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DEVELOPMENT OF ANALYTICAL METHODS FOR PHENOTYPIC CHARACTERIZATION OF ANTIOXIDANT COMPOUNDS AND ANTIOXIDANT ACTIVITY OF MILK

SVILUPPO DI METODI ANALITICI PER LA CARATTERIZZAZIONE FENOTIPICA DI COMPOSTI ANTIOSSIDANTI E PER L'ATTIVITÀ ANTIOSSIDANTE DEL LATTE

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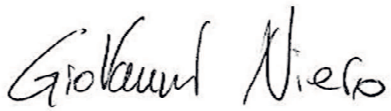
DECLARATION

I declare that the present thesis has not been previously submitted as an exercise for a degree at University of Padova, or any other University, and I further declare that work embodied is my own.



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ABSTRACT

Free radicals are unstable and reactive molecules, with one ore more unpaired electrons in the outer orbit and adverse effects on plant, animal, and human cells. In particular free radicals are responsible of lipids peroxidation, proteins oxidative damages, and DNA cleavage, resulting in increased risk of mutation. Antioxidants represent an important defence against these injuries. Vegetable derived foods are known as important sources of dietary antioxidants such as phenols, anthocyanin, tocopherols, tocotrienols, carotenoids, retinol precursors, and ascorbate.

With this background, the aims of this Ph.D. project were: i) to develop and validate analytical methods for quantification of tocopherols, thiols, and total antioxidant activity (TAA) of milk; ii) to describe the phenotypic variation of thiols in milk of dairy and dual-purpose cow breeds; iii) to describe the phenotypic variation of TAA in milk of cow, buffalo, goat, and sheep; iv) to evaluate the feasibility of mid infrared spectroscopy to predict TAA of cow milk; v) to evaluate the effect of skimming and heat treatments on milk tocopherols and TAA.

In the 1st Chapter, a simplified saponification protocol, followed by cheap HPLC method based on methanol elution and sensible fluorescence detection was developed and validated for the quantification of α-tocopherol and γ-tocopherol, in different types of commercial fluid milk. Chromatograms showed two analytical peaks, corresponding to α-T and γ-T. The method was able to detect the adverse effects of skimming and UHT treatment on α-T and γ-T concentrations. The proposed method could be usefully adopted in future for large scale studies, aiming to investigate phenotypic variation of tocopherols in milk.

The 2nd Chapter represent a first contribution to the characterization of low molecular weight thiols in milk of different cattle breeds. Thiols were extracted from the soluble fraction of milk, and following a derivatization protocol they were separated by reverse phase HPLC and detected

fluorimetrically. Six thiol species were detected and two of them, glutathione (GSH) and cysteine-glycine (Cys-Gly), were identified and quantified. The average concentration of Cys-Gly in milk was greater than that of GSH, and milk from dual-purpose breeds was richer in thiols than milk from dairy cows.

The 3rd Chapter deals with the development and the validation of a robust and fast spectrophotometric method for the determination of TAA of different types of milk. The method was linear, and highly repeatable and reproducible. Preservative added on raw milk had negligible effects on TAA measurement. The greatest TAA was measured on skimmed pasteurised milk, followed by skimmed UHT milk, raw milk, whole pasteurised milk, and whole UHT milk.

The 4th Chapter is the first contribution to the phenotypic characterization of TAA of bovine milk. This phenotype exhibited an exploitable variability, similar to that of other quality traits. Favourable phenotypic correlations of TAA with fat, protein, and casein percentages were observed, as well as with somatic cell score. Total antioxidant activity of milk increased across lactation. Mid-infrared prediction models developed to predict milk TAA were not enough accurate for analytical purposes.

The 5th Chapter deals with the phenotypic characterization of TAA of buffalo, goat, and sheep milk. Sheep milk showed the greatest TAA. This is probably due to its relatively high content in fat, protein, and casein percentages, that are known as compounds contributing to milk antioxidant capacity. Accordingly, buffalo and goat milk had lower TAA as well as lower fat, protein and casein percentages. Milk TAA was unfavourably correlated with milk yield, but the relationship was significant only for buffalo, whereas protein and casein percentages were positively correlated with TAA of goat milk.

1. GENERAL INTRODUCTION

FREE RADICALS AND OXIDATIVE STRESS

Free radicals (**FR**) are generally defined as molecules with one or more unpaired electrons in the outer orbit (Gilbert, 2000). For this reason, they are very unstable and reactive oxidant species, with potential adverse effects on biological substrates, including plant, animal, and human cells. These molecules are primarily generated as by-products of the mitochondrial phosphorylative oxidation (Dröge, 2002), and secondary as a consequence of space radiations on biological tissues (Kovalev, 1983). During these processes, with a relatively low statistical incidence, one or more electrons can be subtracted to molecular oxygen (O_2) and nitrogen (N_2). Therefore, basing on their chemical nature, FR can be divided in two major classes. The first consists of reactive oxygen species (**ROS**) including superoxide, hydroxyl, peroxyl, alcoxyl, and hidroperoxyl radicals. The second class consists of reactive nitrogen species (**RNS**) that includes nitric oxide and nitrogen dioxide radicals (Evans and Halliwell, 2001).

On one hand, both ROS and RNS play important roles in several physiological and regulated processes occurring in animal cells, such as signal transduction, gene transcription (Zheng and Storz, 2000), pathogens killing, vascular smooth muscle relaxation, leukocyte adhesion, and angiogenesis (Ignarro et al., 1999). On the other hand, an uncontrolled production of FR lead to an altered cell homeostasis and red-ox status, with negative effects on essential cell components (Robbins and Cotran, 2010). In particular lipids undergo peroxidation provoking alterations of cell membranes, proteins suffer oxidative damages and loose their structure and enzymatic functions, whereas DNA can be altered or cleaved leading to an increased risk of mutation (Robbins and Cotran, 2010). All these negative effects determine what is generally defined as oxidative stress.

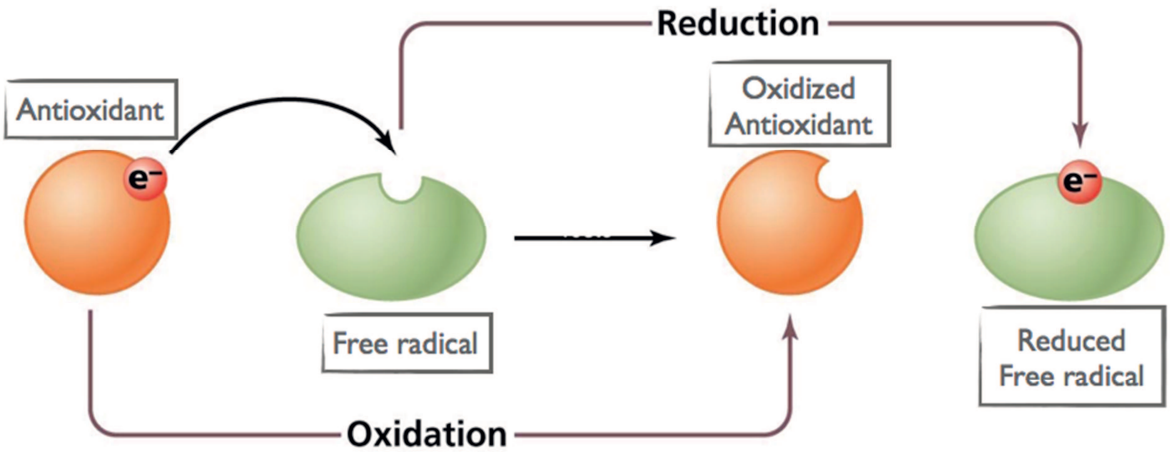
In humans, oxidative stress and FR injuries at biochemical level have been associated with an increased risk of many different clinical diseases, such as cancer, atherosclerosis, rheumatoid arthritis, neurodegeneration, and diabetes (Gilbert, 2000).

ANTIOXIDANTS AND REDOX REACTIONS

Free radicals exert their negative effects on biological substrates through a red-ox reaction: lipids, proteins, and DNA donate electrons to FR, acting as the reducing and the oxidizing species, respectively. This reaction leads to the reduction and deactivation of FR, but also to the oxidation and the damage of essential cellular components.

In this scenario, antioxidants are molecules able to donate electrons to FR, playing as reducing species instead of lipids, protein, and DNA (Figure 1). In other terms antioxidants can be defined as molecules that are able, at relatively low concentrations, to prevent the oxidation of other substrates, being the favourite target of ROS and RNS species (Halliwell and Gutteridge, 1989).

