# UBIQUINONE IN ITALIAN HIGH-QUALITY RAW COW MILK

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## ABSTRACT

The content of ubiquinone (UBN) was evaluated in Italian high-quality (HQ) raw cow milk. Samples were collected from four cowsheds in two different days during summer and winter. The fat content in HQ raw cow milk ranged between 2.86% and 3.46%, while UBN content varied between 0.15 and 0.45 $\mu g/g$ milk. The fat content was significantly influenced by the cowshed only, whereas the UBN content was significantly more affected by both season and sampling days. Although UBN is a lipophilic antioxidant, no statistically significant correlation was found between UBN and fat content in HQ raw cow milk.

## 1. INTRODUCTION

Cow milk is undoubtedly the most frequently consumed dairy product (MERDJI *et al.*, 2015), due to its nutritional composition and properties (ASSOLATTE, 2006). The dairy industry offers many product categories with diverse characteristics, based on consumers' requests and nutritional requirements. The quality of raw milk is greatly affected not only by the technology used for the preservation and diversification of milk products, but also by the characteristics of raw milk itself. The EC Regulation 853/2004 defines raw milk as the product of the mammary gland secretion of farmed animals that has not been heated to more than 40 °C and has not been subjected to any treatment; the same regulation also states the health requirements for raw milk production and its standards of quality. Raw drinking milk can contain pathogenic microflora, so consuming it can pose a significant public health risk (EFSA, 2015). In some EU countries (EC Regulation 853/2004; Intesa Stato-Regioni 25/01/2007), the sale of raw milk is allowed through vending machines, but current law clearly states that it must be heat-treated before being consumed (EC Regulations 852/2004 and 853/2004). The overall quality of raw milk will thus be highly dependent on the breeding conditions and hygienic controls, which will give rise to diverse quality-labelling categories among which that labelled 'high-quality' (HQ) milk is considered the best commercialised one; in fact, the compositional profile of HQ milk is most similar to that of raw milk. The Italian Ministerial Decree 185/1991 imposes rigorous breeder management and hygienic controls aimed to obtain HQ milk, which should fulfil the following stringent sanitary and quality requirements: fat content and protein content $\geq$ 3.50% and 32.0 g/L, respectively; bacterial load $\leq$ 100.000/mL at 30 °C; somatic cells $\leq$ 300.000/mL; lactic acid content $\leq$ 30 ppm; level of non-denatured soluble serum proteins $\geq$ 15.50% of the total protein (when ready for consumption). The Italian Legislation n.169/1989 actually defines HQ fresh pasteurised milk, stating that to further preserve the quality of HQ milk, pasteurization is always necessary and must be performed within 48 h after milking.

Cow milk contains many health promoting compounds, such as vitamins (CLAEYS *et al.*, 2014) and ubiquinone (UBN), also known as coenzyme Q$_{10}$ (MATTILA and KUMPULAINEN, 2001). UBN is present in all cells and membranes, and it has been reported to increase the energy level, to augment the immune system, to act as an antioxidant, to exert a protective effect on the cardiovascular system, and to guard against skin aging and neurodegenerative diseases (HEMAT, 2004; PRAHL *et al.*, 2008; KEWAL, 2011; SAINI, 2011, HECHTMAN, 2011; AMAR-YULI *et al.*, 2009; QUINZII and HIRANO, 2010). As a lipophilic substance, UBN is absorbed following the same process as that of lipids in the gastrointestinal tract, being first incorporated into chylomicrons, followed by absorption and transportation via the lymphatics to the circulatory system (BHAGAVAN and CHOPRA, 2006). Due to its high molecular weight and low water solubility, UBN is poorly and slowly absorbed (Tmax 2-10 h) from the gastrointestinal tract (SEO *et al.*, 2009). PAKAMULA *et al.* (2005) in fact observed different regional permeability of UBN in isolated rat gastrointestinal tracts, suggesting that UBN formulations should target the duodenum to get maximum dosage effect; to compensate for its low absorption rate, diverse strategies can be adopted to enhance UBN bioavailability, such as particle size reduction, solubility improvement (i.e. solid dispersion, complexation, ionization), and use of carriers (i.e. liposomes, microspheres, nanoemulsions, nanoparticles, self-emulsifying systems) (BEG *et al.*, 2010). Once UBN is slowly absorbed from the small intestine, it passes into the lymphatics, and finally to the blood and tissues (GARRIDO-MARAVER *et al.*, 2014). Once UBN reaches the tissues, it is quickly broken down (short half-life of 49-125 h) and degraded by ω-oxidation and β-oxidation of its side-chain (THELIN *et al.*, 1992). The main breakdown product found in tissues, urine and faeces has an intact, fully substituted

