milk, and in particular on dairy products, made use of mass spectrometry. Vitamin B_{12} concentrations found in milk were similar to those described in the literature (Arkbåge *et al*., 2003), although it was not possible to find data com-

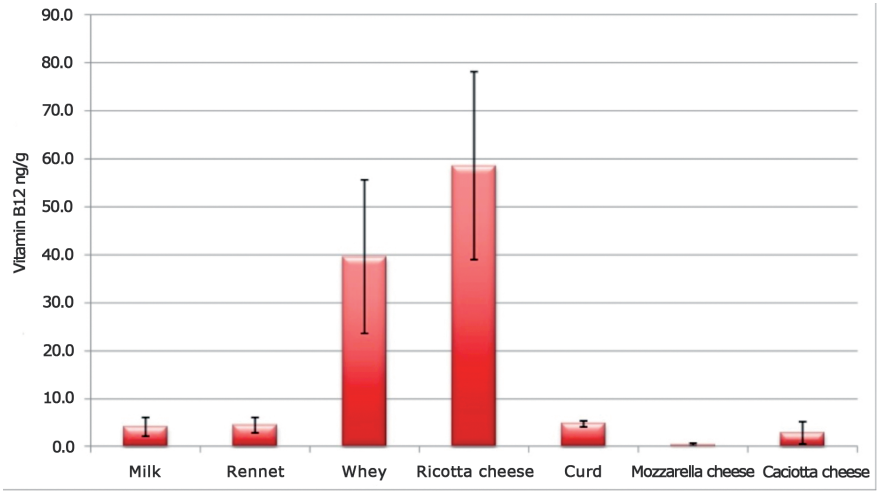

Figure 1. Vitamin B_{12} concentrations in different samples (n=8, mean value±standard deviation).

parable to our cheese making process.

Results showed a level of vitamin B_{12} about 10 times higher in whey and ricotta cheese with respect to the milk they are derived from; at the same time, it is interesting to notice a decrease of cobalamine concentration in curd, caciotta cheese and mozzarella cheese (Figure 1). Vitamin B_{12} affinity for proteins is well known and several kinds of carrier proteins, called transcobalamines, have been identified; these molecules bind cobalamin to protect it from degradation and some analogues are present in bovine milk as well (Fedosov *et al.*, 1995, 1996; Fox and Kelly, 2003; Le *et al.*, 2011).

Conclusions

These data would confirm the tendency of cobalamine to concentrate in the proteic fraction of whey along the cheese production process. The lower value for mozzarella cheese compared to curd is probably due to the process of production, which implies curd draining and a dilution effect related to the water addition in the stretching phase.

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