Figure 1. Red-ox reaction between antioxidant and free radical



In biology, antioxidants are generally classified in enzymatic and non enzymatic. The major enzymatic antioxidants are superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase (Pacher et al., 2007; Halliwell, 2007). Non enzymatic forms are further classified into metabolic, produced by cell and body metabolisms, and dietary, provided through foods and dietary supplements. Non enzymatic metabolic antioxidants include lipoid acid, glutathione, arginine, coenzyme Q10, melatonin, uric acid, bilirubin, metal-chelating proteins, and transferrin (Willcox et al., 2004). Finally, non enzymatic dietary antioxidants are vitamin A (retinol), vitamin C (ascorbate), vitamin E (tocopherols and tocotrienols), carotenoids (carotene, lycopene, and lutein), flavonoids, trace metals (selenium, manganese, zinc), omega-3, and omega-6 fatty acids (Gupta and Singh, 2013).

ANTIOXIDANTS IN HUMAN HEALTH AND DIET

Vegetables are the most well known foods rich in antioxidants: grape, red wines, blueberries, and red fruits have been investigated for great content in phenols and anthocyanin (Vallverdú-Queralt et al., 2015; Timmers et al., 2017), seeds and olive oils are known as major sources of tocopherols and tocotrienols (Duthie et al., 2016), tomatoes and carrots are rich in carotenoids and retinol precursors (Watzl et al., 2003), whereas orange, citrus fruit, kiwi fruit, and broccoli contain great amount of ascorbate (Koh et al., 2009; Ramful et al., 2011; Vissers et al., 2013).

The positive effects of these compounds on human health have been widely discussed in literature, and their protective action towards many different clinical diseases have been proved. Broadly speaking, healthy and nutraceutical properties of antioxidants are mainly associated with their activity as FR scavengers, and thus with their capability on the prevention of cellular oxidative stress. In particular, the strongest evidence for the efficacy of phenols and polyphenols against chronic disease exists in respect to cardiovascular disease risk (Del Rio et al., 2013),

whereas tocopherols consumption and supplementation demonstrated anti-inflammatory activities, especially towards asthma and allergic airway diseases (Jiang, 2014). Retinol and its precursors play a central role in maintaining the lining surface of eyes and respiratory, urinary, and intestinal tracts. Moreover, they are involved in the preservation of skin and mucous membranes by creating a biodefense barrier against bacteria and viruses, and in the regulation of immune function by supporting production and functionality of white blood cells (Darwish et al., 2016). Finally, recent findings of Dixit et al. (2017) demonstrated the protective role of ascorbate in Alzheimer's disease mouse model.

MILK ANTIOXIDANTS

Few studies investigated antioxidants content in animal matrices such as meat, eggs, milk, and derived or processed products (Micke et al., 2002; Pereira et al., 2016). Nevertheless, milk seems to be one of the most promising among animal derived foods, as regard antioxidant content and variety.

Chaveau-Duroit et al. (2010) proposed high pressure liquid chromatography (HPLC) and ultra-high pressure liquid chromatography (UPLC) methods for quantification of tocopherols in cow milk: α-tocopherol was described as the major milk tocopherol, whereas γ-tocopherol was detected in traces, without a precise quantification. Other studies demonstrated that milk antioxidant concentration is influenced by animal feeding and diet as well as by farming strategies. In particular, carotenoids and tocopherols concentration in cow milk rapidly decrease following a switch from grass silage to hay diet (Nozière et al., 2006). Similarly, greater α-tocopherol contents were measured in organic buffalo milk and mozzarella cheese, compared to the same conventional dairy products (Bergamo et al., 2003).

Milk antioxidants have attracted the attention of scientific community for their relevance on human diet and health, but they have been studied also for several applications in the field of food science and technology. In this scenario, the ratio between reduced ascorbate and oxidized ascorbate species can be used as an extremely sensitive marker of oxidative stress in raw milk (Nielsen et al., 2001). Also, tocopherols play an important role in maintaining an oxidative stability of milk and dairy products (Havemose et al., 2006; Bouwstra et al., 2008) and vice versa, processing techniques like skimming and heating may adversely affect the bioavailability of tocopherols (Romeu-Nadal et al., 2008).

Milk proteins, including casein and whey proteins, have been investigated for their antioxidant properties. Zulueta et al. (2009) reported that casein is one of the main compounds contributing to milk antioxidant activity, due to its high content in antioxidant amino acids (Rival et al., 2001). Also casein hydrolysates derived from tryptic digestion showed antioxidant activity by sequestering metal ion catalysts and by direct scavenging of FR (Kitts and Weiler, 2003). Whey proteins have been studied for their antioxidant properties as cysteine donor, and as molecules involved in the maintenance of suitable glutathione concentration in cell (Kent et al., 2003). Micke et al. (2002) demonstrated that whey protein formulas are able to increase glutathione levels in HIV-infected individuals, thanks to their great content in cysteine and glutathione precursor peptides. Moreover, lactoferrin is able to bind iron and to block its pro-oxidant action (Cichosz et al., 2017), and β-lactoglobulin and derivate peptides preserve retinol and α-tocopherol from oxidation along digestive trait (Liang et al., 2011), and deactivate FR through Trp, Tyr, and Met amino acids (Cichosz et al., 2017).

MILK TOTAL ANTIOXIDANT ACTIVITY

The isolation of each single antioxidant compound in milk requires laborious and invasive sample pre-treatment, such as saponification, derivatization, sample drying and concentration (Havemose et al., 2006; Bouwstra et al., 2008; Chauveau-Duriot et al., 2010). Moreover, qualitative and quantitative characterizations are carried out through specific analytical protocols, requiring specialized technical skills, and involving the use of expensive laboratory tools, such as gas chromatography, HPLC, UPLC, and mass spectrometry (Chauveau-Duriot et al., 2010). Such problems can be partially faced considering milk total antioxidant activity (**TAA**), defined as the sum of antioxidant contributions related to the previously mentioned antioxidant molecules (Chen et al., 2003). Milk TAA can be measured through a simple, fast, and cheap spectrophotometric assay. Additionally the study of milk TAA might have higher impact and relevance for human nutrition and health, since it is defined as the total of different antioxidant activities.

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2. AIMS OF THE THESIS

The present thesis aimed to develop analytical methods for phenotypic characterization of antioxidant compounds and total antioxidant activity of milk.

The specific aims were:

i. To develop a fast and non-invasive milk sample preparation protocol, and to validate a cost effective and safety HPLC separation program coupled with fluorescent detection, for quantification of tocopherols in milk;

ii. To characterize soluble thiols in milk of dairy and dual-purpose cow breeds;

iii. To develop and validate a simple, robust, fast and cost-effective spectrophotometric assay for the determination of TAA on cow milk;

iv. To assess the associations between milk TAA and traditional milk quality traits, to investigate sources of variation of milk TAA, and to evaluate the feasibility of MIRS to predict TAA of cow milk;

v. To describe the phenotypic variation of TAA of buffalo, goat, and sheep milk, and to assess correlations between total antioxidant activity and milk yield and quality traits.

3. CHAPTER 1st

Development and validation of a HPLC method for the quantification of tocopherols in different types of commercial cow milk

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ABSTRACT

In the present study a methanol-fluorescence based HPLC method was validated for its utility to quantify α-tocopherol (α-T) and γ-tocopherol (γ-T) in raw milk (RM), whole UHT milk (WUM), partially skimmed UHT milk (SUM), whole pasteurised milk (WM), and partially skimmed pasteurised milk (SM). Repeatability and reproducibility, calculated as relative standard deviation of ten measurements within a day and thirty measurements across 3 days, respectively, were always below 5% for both tocopherols concentrations and retention times. Recovery was assessed through three spiking levels and it ranged from 89 to 107%. The method was able to detect the expected declines in tocopherols in milk exposed to UHT treatment or being skimmed. Vitamin E, calculated as the sum of α-T and γ-T, was similar in WM and RM, averaging 1.57 and 1.56 mg L^{-1} followed by WUM (1.33 mg L^{-1}), SM (0.77 mg L^{-1}), and SUM (0.61 mg L^{-1}).

Key words: milk, tocopherols, HPLC, ultra high temperature, skimming

INTRODUCTION

Tocopherols are among the major fat soluble antioxidants and they exist in nature in four variants, namely α-, β-, γ-, and δ-tocopherol (**T**). These molecules belong to a wider class of organic compounds, commonly known as vitamin E that includes also α-, β-, γ-, and δ-tocotrienols (Ju et al., 2009). Both *in vivo* and *in vitro* studies demonstrated that tocopherols have direct and indirect antioxidant activity, exerted through the interruption of free radicals chain reactions (Traber, 2007) and the activation of antioxidant enzymes as cofactors (Ogawa et al., 2008), thus preventing DNA cleavage, and protein and lipid oxidative alteration (Wu and Cederbaum, 2003). Human epidemiological studies showed that there is an association of a low vitamin E status with an increased risk of cancer and heart diseases (Dietrich et al., 2006; Ju et al., 2009).