ring, a short side-chain (5-7 carbon atoms) and a carboxylated ω-terminus (NAKAMURA *et al.*, 1999). By using labelled UBN, it has been demonstrated that UBN is metabolised in all tissues. The metabolites are converted into more hydrophilic compounds (mostly in their phosphorylated form) in the cells, transported in the blood to the kidney, and excreted into the urine (BENTINGER *et al.*, 2003); however, other minor metabolites were also detected in the faeces, which contained non-metabolised labelled UBN, excreted through the bile. Under certain physical conditions (such as aging, cardiomyophathies, degenerative muscle diseases, and carcinogenesis), UBN concentration can diminish greatly (BENTINGER *et al.*, 2003). Ubiquinone deficiency may be due to insufficient dietary intake, impairment in UBN biosynthesis, excessive utilization by the body or a combination of any of these three (FEDACKO *et al.*, 2011). UBN deficiencies are clinically and genetically heterogeneous. This syndrome has been associated with five major clinical phenotypes: (1) encephalomyopathy, (2) severe infantile multisystemic disease, (3) cerebellar ataxia, (4) isolated myopathy, and (5) nephrotic syndrome (QUINZII and HIRANO, 2011).

Several studies show that milk is a natural source of UBN, whose concentration level varies from species to species and is influenced by the lactation stage and the heat treatment during processing (MATTILA and KUMPULAINEN, 2001; STRAZISAR *et al.*, 2005; NIKLOWITZ *et al.*, 2005; TANG *et al.*, 2006; QUILES *et al.*, 2006). MATTILA and KUMPULAINEN (2001) assessed the levels of coenzyme $Q_9$ (CoQ$_9$) and UBN in milk purchased from major dairies, finding only UBN at a concentration level equal to 0.1 $\mu$g/g in milk (1.5% fat). STRAZISAR *et al.* (2005) also evaluated the UBN content of fresh cow milk produced in a Slovenian farm (3.6% fat), cow milk from the alpine region (3.5% fat), and ultra-heat-treated homogenised milk (3.5% fat), finding a UBN content equal to 1.90 $\mu$g/g, 1.57 $\mu$g/g, and 1.70 $\mu$g/g, respectively. As reported earlier, most studies have been carried out on pasteurised milk, but to the best of our knowledge, there is no report available in the literature about the UBN content in HQ raw cow milk.

The aim of this survey was to evaluate the level of UBN in Italian HQ ("Alta Qualità") raw cow milk. To this purpose, milk samples were collected from four different Italian cowsheds producing HQ milk, in two different days during both summer and winter. Raw cow milk samples were analysed for both fat and UBN content.


## 2. MATERIALS AND METHODS

### 2.1. Chemicals

All reagents and solvents used were analytical grade chemicals. Potassium hydroxide pellets ($\geq$ 85%) and pyrogallol ($\geq$ 98%), were purchased from Carlo Erba (Milan, Italy). Commercial standards of UBN ($\geq$ 98%, HPLC) and CoQ$_9$ ($\geq$ 96%, HPLC) were supplied by Sigma-Aldrich Co. (St. Louis, MO, USA), whereas bidistilled water (100%) was purchased from Panreac (Barcelona, Spain). Petroleum ether with a boiling range 40-60°C ($\geq$ 95.0%), diethyl ether ($\geq$ 99.5%), ethanol ($\geq$ 99.9%), acetonitrile ($\geq$ 99.9%, HPLC), *n*-pentane ($\geq$ 99.0%), anhydrous sodium sulphate ($\geq$ 99.9%), ammonia solution 14 M, and 2-propanol ($\geq$ 99.8%, HPLC), were supplied by Merck KGaA (Darmstadt, Germany). Acetonitrile and 2-propanol were degassed before use by filtering under vacuum through a 0.20 $\mu$m nylon membrane filter (Phenomenex, Westboro, MA, USA).

## 2.2. Sampling and experimental design

Milk samples were obtained from Holstein-Friesian cows that were bred in four different cowsheds. The latter belonged to medium/large-sized Italian farms focusing on the production of HQ milk and located in the valley area between the provinces of Bologna, Mantova, and Modena (Italy). The management of these farms is characterised by a particular attention to hygienic conditions, nutrition and animal welfare, which are the basic requirements for obtaining HQ milk as regulated by the Italian D.M. 185/1991. In these farms, *unifeed* or "single pot" is used for feeding, with the objective of providing a food ration that is homogeneously mixed, properly formulated, and nutritionally balanced. The daily dose administered consisted of 20-25 Kg of unifeed per milk cow. UBN in the food ration is provided mainly by the forage, which is almost exclusively hay from *Medicago sativa L.* UBN was evaluated in hay specimens at different mowing times and was found to be present in an average content of 11 mg/Kg; therefore, the amount of presumed UBN taken in the daily ration could be estimated around 46-58 mg.

Two milk samples were collected from each cowshed (CS) in two different sampling days (SD, i and ii) and two diverse seasons (S, summer and winter), thus giving a total of 16 (4x2x2) samples that were analysed in duplicate (32 analysis in total). Raw cow milk samples were collected from bulk tanks containing the morning and evening milk of the entire herd. The collected milk was placed in 1-L PET bottles and kept at 2-6°C during sample delivery. The milk samples were then divided into 100-mL PET bottles, frozen and stored at -20°C until subsequent analysis. All milk samples were analysed for fat content (by cold extraction), as well as for UBN content (by direct cold saponification followed by extraction of the unsaponifiable matter and high-performance liquid chromatography-ultraviolet diode-array (HPLC-UV/DAD) analysis.

## 2.3. Lipid extraction

Lipids were extracted according to the ISO 14156:2001 method (IDF 172:2001). An aliquot of 100 mL (at 20 °C) milk was introduced into a 500-mL separatory funnel. Eighty mL of ethanol, 20 mL of 14 M ammonia aqueous solution and 100 mL diethyl ether were added, and the funnel was shaken vigorously for 1 min. Thereafter, 100 mL of *n*-pentane were added and the funnel was gently shaken. After phase separation, the aqueous phase was discarded. The organic phase (lipid-containing one) was washed twice with 100 mL of 10% (w/v) sodium sulphate aqueous solution. The organic phase was transferred into a 250-mL Erlenmeyer flask fitted with a ground glass stopper and approximately 10 g of anhydrous sodium sulphate were added. The flask was stoppered, well shaken, and allowed to stand for 10 min. The organic phase was then filtered into a 100-mL round-bottom flask through a Whatman No. 1 filter paper, dried at 40°C using a vacuum rotary evaporator and at the end dried under a gentle stream of nitrogen. The fat content was gravimetrically determined. Two replicates for each sample were performed.

## 2.4. Saponification and extraction of the unsaponifiable matter

The sample preparation method for the quantification of UBN in milk consisted of a direct cold saponification of milk followed by the extraction of the unsaponifiable matter. The direct cold saponification was performed according to the modified method of RENKEN and WARTHESEN (1993). About 35 g of milk were weighed into a 100-mL glass bottle with screw cap. Fifteen micrograms of CoQ₉ (internal standard), 35 mL of 2 N potassium hydroxide in 85% ethanol, and 20 mL of 1% (w/v) pyrogallol in ethanol were added. The headspace of the bottle was flushed with a nitrogen stream to remove the oxygen,

stoppered, and kept at room temperature for 18-20 h, in the dark and under continuous agitation (180 oscillations/min). After the saponification had taken place, the alcoholic soap solution was transferred into a 250-mL separatory funnel to extract the unsaponifiable matter containing UBN. The unsaponifiable portion was extracted by adding consecutively 15 mL of bidistilled water, 5 mL of ethanol, and 35 mL of a petroleum ether:diethyl ether mixture (9:1, v/v), under shaking. After phase separation, the aqueous phase was transferred into a 150-mL separatory funnel. Five mL of ethanol and 35 mL of a petroleum ether:diethyl ether mixture (9:1, v/v) were added, shaken, and allowed to stand until phase separation. The two ethereal fractions were combined in the 250-mL separatory funnel and washed until neutrality was reached by using cold bidistilled water (approximately 3 x 30 mL). The ethereal extract was transferred into a 100-mL Erlenmeyer flask fitted with a ground glass stopper; next, approximately 5 g of anhydrous sodium sulphate were added. The flask was stoppered, shaken well, and allowed to stand for 60 min. The ethereal extract was then filtered into a 100-mL round-bottom flask through a Whatman No. 1 filter paper and dried at 40 °C using a vacuum rotary evaporator. The unsaponifiable matter was dissolved in 1 mL of 2-propanol and filtered through a 0.45 $\mu$m nylon syringe-type filter (Econofilter, 25-mm diameter, Agilent Technologies, Wilmington, DE, USA), before injection into a HPLC system. Two replicates for each sample were performed.