Tocopherols cannot be synthesized either in human or in animal metabolism, and therefore dietary sources are essential to reach the recommended daily requirement (Ford et al., 2006). In particular, α-T and γ-T are the major dietary tocopherols, being the most biologically active and those which are the most prevalent in the diet among the four tocopherols (Ford et al., 2006; Ju et al., 2009). Vegetable oils, nuts, and seeds as major sources of tocopherols have been widely studied for their contents of tocopherols (Lu et al., 2015). Among the animal derived foods, cow milk and milk products are good sources of these compounds as well (Guo et al., 2014), and thus they are worthy of scientific investigation for two main reasons. Firstly, tocopherols are frequently used as feed additive for dairy cows in order to cover their requirements and to improve milk quality, health status, fertility and productive performance (Allison and Laven, 2000; Baldi et al., 2000). Secondly, from a technological point of view, tocopherols play an important role in maintaining an oxidative stability of milk and dairy products (Havemose et al., 2006; Bouwstra et al., 2008) and vice versa, processing techniques like skimming and heating may adversely affect the bioavailability of the tocopherols (Romeu-Nadal et al., 2008).

In the literature, several alkaline saponification procedures for milk tocopherols extraction, as well as HPLC protocols for tocopherols separation and detection have been described. Nevertheless, the majority of these procedures involves a time-consuming samples pre-treatment, that might in addition affect accuracy of the results (Ramalho et al., 2012). Moreover, the chromatographic conditions are mainly based on toxic and expensive mobile phases such as heptane (Meglia et al., 2006), hexane (Ellis et al., 2007), acetonitrile, dichloromethane (Chauveau-Duriot et al., 2010) or triethylamine (Ramalho et al., 2012). Finally, UV and visible wavelengths are often used for detection, resulting in a weak signal-to-noise ratio and a low sensibility, which makes the accurate quantification of traces tocopherols such as γ-T difficult (Chauveau-Duriot et al., 2010; Guneser and Yuceer, 2012; Ramalho et al., 2012). The present

work aimed to develop and validate a fast milk sample preparation protocol, and to validate a cost effective HPLC separation program coupled with sensible fluorescence detection, for quantification of tocopherols in different types of commercial cow milk.

MATERIALS AND METHODS

Chemicals and Equipment

Methanol (purity ≥ 99.9%), ethanol (purity ≥ 99.8%), hexane (purity ≥ 95%), toluene (purity ≥ 99.9%), KOH pure pellets, ascorbate (purity ≥ 99.0%), α-T (purity ≥ 96%), β-T (purity ≥ 96%), γ-T (purity ≥ 96%), and δ-T (purity ≥ 90%) were purchased from Sigma Aldrich (St Louis, MO, USA). Ultrapure water was used for the preparation of the solutions. Milk tocopherols were detected and quantified through a quaternary pump HPLC device (Waters Alliance 2695; Waters Corporation, Milford, USA), equipped with a fluorescence detector (Waters Multi Fluorescence Detector 2475; Waters Corporation, Milford, USA) and a reverse phase C18 column (Nucleodur PolarTec 250 mm, 3-µm particle size; Macherey-Nagel, Düren, Germany).

Sample Collection and Preparation

Raw milk (**RM**) sample was purchased in a vending machine (Zurich, Switzerland). Four milk samples of the same dairy brand, including whole UHT milk (**WUM**), partially skimmed UHT milk (**SUM**), whole pasteurised milk (**WM**), and partially skimmed pasteurised milk (**SM**) were purchased in a single local commercial store (Zurich, Switzerland). According to the nutritional labels, the fat contents were 3.7% (RM), 3.9% (WM and WUM), and 2.5% (SM and SUM). All samples were kept at 4°C and warmed at room temperature for 2 h before analysis. Aliquots of 10 mL milk were poured into screw cup glass vessels, and complemented with 1 g of ascorbate, 10 mL of methanol and 10 mL of 0.18 M KOH in methanol and water (1:2). The

resulting mixture was cooked in a water bath at 100°C for 1 h in hermetically closed vessels. Samples were cooled at room temperature, rinsed with 35% ethanol and transferred to a volumetric flask up to a volume of 100 mL. An aliquot of 10 mL of this mixture was added with 3 mL of hexane and toluene (1:1), and gently shake for 5 min to promote tocopherols extraction. Samples were centrifuged for 5 min at 2500 g and 2 mL of the organic layer were dried under nitrogen. Finally, samples were resuspended in 1 mL of methanol and analysed in HPLC.

Determination of Tocopherols by HPLC

Sample vials were kept at 10°C in a refrigerated auto sampler. Gradient elution was carried out with a mixture of three solvents (A, B and C), which consisted of 85% (A), 95% (B) and 100% (C) methanol, respectively. The solvents were flushed with He and sonicated in ultrasonic bath to prevent bubble development. Injection volume was 25 μL. The elution program is depicted in the background of Fig. 1 and consisted of a linear gradient from 100 to 50% A and from 0 to 50% B in 30 min, a linear gradient from 50 to 0% A, from 50 to 0% B and from 0 to 100% C in 1 min, an isocratic elution at 100% C for 9 min, and a linear return to the starting condition within 1 min. Before injecting the following sample, the column was re-equilibrated under the starting conditions for 9 min. Therefore, the total analysis lasted 50 min per sample. The flow rate was 0.4 mL min^{-1}, the column temperature was kept at 30°C and the detection was carried out in fluorescence with an excitation wavelength of 295 nm and an emission wavelength of 330 nm.

Tocopherol Quantification and Recovery

The chromatogram peaks were identified by comparing retention times with external standards and verifying the increasing of fluorescence signal with internal standard.

Quantification of each single peak was obtained with five points calibration curves, made up with increasing standard concentrations. For all calibration curves the coefficient of determination was ≥0.99. Results were corrected by the recovery rate of δ-T, used as internal standard, in order to cope with the underestimation due to the losses of target molecules.

Recovery of the method was assessed separately for RM, WUM, SUM, WM, and SM through three spiking levels of the starting milk (Biswas et al., 2011). The final concentrations of the α-T and the γ-T standards were 1.00 and 0.25 mg L^{-1}, 2.00 and 0.50 mg L^{-1} and 4.00 and 1.00 mg L^{-1}, for spiking 1, 2 and 3, respectively. The aliquots were mixed and split in ten sub-aliquots processed separately as previously described.

Specificity, Resolution, and Sensitivity

According to the ICH Harmonised Tripartite Guidelines (2005), the specificity of the method is the ability to assess unequivocally the analyte in the presence of impurities, interferences and matrix components which may be expected to be present. Resolution (R) of the method, defined as the quality of the separation of two neighbour peaks (1^{st} and 2^{nd}) was calculated according to Chauveau-Duriot et al. (2010) as:

$$R = 2 \times (Rt_{2nd} - Rt_{1st}) / (W_{1st} + W_{2nd}),$$

where Rt_{1st} and Rt_{2nd} (min) are retention times of the first and the second peak, and W_{1st} and W_{2nd} (min) are widths of the first and the second peak at the baseline.

The sensitivity of the method was calculated as limit of detection (LOD) and limit of quantification (LOQ). Limit of detection, defined as the lowest analytic concentration which can be reliably distinguished from the limit of blank (Armbruster and Pry, 2008), was set at a signal-to-noise ratio of 3:1 (Chauveau-Duriot et al., 2010). The limit of quantification, also called functional sensitivity, defined as the lowest concentration at which the analyte can be reliably

detected excluding bias (Armbruster and Pry, 2008), was set at a signal-to-noise ratio of 10:1 (Chauveau-Duriot et al., 2010).

Statistical Analysis

The assumption of normality of milk tocopherols content was checked using Shapiro-Wilk's test and by visual inspection of the distributions. Repeatability was calculated as the relative standard deviation (RSD_r) of ten consecutive measures of the RM, WUM, SUM, WM, and SM samples within the same day, both for tocopherol concentration and retention time. Similarly, reproducibility was calculated as the relative standard deviation (RSD_R) of thirty measures obtained across 3 days of analyses, both for tocopherols concentration and retention time, as proposed by Sturaro et al. (2016) and Niero et al. (2017) in chromatographic and spectrophotometric applications, respectively.