## 2.5. HPLC-UV/DAD determination of UBN

The separation of the compounds of interest (UBN and CoQ$_9$) was performed as suggested by RAO *et al.* (2008), with minor modifications. A HPLC system (HP 1050 series; Hewlett-Packard, Palo Alto, CA, USA) consisting of an autosampler (Series 1100), a quaternary pump, a UV/DAD detector and a Poroshell 120 EC-C18 (3.0 x 50 mm x 2.7 µm particle size) analytical column (Agilent Technologies, Santa Clara, CA, USA), was used. Ten microliters of the sample solution were injected in isocratic mode, using a mixture of acetonitrile:2-propanol (70:30, v/v) at a flow rate of 1.2 mL/min. The column temperature was maintained at room temperature (25°C). The detection wavelength of the UV/DAD detector was set at 275 nm as suggested by KOMMURU *et al.* (1998). Data were acquired using Chemstation for LC3D software (Agilent Technologies, Palo Alto, CA, USA). Standard solutions (CoQ$_9$ and UBN) were prepared in 2-propanol and stored at -20°C in amber vials until further analysis. Their concentrations were periodically checked by measuring the absorbance at 275 nm using a UV-Visible spectrophotometer (V-550; Jasco, Tokyo, Japan) and using the known molar extinction coefficients for CoQ$_9$ ($E^{1\%}_{1cm}$ 185) and UBN ($E^{1\%}_{1cm}$ 165) as reference (HATEFI, 1963; SOUCHET and LAPLANTE, 2007). Two replicates were performed for each sample. The limits of detection (LOD) and quantification (LOQ) were determined according to VIAL and JARDY (1999), with a signal-to-noise ratio equal to 3 and 10, respectively. LOD of UBN was 0.35 $\mu$g/mL, while its LOQ was equal to 1.18 $\mu$g/mL.
The content of UBN was calculated using the following equation [1]:

$$\text{UBN } (\mu\text{g/g milk}) = \frac{A_a \times C_{IS}}{A_{IS}} \times \frac{1}{w_m} \tag{1}$$

Where:
A$_a$ is the peak area of the analyte;
A$_{IS}$ is the peak area of the internal standard;
C$_{IS}$ is the concentration of the internal standard, in $\mu$g;
W$_m$ is the weight of the milk sample, in g.

To identify $CoQ_9$ and UBN in milk samples, commercial standards of both compounds were individually injected into the HPLC system and their corresponding chromatographic retention times were compared with those of the unknown peaks in milk samples. The identification of UBN in milk samples was further confirmed by LC-MS analysis. An analytical column Kinetex 5 $\mu$m (C18 100A) (Phenomenex, Torrance, CA, USA) was used. Fifty microliters of the sample solution were injected in isocratic mode. The eluent mixture was made up of methanol:2-propanol (70:30, v/v) at a flow rate of 1.0 mL/min. The HPLC flow was split in two detectors in parallel, DAD and electrospray interface (ESI), through a three-way valve. ESI was used in positive mode at a voltage of 4.4 kV (LCQ Duo Mass Spectrometer, Thermo Finnigan, San José, CA, USA). The flow was 0.1 mL/min and the temperature was 200°C. The presence of UBN was monitored at $m/z$ 880, which corresponds to the molecular weight of UBN (863.3) + $NH_4$–H. The DAD detector (Varian mod. 330) was set at 275 nm.

## 2.6. Statistical analysis

To perform the statistical tests, Minitab software (version 16.1.0; LEAD Technologies, Inc., Charlotte, NC, USA) was used. The analysis of variance (ANOVA) on the whole set of sample data was assessed, as well as the effects of season (S), cowshed (CS), sampling day (SD), their first-degree interactions (S × CS, S × SD, and CS× SD), and second-degree interaction (S × CS × SD) on fat and UBN contents. Tukey's honest significance test was carried out at a 95% confidence level ($p < 0.05$). The percentage contribution of each factor and interaction was calculated using eta-squared values from the ANOVA summary table. The Pearson's correlation ($\alpha = 0.05$) with two-tailed probability value was used to estimate the strength of association between fat content and UBN content.

## 3. RESULTS

The fat and UBN contents of sixteen samples of HQ raw cow milk were analysed in duplicate.
Table 1 reports the fat content (%) and the UBN content (expressed as $\mu$g/g milk) of HQ raw cow milk samples. Data correspond to the mean of two analytical determinations. A three-factor experimental design was used. Each factor was set at different levels (season (S)-2 levels, cowshed (CS)-4 levels, and sampling day (SD)-2 levels).
The fat content ranged from 2.9% to 3.5%, whereas the UBN content varied from 0.15 to 0.45 $\mu$g/g milk.

**Table 1**. Contents of fat (%) and UBN ($\mu$g/g milk) in raw cow milk.

| Season (S) | Cowshed (CS) | Sampling Day (SD) | Fat | UBN |
|---|---|---|---|---|
| Summer | 1 | i | 2.89±0.08[d] | 0.177±0.004[hi] |
| | | ii | 3.22±0.00[bc] | 0.149±0.001[i] |
| | 2 | i | 3.34±0.02[ab] | 0.198±0.013[gh] |
| | | ii | 2.95±0.02[d] | 0.451±0.000[a] |
| | 3 | i | 2.97±0.03[d] | 0.236±0.019[fg] |
| | | ii | 3.43±0.02[a] | 0.274±0.015[ef] |
| | 4 | i | 3.01±0.05[d] | 0.192±0.009[ghi] |
| | | ii | 2.94±0.02[d] | 0.312±0.002[de] |

| | | | | |
|---|---|---|---|---|
| | 1 | i | 3.00±0.10$^d$ | 0.351±0.007$^{cd}$ |
| | | ii | 2.98±0.06$^d$ | 0.378±0.010$^{bc}$ |
| | 2 | i | 3.46±0.03$^a$ | 0.355±0.020$^{cd}$ |
| | | ii | 3.31±0.00$^{abc}$ | 0.413±0.003$^{ab}$ |
| Winter | 3 | i | 2.93±0.00$^d$ | 0.294±0.015$^e$ |
| | | ii | 2.92±0.01$^d$ | 0.236±0.006$^{fg}$ |
| | 4 | i | 3.18±0.03$^c$ | 0.421±0.019$^{ab}$ |
| | | ii | 2.86±0.01$^d$ | 0.420±0.003$^{ab}$ |

Values are expressed as mean±standard deviation of two replicates. i and ii correspond to 2 independent sampling days. Different letters within the same column denote statistically significant differences at $p < 0.05$ (Tukey's test) between the milk samples; the letter "a" indicates the highest value and "e" the lowest one.