RESULTS AND DISCUSSION

Specificity and Sensitivity

The chromatographic track obtained from RM (Fig. 1), WUM, SUM, WM, and SM revealed two major peaks, corresponding to γ-T and α-T, being the first and the second in elution order and the minor and the major in signal abundance, respectively. On the other hand, chromatograms did not show any signal related to β-T and δ-T, meaning that these tocopherols species are absent in milk. The findings of the present study are in agreement with results of Chauveau-Duriot et al. (2010) who reported that α-T is the major tocopherol in milk, whereas γ-T was only detected in traces, without detailed quantification. Considering the cleanness of the whole chromatogram, the almost total absence of background noises and interference signals, and the complete separation of the two chromatographic peaks corresponding to the target molecules,

we can state that the proposed method shows a high specificity for these two milk tocopherols (ICH Harmonised Tripartite Guideline, 2005) (Fig. 1). The resolution between α-T and γ-T chromatographic peaks in RM, WUM, SUM, WM, and SM ranged from 1.83 to 2.12, thus exhibiting greater separation performances compared with HPLC and UPLC applications proposed by Chauveau-Duriot et al. (2010), which ranged from 1.43 to 1.93.

Limit of detection and LOQ calculated through standard solutions were 0.15 and 0.50 ng mL^{-1} for α-T and 0.24 and 0.80 ng mL^{-1} for γ-T, respectively. The minimum concentration of α-T found in the present study was 85 fold the LOD, and the lowest concentration of γ-T found was 12 times the LOD. Thus optimal confidential level and sensitivity were provided.

The present method showed greater specificity and sensitivity compared with HPLC and UPLC based protocols developed by Chauveau-Duriot et al. (2010). The improvements reached in the present study could be partially due to several analytical precautions that were adopted: 1) the less invasive procedure for milk sample preparation, compared to other laborious saponification protocols involving repeated extractions phases and rotary evaporator (Ellis et al., 2007; Guneser and Yuceer, 2012), and 2) the high specificity and sensitivity of fluorescence used for tocopherols detection (Niero et al., 2015) instead of the less specific UV and visible wavelengths. The method is relatively cheap in terms of both analytical apparatus and reagents: 1) HPLC equipment is cheaper compared to UPLC (Chauveau-Duriot et al., 2010), and 2) methanol is considerably less expensive than other mobile phases such as heptane (Meglia et al., 2006), hexane (Ellis et al., 2007), acetonitrile, dichloromethane (Chauveau-Duriot et al., 2010), and triethylamine (Ramalho et al., 2012) that have been proposed as eluents for tocopherols chromatographic analyses. Overall, about 20 milk samples can be prepared and analysed in 24 hours, at a cost of about 2.20-2.50 € per sample.

Repeatability and Reproducibility

Table 1 shows repeatability and reproducibility obtained for α-T and γ-T quantification. Values of RSD_r indicated a high repeatability for each type of commercial milk, ranging from 1.65% for RM γ-T on day 3, to 4.09% for SUM γ-T on day 3, and values of RSD_R suggested a high reproducibility, ranging from 2.62% (RM) to 4.45% (SUM). Therefore, in accordance with results of Sturaro et al. (2016) and Niero et al. (2017), a slight decline in accuracy was found between days compared with the accuracy found within day (Fig. 1).

Repeatability and reproducibility were even more accurate for retention times of α-T and γ-T (Table 2). For repeatability, the RSD_r ranged from 0.02% to 1.14%, and for reproducibility the RSD_R remained always below 1%, ranging from 0.04% to 0.87%. The overall accuracy of the method is illustrated in Fig. 2.

Recovery

Recovery tests reached the greatest values for the first spiking level in SUM, scoring 105% and 107% for α-T and γ-T, respectively, whereas the lowest recoveries were observed in the third spiking level for γ-T, averaging 89% and 91% in SUM and WUM, respectively (Table 3). Therefore, the maximum error in overestimation was +6.6% whereas the major bias in underestimation was -10.8%. On average, repeatability of recovery tests was in the range between 1.77% for α-T in spike level 1 of WM, and 3.78% for α-T in spike level 2 of WUM. These values were similar to recoveries reported by other authors for HPLC and spectrophotometric applications (Bonfatti et al., 2008; Biswas et al., 2011).

For RM, WUM, SUM, and SM a slight decline in recovery percentages was observed for higher spiking levels. Accordingly, the recovery was most generally efficient for low spiking

levels. This phenomenon might be partially due to the saturation of the solvents used for milk sample preparation at the high spiking levels.

Means and Variation of Tocopherols in Milk

Descriptive statistics of α-T and γ-T content in RM, WUM, SUM, WM and SM are shown in Table 4. The α-T was numerically most concentrated in WM (mean of 1.41 mg L^{-1}), followed by RM (1.35 mg L^{-1}), WUM (1.18 mg L^{-1}), SM (0.65 mg L^{-1}), and SUM (0.47 mg L^{-1}). The average concentration of α-T in RM observed in the present work is close to values reported by Baldi et al. (2000), Nozière et al. (2006), and Guneser and Yuceer (2012). On the other hand, lower milk tocopherol concentrations were reported by Havemose et al. (2006) and Marino et al. (2014). Comparisons with other papers dealing with tocopherols and vitamin E in milk is difficult because of the different units of measure applied (Butler et al., 2008; Ramalho et al., 2012).

The greatest numerical concentration of γ-T was measured in RM (mean of 0.21 mg L^{-1}), followed by WM (0.16 mg L^{-1}), WUM (0.15 mg L^{-1}), and SUM (0.14 mg L^{-1}). The milk numerically poorest in γ-T content was SM (0.12 mg L^{-1}). The overall amount of vitamin E in milk, calculated as the sum of α-T and γ-T concentrations, was numerically similar in WM (mean of 1.57 mg L^{-1}) and RM (mean of 1.56 mg L^{-1}), and was numerically higher than that of WUM (1.33 mg L^{-1}) and SM (0.77 mg L^{-1}). The numerically lowest vitamin E content was observed in SUM, with mean of 0.61 mg L^{-1}. The vitamin E content of milk from cows is considerably higher than that of buffaloes (Spagnuolo et al., 2003) but lower than that of goats (Guneser and Yuceer, 2012) and yaks (Guo et al., 2014). These differences might be related to the different fat content of the milk samples, as well as to the different physiological mechanisms and efficiencies involved in vitamin E transportation from feed to blood, and from blood to milk (Kalač, 2012).

Recovery of Expected Effects of Skimming and UHT Treatment on Tocopherols Concentration

The expected effect of skimming treatment on the lipophilic milk tocopherols concentration (Manzi and Pizzoferrato, 2010) was found when comparing WM with SM (declines of 54% and 25% in α-T and γ-T, respectively), and WUM with SUM (declines of 60% and 6.7% in α-T and γ-T, respectively) (Table 4). The adverse effect of UHT treatment on vitamin E (Guneser and Yuceer, 2012) was found also in whole milk and skim milk with declines of 16% and 6.3% in α-T and γ-T concentration with whole milk and 28% in α-T concentration in skim milk, where no difference in γ-T concentration was found.

CONCLUSIONS

A simple and fast saponification protocol, followed by cheap HPLC method based on methanol elution and sensible fluorescence detection was validated for the quantification of α-T and γ-T in different types of commercial fluid milk. Good validation results (specificity, sensibility, reproducibility, repeatability and recovery) were obtained for both tocopherols concentrations and retention times. Chromatograms obtained from RM, WUM, SUM, WM, and SM showed two analytical peaks, corresponding to α-T and γ-T. α-tocopherol was the major tocopherol in milk, ranging from 43.8 mg L^{-1} (SUM) to 154.0 mg L^{-1} (WM), and γ-T showed the lowest and the greatest concentration in SUM (9.5 mg L^{-1}) and RM (22.0 mg L^{-1}). The method was able to detect the adverse effects of skimming and UHT treatment on α-T and γ-T. For these reasons, the present method could be usefully adopted in future for large scale studies, aiming to investigate phenotypic variation of tocopherols in milk. At the same time, this would allow to assess the ability of mid infrared spectroscopy to predict vitamin E content in milk, and to propose specific breeding strategies for the enhancement of this nutraceutical item.

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Table 1. Relative standard deviation of repeatability (RSD_r, %; n = 10) and relative standard deviation of reproducibility (RSD_R, %; n = 30) for mean concentrations (mg L^{-1}) of α-tocopherol and γ-tocopherol in milk

Milk [a]	Day 1		Day 2		Day 3		
	Mean	RSD_r	Mean	RSD_r	Mean	RSD_r	RSD_R
RM							
α-tocopherol	1.37	2.26	1.37	2.69	1.32	3.46	3.21
γ-tocopherol	0.21	2.89	0.21	3.13	0.21	1.65	2.62
WUM							
α-tocopherol	1.22	2.36	1.17	3.88	1.15	2.74	3.82
γ-tocopherol	0.15	3.01	0.15	3.01	0.14	3.19	3.41
SUM							
α-tocopherol	0.46	3.72	0.47	2.74	0.49	4.52	4.45
γ-tocopherol	0.11	3.52	0.10	3.36	0.10	4.09	4.36
WM							
α-tocopherol	1.45	3.59	1.38	3.28	1.40	2.24	3.70
γ-tocopherol	0.16	2.87	0.16	3.86	0.16	3.88	3.60
SM							
α-tocopherol	0.65	3.54	0.65	3.64	0.66	3.14	3.42
γ-tocopherol	0.12	3.04	0.12	3.36	0.12	3.33	3.61

[a] RM = raw milk; WUM = whole UHT milk; SUM = partially skimmed UHT milk; WM = whole pasteurised milk; SM = partially skimmed pasteurised milk.

Table 2. Relative standard deviation of repeatability (RSD_r, %; n = 10) and relative standard deviation of reproducibility (RSD_R, %; n = 30) for mean retention time (min) of α-tocopherol and γ-tocopherol

Milk [a]	Day 1		Day 2		Day 3		
	Mean	RSD_r	Mean	RSD_r	Mean	RSD_r	RSD_R
RM							
α-tocopherol	34.0	1.14	34.1	0.02	34.1	0.14	0.65
γ-tocopherol	32.9	0.64	32.8	0.03	32.9	0.16	0.39
WUM							
α-tocopherol	32.9	0.06	32.9	0.07	33.0	0.02	0.11
γ-tocopherol	31.6	0.06	31.6	0.08	31.7	0.03	0.11
SUM							
α-tocopherol	33.0	0.03	33.0	0.03	32.9	0.02	0.04
γ-tocopherol	31.7	0.03	31.7	0.03	31.7	0.03	0.05
WM							
α-tocopherol	34.1	0.06	34.0	0.03	33.5	0.07	0.79
γ-tocopherol	32.9	0.07	32.8	0.03	32.3	0.08	0.87
SM							
α-tocopherol	33.5	0.32	33.2	0.02	33.2	0.06	0.51
γ-tocopherol	32.3	0.34	31.9	0.03	31.9	0.06	0.56

[a] RM = raw milk; WUM = whole UHT milk; SUM = partially skimmed UHT milk; WM = whole pasteurised milk; SM = partially skimmed pasteurised milk.

Table 3. Recovery (%) and relative standard deviation of repeatability (RSD_r, %; n = 10), for α-tocopherol and γ-tocopherol in milk

Milk [a]	Spike level 1		Spike level 2		Spike level 3	
	Recovery	RSD_r	Recovery	RSD_r	Recovery	RSD_r
RM recovery						
α-tocopherol	98.5	2.56	95.3	1.89	93.7	3.21
γ-tocopherol	96.2	3.63	96.0	3.37	92.1	2.62
WUM recovery						
α-tocopherol	101.5	1.98	93.1	3.78	95.9	3.48
γ-tocopherol	94.6	3.67	94.3	2.24	91.4	2.76
SUM recovery						
α-tocopherol	104.8	2.97	101.2	2.17	96.3	2.82
γ-tocopherol	106.6	3.31	96.3	1.86	89.2	2.75
WM recovery						
α-tocopherol	92.3	1.77	92.2	2.20	94.0	2.02
γ-tocopherol	95.3	2.73	95.6	2.13	95.2	3.81
SM recovery						
α-tocopherol	103.2	3.00	95.4	2.99	95.6	3.50
γ-tocopherol	104.8	1.92	100.3	2.12	100.8	2.43

[a] RM = raw milk; WUM = whole UHT milk; SUM = partially skimmed UHT milk; WM = whole pasteurised milk; SM = partially skimmed pasteurised milk.

Table 4. Descriptive statistics of α-tocopherol and γ-tocopherol in milk (mg L^{-1}; n = 30)

Milk [a]	Mean	SD [b]	Minimum	Maximum
RM				
α-tocopherol	1.35	4.34	1.25	1.42
γ-tocopherol	0.21	0.54	0.20	0.22
WUM				
α-tocopherol	1.18	4.51	1.10	1.26
γ-tocopherol	0.15	0.50	0.14	0.16
SUM				
α-tocopherol	0.47	2.11	0.44	0.52
γ-tocopherol	0.14	0.52	0.10	0.12
WM				
α-tocopherol	1.41	5.21	1.33	1.54
γ-tocopherol	0.16	0.57	0.15	0.17
SM				
α-tocopherol	0.65	2.23	0.59	0.69
γ-tocopherol	0.12	0.43	0.11	0.13

[a] RM = raw milk; WUM = whole UHT milk; SUM = partially skimmed UHT milk; WM = whole pasteurised milk; SM = partially skimmed pasteurised milk.

[b] SD = standard deviation.

Figure 1. Elution program applied for the separation of milk tocopherols in HPLC (solvent A 85% methanol, solvent B 95% methanol, solvent C 100% methanol), overlaid with the chromatogram obtained with untreated raw milk

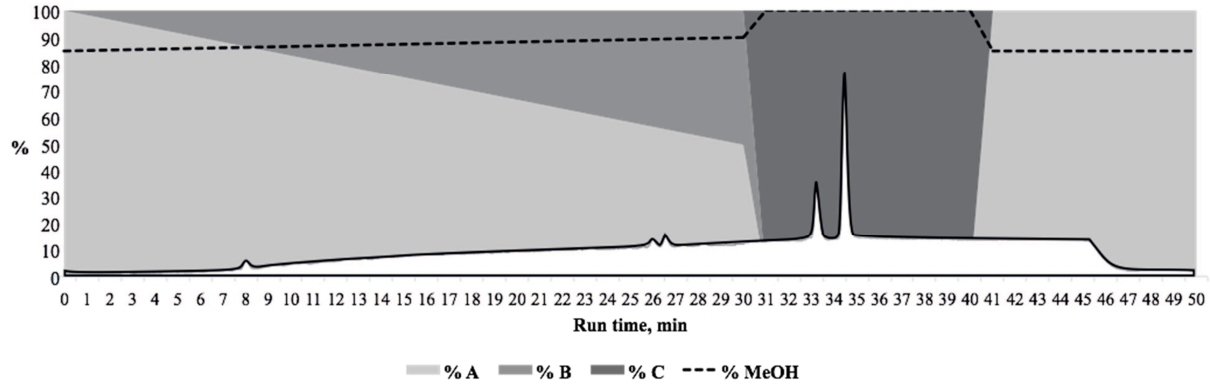
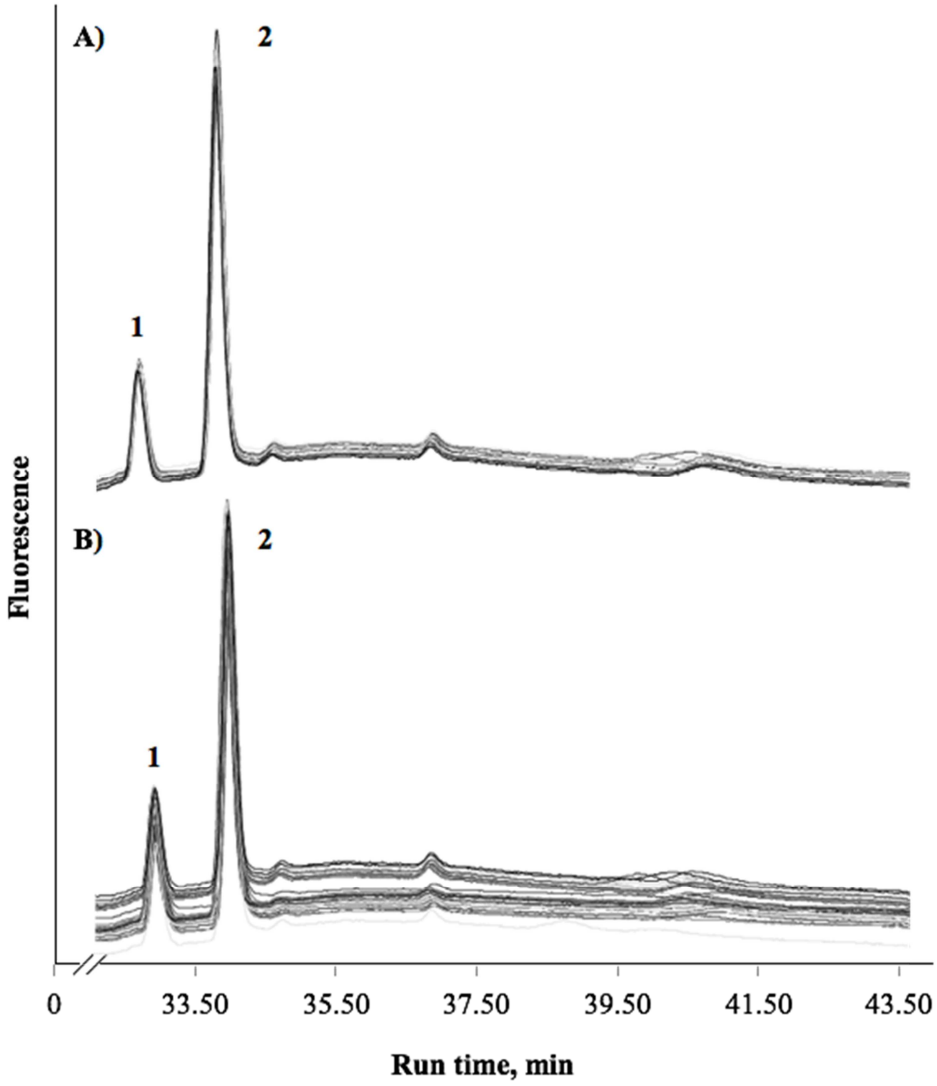