Figure 1 shows the HPLC-UV/DAD chromatograms (at 275 nm) of CoQ$_9$ standard solution, the unspiked and spiked (with UBN) unsaponifiable fraction of HQ raw cow milk. Since CoQ$_9$ was absent in HQ milk, it was therefore used as internal standard (IS) in the quantitative determination of UBN.
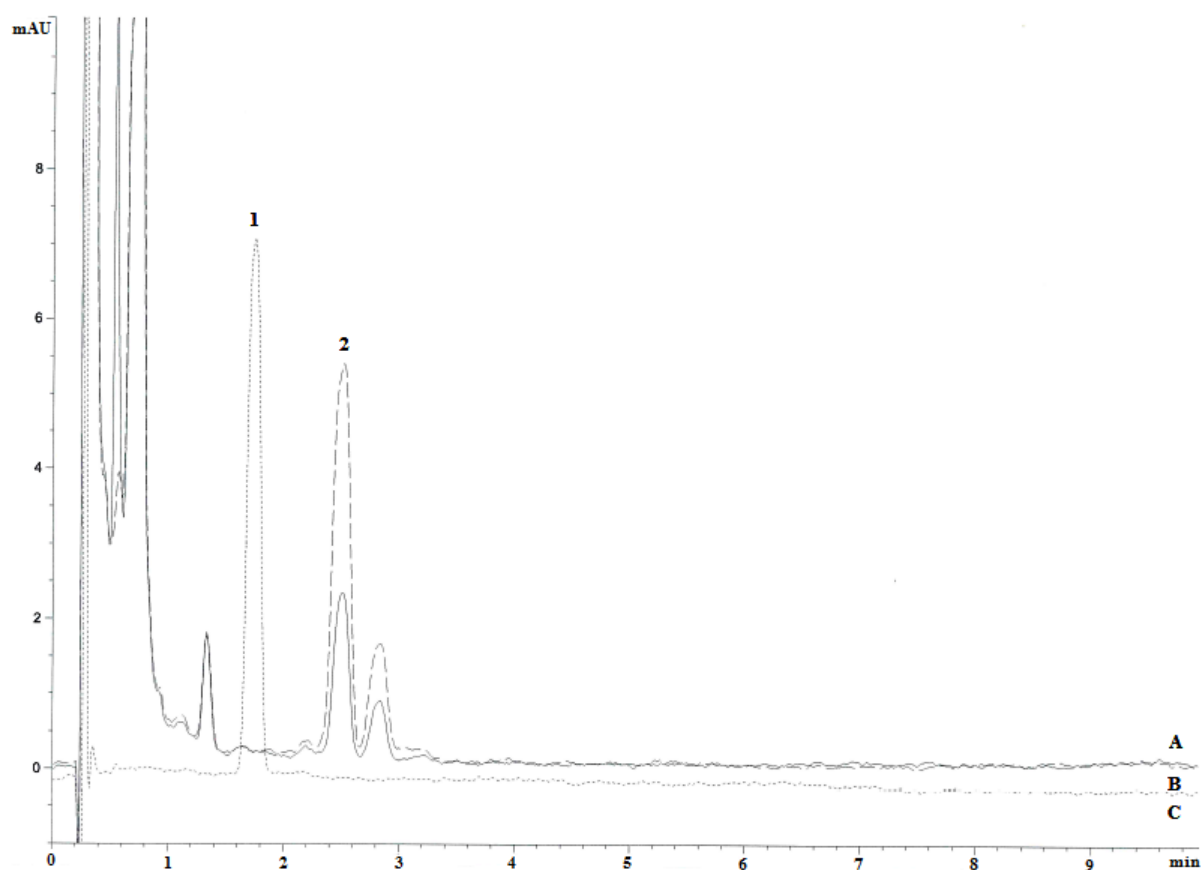


**Figure 1**. Overlaid HPLC-UV/DAD chromatograms (at 275 nm) of the unspiked (A) and the spiked (with UBN) unsaponifiable fraction of raw cow milk (B), as well as the CoQ$_9$ standard solution (C). Peak identification: 1, CoQ$_9$ internal standard; 2, UBN.

## 4. DISCUSSION

The fat content was determined to find out whether there was a correlation with the level of UBN in the HQ milk samples. Data analysis (Table 2) was carried out to emphasise the effect of each individual factor and their first-and second-degree interactions on fat and UBN contents of HQ raw cow milk.

Regarding milk fat content, data processing showed that it was significantly influenced by cowshed (CS) (28.1%) and by all interactions. However, season (S) and sampling day (SD) factors had no significant effects on the HQ raw cow milk fat content. The fat content of HQ milk significantly varied among cowsheds; in general, the highest milk fat content was found at CS no. 2, followed by 3, 1, and 4. This variation may be due to feeding with different fodders and concentrates, the lactation stage of individual animals from herds, the variability among animals and/or the CS location. The average fat content of milk varies considerably through lactation, from approximately 3% in early lactation to more than 4.5% in late lactation, and among individuals (FOX and KELLY, 2012).

Concerning the UBN content of HQ milk, it was influenced by all factors and their interactions (Table 2); however, the factors that exerted the greatest influence were S (32.9%) and CS (19.5%). The highest content of UBN was found in milk from cowshed no. 2, in which the CS x SD interaction indicated that the UBN milk content varied according to the sampling day.