Figure 2. Overlaid chromatograms relative to (A) repeatability (n = 10) and (B) reproducibility (n = 30) for tocopherols in raw milk: γ-tocopherol (1) and α-tocopherol (2)



4. CHAPTER 2nd

Short communication: **Characterization of soluble thiols in bovine milk**

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ABSTRACT

Antioxidants are molecules essential for the maintenance of cell homeostasis and their intake through the diet has positive effects on human health. Among antioxidants, low molecular weight (LMW) thiols represent an important class of compounds. The aim of this study was to identify LMW thiols in bovine milk. A total of 96 individual milk samples from Brown Swiss, Holstein-Friesian, Alpine Grey, and Simmental cattle breeds were collected in 8 herds. LMW thiols were extracted from the soluble fraction of milk, and following a derivatization protocol they were separated by reverse phase high performance liquid chromatography and detected fluorimetrically. Six thiol species were detected and two of them, glutathione (GSH) and cysteine-glycine (Cys-Gly), were identified and quantified. Regardless of the breed, the average concentration of Cys-Gly in milk was greater than that of GSH. Overall, milk from dual-purpose breeds (Simmental and Alpine Grey) was richer in LMW thiols than milk from dairy cows (Holstein-Friesian and Brown Swiss). Glutathione and Cys-Gly, closely linked metabolically, were strongly correlated. Pearson correlations of Cys-Gly with protein and casein contents were moderately low, and no relationship was found between GSH and milk chemical composition. Future research should focus on the identification of all detected LMW thiol species.

Key words: thiol, antioxidant, milk, cattle breed

SHORT COMMUNICATION

Free radicals (**FR**) are molecules with an unpaired electron in the outer orbit (Gilbert, 2000). For this reason they are very unstable and reactive, and may have adverse effects on animal and human cells. Free radicals can be divided in two major classes. The first includes the reactive oxygen species (**ROS**) such as superoxide, hydroxyl, peroxyl, alcoxyl and hidroperoxyl radicals, and the second includes the reactive nitrogen species (**RNS**) such as nitric oxide and

nitrogen dioxide radicals (Evans and Halliwell, 2001). These molecules are produced in animal cells as a consequence of space radiations on biological substrates (Kovalev, 1983) or as by-products, during the mitochondrial phosphorylative oxidation (Dröge, 2002). Both ROS and RNS play an important role in physiological processes that occur in animal cells, such as signal transduction, gene transcription (Lander, 1997; Zheng and Storz, 2000), pathogens killing, vascular smooth muscle relaxation, leukocyte adhesion and angiogenesis (Ignarro et al., 1999). However an excessive production of FR has negative effects on animal cell molecular structures such as lipid peroxidation, oxidative alterations of proteins, and DNA cleavage (Robbins and Cotran, 2010). These biochemical injuries may lead to clinical diseases such as cancer, atherosclerosis, rheumatoid arthritis, neurodegeneration, and diabetes (Jackson, 1999; Gilbert, 2000).

Antioxidants acting against FR are essential for the maintenance of cell homeostasis. Antioxidants are defined as substances that are able, at relatively low concentration, to compete with other oxidizable substrates, thus preventing their oxidation. This definition includes antioxidant enzymes and molecules acting as antioxidants themselves, such as α-tocopherol, β-carotene, ascorbate, and glutathione (**GSH**), a tripeptide composed by cysteine, glycine, and glutamate (Halliwell and Gutteridge, 1989). In particular, GSH belongs to the class of low molecular weight (**LMW**) thiols, highly reactive molecules, massively involved in the maintenance of cellular redox homeostasis (Pivato et al., 2014) and in FR scavenge by formation of disulphide bond (Fang et al., 2002). Other LMW thiols, biosynthetically connected with GSH, are cysteine, cysteine-glycine (**Cys-Gly**), cysteamine, and γ-glutamylcysteine.

The daily intake of these compounds through the diet plays an important role against FR and contributes positively to diseases preventing. The most well known dietary matrices rich in thiols and antioxidants are of vegetable origins (Ames et al., 1993; Halliwell, 1996; Sies, 1997;

Beckman and Ames, 1998; Gutteridge and Halliwell, 2000). Few studies have investigated antioxidants and thiols content in animal matrices such as milk and milk whey (Hakkak et al., 2000; Micke et al., 2002; Niero et al., 2014). Moreover, most of the literature addressed the problem in a general sense, investigating the total antioxidant capacity of milk (Chen et al., 2003), and the antioxidant properties of peptides, caseins, and whey proteins (Chiang and Chang, 2005; Pihlanto, 2006) or the total content of sulfhydryl groups (Cosio et al., 2000). Therefore, the aim of this work was to characterize soluble thiols in milk of dairy and dual-purpose cattle breeds.

Individual milk samples (n = 96) of Brown Swiss (**BS**), Holstein-Friesian (**HF**), Simmental (**SI**), and Alpine Grey (**AG**) cows from parity 1 to 8 and from 5 to 524 DIM were collected in 8 herds between April and May 2014 by the South Tirol Dairy Association (Bolzano, Italy). The same number of milk samples (n = 24) was available for each breed. Immediately after sampling, milks were added with preservative and transferred at 4°C to the laboratory of the South Tirol Dairy Association. Milk chemical composition was determined using a MilkoScan FT6000 (Foss Electric A/S, Hillerød, Denmark) and SCC was assessed by Fossomatic (Foss Electric A/S, Hillerød, Denmark). Values of SCC were transformed to SCS through the formula $SCS = 3 + \log_2(SCC/100{,}000)$. An aliquot of each individual sample was transferred to the Department of Agronomy, Food, Natural resources, Animals and Environment of the University of Padova (Legnaro, Italy), and stored at -20°C.

Milk samples were thawed at room temperature and centrifuged for 10 min at 12,000 rpm to promote the separation of fat from the soluble fraction. To ensure protein precipitation, 250 µl of the soluble fraction were added with 34 µl of HCl 4M and centrifuged for 10 min at 14,000 rpm. Following this step, a pellet containing high molecular weight proteins and a supernatant, composed of LMW proteins and thiols, was obtained. An aliquot of 50 µl of supernatant was

added with 117 µl of 1M borate buffer (pH 10.5), 33 µl of 1% tributylphosphine and 33 µl of 0.3% ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F). The solution obtained was kept in water bath at 60°C for 1 h. Samples were cooled in ice for 2 min. The reaction was stopped and the fluorescent products were stabilized adding 17 µl HCl 4M. Before HPLC analysis, samples were filtered with a 0.22 nm filters (Masi et al., 2002).

An aliquot of derivatized sample was analysed in HPLC (Beckman Coulter System Gold 126), equipped with a reverse phase (**RP**)-C18 column (Luna Phenomenex, 150x4.6, 3 µm particle size). The HPLC analysis was carried out in isocratic conditions (0.3 ml/min), with a mobile phase of 75 mM NH4-formiate (pH 2.9) – metanol (97:3). Fluorescent compounds were detected with a fluorimetric detector (Yasco 821FP, λex 386 nm, λem 516 nm). Chromatograms peaks were identified by comparing retention times with standards. Quantification of each single peak was obtained with calibration curves, made up with different standard concentrations.

The normal distribution of LMW thiols in milk were checked using Shapiro-Wilk's test. Pearson correlations between traits were estimated through the CORR procedure of SAS (SAS Institute Inc., Cary, NC, USA). Sources of variation of LMW thiols concentration in milk were investigated using the GLM procedure of SAS. The model included the fixed effects of breed, herd nested within breed, parity (4 classes: parity 1, parity 2, parity 3, and parities 4 to 8), DIM (5 classes: 5 to 60 d, 61 to 120 d, 121 to 180 d, 181 to 240 d, and 241 to 524 d), and first-order interactions between breed and parity, and breed and DIM. Significance of breed effect was tested on the herd within breed variance. A multiple comparison of means was performed for the breed effect using Bonferroni's test ($P < 0.05$).

For each milk sample, RP-HPLC analysis showed six chromatographic peaks, which corresponded to six LMW thiols species. Only two of them (Cys-Gly and GSH) were identified by comparing the retention time of samples peaks with peaks obtained using internal and external

standards (Figure 1). Glutathione is the main cellular LMW thiol and it protects cell components by acting as a redox buffer to quench a variety of reactive chemical species, such as reactive oxygen and nitrogen species, or to scavenge or conjugate metals, xenobiotics, and other reactive electrophilic species (Messens et al., 2013). Glutathione is degraded by γ-glutamyl transferases to yield glutamic acid and Cys-Gly, which is further converted to cysteine and glycine by a dipeptidase. Cysteine, which could not be detected in this study, is indeed generally found at low concentrations because it is known to be either rapidly converted into other compounds or incorporated in proteins.

The mean (SD) concentrations of Cys-Gly and GSH were 0.087 (0.068) and 0.067 (0.064) μM, respectively (Table 1), which are comparable with results of Niero et al. (2014) in a study that aimed at investigating different concentrations of microparticulated whey proteins, added during cheese-making process, on the recovery of LMW thiols in cheese. Means (SD) of fat content, protein content, casein content, and SCS were 3.98 (0.61)%, 3.56 (0.46)%, 2.79 (0.37)%, and 2.58 (1.84), respectively (Table 1). These values are similar to findings of Penasa et al. (2014), who compared three cattle breeds in mixed dairy herds for predicted milk coagulation properties and composition traits. Finally, pH and urea content averaged 6.66 (SD: 0.06) and 22.8 mg/dL (SD: 7.1), respectively (Table 1). Urea concentration reported in this work is slightly greater than findings of other studies (Jonker et al., 2002; Nousiainen et al., 2004).

Cysteine-glycine exhibited moderately low Pearson correlations with protein content (0.290; $P < 0.01$), casein content (0.268; $P < 0.01$), and urea content (0.205; $P < 0.05$), and GSH did not show any significant relationships ($P > 0.05$) with traditional milk components (Table 2). Glutathione and Cys-Gly, which are closely linked metabolically (Wu et al., 2004), were strongly correlated (0.653; $P < 0.001$). To our knowledge, no other studies have estimated phenotypic relationships between LMW thiols and traditional milk quality characteristics.

Fixed factors included in statistical model explained approximately 60 and 40% of total phenotypic variance of Cys-Gly and GSH, respectively, and breed and herd nested within breed were the most important effects contributing to the variation of LMW thiols (results not shown). The structure of data analyzed in the present work was based on single-breed farms, which means that herd effect was nested within breed. Despite all farms were located in the same alpine area (Bolzano province) and all milk samples were collected within a short period (3 wk), it is likely that managerial and feeding strategies were different among herds and thus the breed-estimated effect could also include a part of the rearing conditions effect. Overall, the concentration of Cys-Gly in milk was greater than that of GSH across all cattle breeds. Moreover, LMW thiols were richer in milk from dual-purpose (SI and AG) than dairy (HF and BS) cows (Figure 2). However, breeds did not differ significantly ($P > 0.05$) in terms of GSH concentration in milk, and only BS exhibited a significantly ($P < 0.05$) lower Cys-Gly content than SI and AG cows (Figure 2).

Broadly speaking, the intrinsic reactivity of the nucleophilic sulfhydryl group makes LMW thiol molecules biologically important: as antioxidant molecules, they participate in several reversible redox reactions, they can conjugate and thus deactivate xenobiotics and toxic compounds, and they may also have a technological role, with implications for food quality and safety. These aspects may have a possible fallout on human health. The present study is a first contribution to the characterization of LMW thiols in milk of different cattle breeds. Further studies should be performed in order to characterize all detected LMW thiols and to quantify total antioxidant milk activity. Moreover, information of the present work will be used to build mid-infrared spectroscopy models for the *a posteriori* prediction of historical spectral data (De Marchi et al., 2014). This would allow to collect LMW thiols at population level, to estimate covariance components (if any), and to propose breeding strategies to enhance these traits.

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Table 1. Descriptive statistics of low molecular weight thiols and milk quality traits (n = 96)

Trait[1]	Mean	SD	Minimum	Maximum
Cys-Gly, μM	0.087	0.068	0.002	0.288
GSH, μM	0.067	0.064	0.000	0.262
Milk yield, kg/milking	12.4	4.1	5.0	24.0
Fat, %	3.98	0.61	2.51	5.53
Protein, %	3.56	0.46	2.61	5.50
Casein, %	2.79	0.37	1.98	4.38
Lactose, %	4.82	0.20	4.20	5.35
SCS	2.58	1.84	-3.00	6.06
pH	6.66	0.06	6.48	6.92
Urea, mg/dL	22.8	7.1	5.9	37.7

[1]Cys-Gly = cysteine-glycine; GSH = glutathione; SCS = somatic cell score.

Table 2. Pearson correlations between low molecular weight thiols and milk quality traits

	Thiol	
Trait[1]	Cys-Gly	GSH
Cys-Gly		0.653***
Milk yield	-0.154	0.117
Fat	-0.003	-0.170
Protein	0.290**	-0.048
Casein	0.268**	-0.054
Lactose	-0.166	0.006
SCS	-0.135	-0.103
pH	-0.092	-0.016
Urea	0.205*	0.176

[1]Cys-Gly = cysteine-glycine; GSH = glutathione; SCS = somatic cell score.

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Figure 1. Example of low molecular weight thiols chromatogram of milk (1 = unidentified chromatographic peaks; 2 = cysteine-glycine; 3 = glutathione).

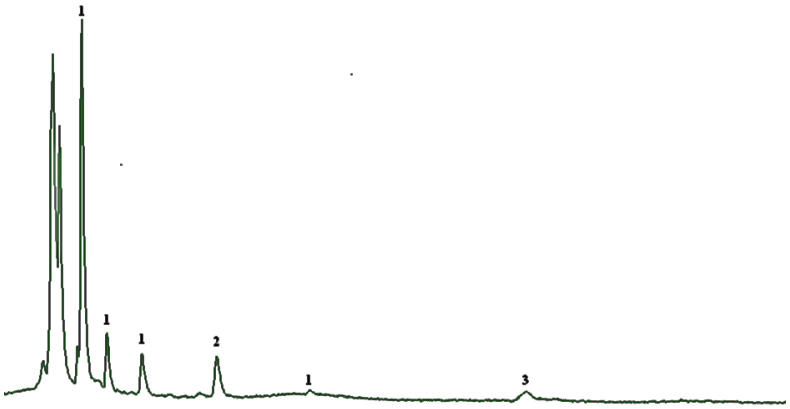
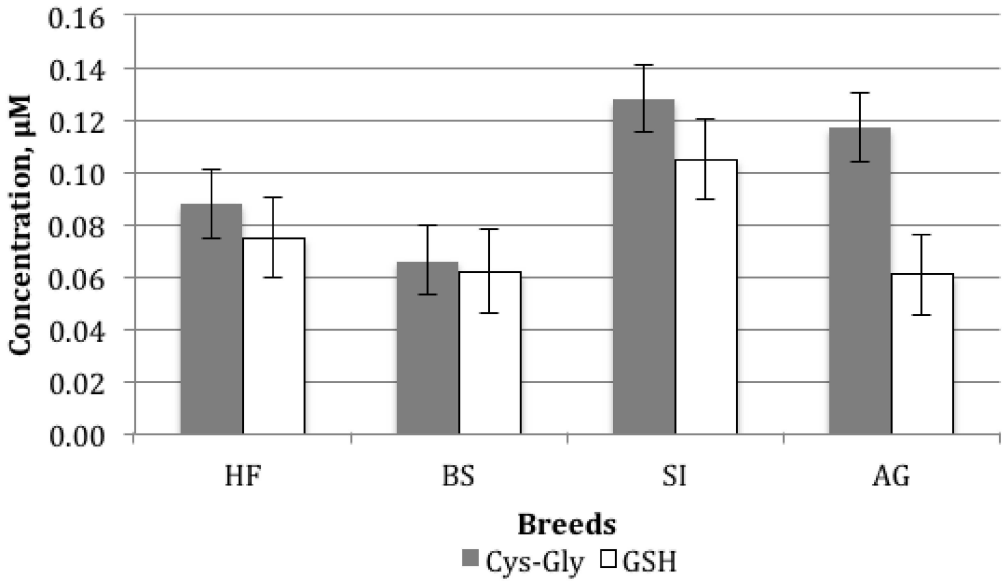


Figure 2. Least squares means (with standard errors) of cysteine-glycine (Cys-Gly) and glutathione (GSH) concentration across breeds (HF = Holstein-Friesian; BS = Brown Swiss; SI = Simmental; AG = Alpine Grey).