**Table 2**. Effects of season, cowshed, sampling day, and their first- and second-degree interactions on fat (%) and UBN contents ($\mu$g/g milk).

| Factor | Fat | UBN |
|---|---|---|
| **Season (S)** | | |
| Summer | 3.09 | 0.249[b] |
| Winter | 3.08 | 0.358[a] |
| *p/contribution (%)* | *0.353 n.s./0.1* | *< 0.001***/32.9* |
| **Cowshed (CS)** | | |
| Cowshed 1 | 3.02[bc] | 0.264[c] |
| Cowshed 2 | 3.27[a] | 0.354[a] |
| Cowshed 3 | 3.07[b] | 0.260[c] |
| Cowshed 4 | 3.00[c] | 0.336[b] |
| *p/contribution (%)* | *< 0.001***/28.1* | *< 0.001***/19.5* |
| **Sampling Day (SD)** | | |
| i | 3.10 | 0.278[b] |
| ii | 3.08 | 0.329[a] |
| *p/contribution (%)* | *0.148 n.s./0.3* | *< 0.001***/7.1* |
| **S x CS** | | |
| Summer x cowshed 1 | 3.06[cd] | 0.163[e] |
| Summer x cowshed 2 | 3.15[bc] | 0.324[c] |
| Summer x cowshed 3 | 3.20[b] | 0.255[d] |
| Summer x cowshed 4 | 2.98[de] | 0.252[d] |
| Winter x cowshed 1 | 2.99[de] | 0.364[b] |
| Winter x cowshed 2 | 3.38[a] | 0.384[b] |
| Winter x cowshed 3 | 2.93[e] | 0.265[d] |
| Winter x cowshed 4 | 3.02[de] | 0.420[a] |
| *p/contribution (%)* | *< 0.001***/21.6* | *< 0.001***/16.7* |

| S x SD | | |
|---|---|---|
| Summer x i | 3.06$^b$ | 0.201$^c$ |
| Summer x ii | 3.13$^a$ | 0.297$^b$ |
| Winter x i | 3.14$^a$ | 0.355$^a$ |
| Winter x ii | 3.02$^b$ | 0.361$^a$ |
| *p/contribution (%)* | *< 0.001\*\*\*/6.5* | *< 0.001\*\*\*/5.5* |
| **CS x SD** | | |
| Cowshed 1 x i | 2.94$^c$ | 0.264$^d$ |
| Cowshed 1 x ii | 3.10$^b$ | 0.263$^d$ |
| Cowshed 2 x i | 3.40$^a$ | 0.276$^d$ |
| Cowshed 2 x ii | 3.13$^b$ | 0.432$^a$ |
| Cowshed 3 x i | 2.95$^c$ | 0.265$^d$ |
| Cowshed 3 x ii | 3.18$^b$ | 0.255$^d$ |
| Cowshed 4 x i | 3.10$^b$ | 0.306$^c$ |
| Cowshed 4 x ii | 2.90$^c$ | 0.366$^b$ |
| *p/contribution (%)* | *< 0.001\*\*\*/29.6* | *< 0.001\*\*\*/11.9* |
| **S x CS x SD** | | |
| *p/contribution (%)* | *< 0.001\*\*\*/11.7* | *< 0.001\*\*\*/5.6* |

Values are expressed as mean of two replicates. Abbreviations: CS, cowshead; S, season; SD; sampling day; i and ii correspond to 2 independent sampling days. Different letters within the same column denote statistically significant differences (Tukey's test $p < 0.05$) between the milk samples; the letter "a" indicates the highest value and "e" the lowest one. Significant differences are denoted by asterisks: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; $p \geq 0.05$, non-significant.

The level of UBN in milk may be influenced by genetic characteristics, diet, duration of lactation, length of gestation (TANG *et al.*, 2006), and metabolic changes (SOUCHET and LAPLANTE, 2007). Previous studies on the quantification of UBN were carried out using various dairy product categories that contained different fat percentages and that underwent diverse heat treatments (MATTILA and KUMPULAINEN, 2001; STRAZISAR *et al.*, 2005). Thus, the comparison with our results is difficult. STRAZISAR *et al.* (2005) reported a higher UBN content (1.90 $\mu$g/g milk) in fresh cow milk (3.6% fat) than that detected in the present study (0.15-0.45 $\mu$g/g milk). The same authors noticed that UBN levels were lower in UHT milk having diverse fat content (0.46 $\mu$g/g milk in skimmed milk (0.5% fat), 1.16 $\mu$g/g milk in semi-skimmed milk (1.6% fat), and 1.70 $\mu$g/g milk in whole milk (3.5% fat)). However, MATTILA and KUMPULAINEN (2001) found a significantly lower UBN content (0.1 $\mu$g/g milk) in commercially available semi-skimmed milk (1.5% fat), probably due to the heat treatment. As already mentioned, UBN is known to be a temperature sensitive molecule; MILIVOJEVIC FIR *et al.* (2009) in fact showed that pure UBN is degraded by 72.3% after being exposed at 80°C for 120 min in the presence of UV light.

Regarding the correlation of the fat and UBN contents in HQ raw cow milk samples, the results of the current study did not show any significant relationship ($r= - 0.034$, $p = 0.855$). This lack of correlation is further confirmed by the scatter plot (**Figure 2**) developed with the fat and UBN contents (linear regression and no fit intercept). This is in contrast to the data of STRAZISAR *et al.* (2005), who noticed a positive correlation trend between the UBN and the fat contents of milk, even though it was not statistically confirmed. On the other hand, NIKLOWITZ *et al.* (2005) found a high level of UBN in human colostrum, even though its fat content was low.
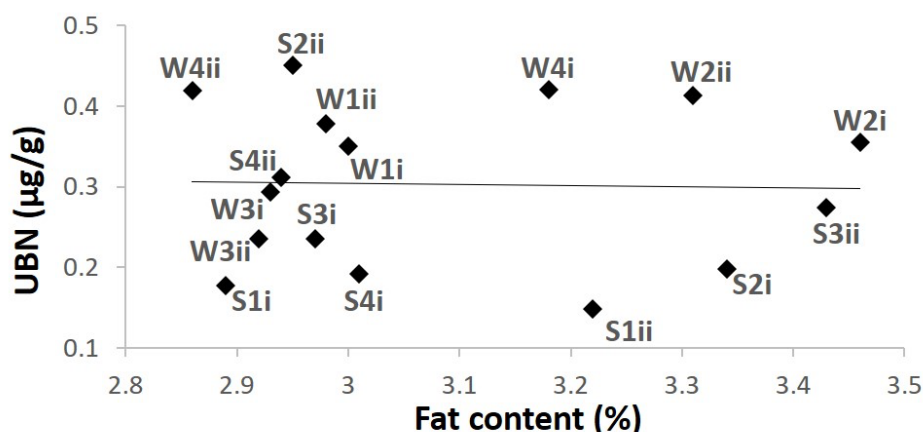
**Figure 2**. Scatter plot of fat content vs. UBN content. Abbreviations: S, summer; W, winter; 1-4, number of single cowsheds; i, sampling day 1; ii, sampling day 2.

The scatter plot of Fig. 2 also displays the distribution of the samples according to both variables (UBN and fat content). Only two groups can be distinguished in terms of fat content: one below 3.02% (10 samples) and the other above 3.17% (6 samples). No more clustering was evident as related to the rest of variables (cowshed, season, sampling day).

## 5. CONCLUSIONS

The content of UBN in Italian HQ ("Alta Qualità") raw cow milk was for the first time determined in the present study, ranging from 0.15 to 0.45 $\mu$g/g milk. Although health authorities have not yet established specific dietary intake recommendations for UBN, some researchers suggest a daily dose of 30-200 mg UBN for 19-year adult and older (EFSA, 2010). Considering that the guidelines for healthful Italian food habits published by the Italian National Institute for Research on Food and Nutrition (INRAN, 2003) recommend an average consumption of three 125-mL portions of milk and/or yogurt a day, a consumption of 250 mL of HQ raw milk would potentially provide an intake of 0.04-0.11 mg UBN. However, UBN content in pasteurised HQ milk may suffer a decline due to the heat treatment (especially UHT) and storage, even though modern pasteurization technologies applied to HQ milk with relatively low temperatures and short times (72°C for 15 sec) should limit significant thermal degradation of UBN. The results of this study indicate that the UBN content in HQ raw cow milk was significantly affected by both seasons and sampling days within the same cowshed. However, no significant correlation was found between UBN and fat content in HQ raw cow milk, even though UBN is a lipophilic antioxidant. Although an investigation involving a greater number of different cowshed types from diverse locations and product categories would be necessary to better understand the contribution level of the various factors on the increase of UBN content in milk, the adoption of the Italian HQ regulation by the EU could be a way to further improve the nutritional quality of European milk, including its UBN content.

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