5. CHAPTER 3rd

Development and validation of a near infrared spectrophotometric method to determine total antioxidant activity of milk

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ABSTRACT

In the present study a spectrophotometric method for the determination of total antioxidant activity (TAA) based on ABTS assay was developed and validated on raw milk (RM), whole UHT milk (WUM), partially skimmed UHT milk (SUM), whole pasteurised milk (WM), and partially skimmed pasteurised milk (SM). The most suitable solvent for antioxidants extraction was 80% acetone. Regardless the type of milk, the coefficient of determination from linearity test was greater than 0.95. The limit of detection ranged from 0.74 to 6.07 μmol L^{-1} Trolox equivalents. Repeatability, calculated as relative standard deviation of twenty measures within a day, and reproducibility, calculated as relative standard deviation of sixty measures across three days, ranged from 1.24 to 4.04% and from 2.18 to 3.52%, respectively. Preservative added on RM had negligible effects on TAA of milk. The greatest TAA was measured on SM followed by SUM, RM, WM, and WUM.

Key words: milk, free radical, spectrophotometer, repeatability, reproducibility, nutraceutical

INTRODUCTION

Free radicals (**FR**) are defined as highly reactive and unstable molecules, having an unpaired electron in their outer orbit (Gilbert, 2000). These molecules originate in animal cells due to space radiations (Kovalev, 1983) or as by-products of mitochondrial phosphorylative oxidation (Dröge, 2002), in the form of reactive oxygen species (**ROS**) and reactive nitrogen species (**RNS**). Although FR have some important roles in animal and human physiology (Valko et al., 2007), in a long term and at high concentrations they can damage or cause complete degradation of essential molecules in cells, including fat molecules, proteins, and DNA, leading to several clinical diseases (Wu and Cederbaum, 2003). Therefore, FR scavenging is essential for human health and it involves a wide array of antioxidant substances, defined as molecules able to

compete with other oxidizable substrates, preventing their oxidation (Halliwell and Gutteridge, 2015).

Consumers are more incline to intake natural antioxidants through the daily diet rather than assuming chemicals, pharmaceutical or dietary supplements (Gülçin, 2012). Regarding food and diet antioxidants consumption, scientific studies almost entirely investigated antioxidant properties of vegetable foodstuffs, whereas very few dealt with livestock products (e.g. dairy and meat) (Horita et al., 2016; Pereira et al., 2016a,b). However, among the latter, milk is one of the most interesting and promising product for its potential antioxidant activity, due to a wide variety of antioxidant molecules. First, milk caseins and milk whey proteins have been investigated in the literature for their antioxidant activity (Suetsuna et al., 2000; Pihlanto, 2006). Second, milk contains a variety of antioxidant molecules in traces, such as low molecular weight thiols (Niero et al., 2014; Niero et al., 2015), ascorbate (Nielsen et al., 2001), tocopherol, retinol, and carotenoids (Jensen and Nielsen, 1996; Nozière et al., 2006). The study of these molecules is difficult, because it implies the fine-tuning of time consuming and expensive HPLC and mass spectrometry methodologies. Moreover, it would be difficult to attribute to each antioxidant molecule its own contribute to the antioxidant power of milk. As reported by Chen et al. (2003), it would be more convenient to consider the total antioxidant activity (**TAA**) of milk, defined as the sum of each antioxidant contributions related to the aforementioned molecules. Various analytical methods for TAA determination of different food matrices have been developed, and all were based on hydrogen atom transfer (**HAT**) or on electron transfer (**ET**) assays. Among HAT assays, the most common are the oxygen radical absorbance capacity and the radical trapping antioxidant parameter, while among ET assays the main are the folin ciocalteu reagent, the ferric ion reducing antioxidant power, the 2,2-Diphenil-1-picrylhydrazil radical scavenging capacity and the 2,2'-Azino-Bis(3-Ethilbenzotiazolina-6 Sulfonic Acid)Diammonium Salt test

(ABTS) (Huang et al., 2005). The last method, involving a colorimetric reaction, is based on the deactivation of ABTS radical solution and is described in literature as the most effective assay for milk TAA measurement (Chen et al., 2003).

Total antioxidant activity in milk is a new and unexplored trait, and it could have relevant economic and practical applications in dairy sector. The development of an accurate gold standard method for the quantification of TAA in milk is essential also to evaluate the effectiveness of alternative methods such as infrared techniques (Tiezzi et al., 2013; De Marchi et al., 2014; Penasa et al., 2014; Revilla et al., 2016) for the prediction of TAA on large data, to assess its phenotypic and genetic variation. Therefore, the aim of this study was to develop and validate a simple, robust, fast and cost-effective spectrophotometric assay for the determination of TAA on milk.

MATERIALS AND METHODS

Chemicals and Equipment

Acetonitrile (purity 99.9%), HCl (purity ≥ 37%), ABTS (2,2'-Azino-Bis[3-Ethilbenzotiazolina-6 Sulfonic Acid]Diammonium Salt, purity > 98%), $K_2S_2O_8$ (purity ≥ 99%), Trolox ([±]-6-Hidroxy-2,5,7,8-Tetra-Methylchoromane-2-Carboxilic Acid, purity 97%) were purchased from Sigma Aldrich (St Louis, MO, USA). Bronopol (2-bromo-2-nitropropan-1,3-diol; Knoll Pharmaceuticals, Nottingham, UK) was used as milk preservative. Acetone (purity 99.8%) and ethanol (purity 99.9%) were purchased from Carlo Erba (Cornaredo, MI, Italy). Ultrapure water produced by Arium 611UV Sartorius (Sartorius, MB, Italy) was used for the preparation of all solutions. Biospectrometer Kinetic 1.3.6.0 (Eppendorf, Hamburg, Germany) and 1 cm length path plastic cuvettes (Ratiolab, Dreieich, Germany) were used for spectrophotometric assays.

All analyses were carried out in the laboratories of the Department of Agronomy, Food, Natural Resources, Animals and Environment of the University of Padova (Legnaro, Italy).

Solutions and Blank

Stock of ABTS 14 mM (solution A) and stock of 4.9 mM $K_2S_2O_8$ (solution B) were prepared in water. These solutions can be stored safely at 4 °C for 3 months. Before TAA assays, solutions A and B were mixed (1:1) and dark stored for 12 hours at room temperature to activate ABTS radical, obtaining a dark blue solution (Huang et al., 2005).

In order to compare different analytical conditions and to find the most effective protocol for milk TAA quantification, four different extraction solvents (ES) were tested by diluting the activated ABTS radical with acetone in water (80:20), ethanol, acetonitrile or HCl 1M, until reaching an absorbance of 1.10 ± 0.05 at 730 nm (Chen et al., 2003).

For each analytical trial, blank samples were obtained by adding 0.1 mL of water to 1 mL of each ES added with ABTS, and measuring their absorbance at 730 nm.

Sample Collection and Preparation

One sample of raw milk (**RM**), whole UHT milk (**WUM**), partially skimmed UHT milk (**SUM**), whole pasteurised milk (**WM**), and partially skimmed pasteurised milk (**SM**), for a total of 5 milk samples, were purchased in local commercial stores. Four individual RM samples of Simmental cows were collected in the experimental farm "L. Toniolo" of the University of Padova (Legnaro, Italy), and each sample was divided in two subsamples of 40 mL. One aliquot was added with 200 µl of preservative (Bronopol). All samples were kept at 4 °C until the beginning of analyses.

Before TAA analyses, milk was thawed at room temperature for 1 hour and diluted in water (1:20). In order to assess the best antioxidant extraction procedure, 0.1 mL of diluted milk was added with 1 mL of each ES added with ABTS. Immediately after milk addition, samples were vortexed to promote antioxidants extraction and incubated at room temperature for 10 min. Samples were then centrifuged at 18,000 g for 5 min, to promote milk proteins precipitation.

Determination of TAA by ABTS Method

The ABTS radical deactivation, depending on milk antioxidants content and on the efficiency of the ES, is appreciable through the clarification of the ABTS solution. Thus, after centrifugation, 1 mL of surnatant was transferred in a cuvette and the sample absorbance was read at 730 nm, in the near infrared spectrum. The difference between the absorbance of blank samples prepared in four different ES and the absorbance of milk samples, treated respectively with the same ES, is directly proportional both to the ABTS radical deactivation and the TAA. Finally this difference was expressed in μmol L^{-1} of Trolox equivalents (TE) that is the most common way to express TAA.

Linearity, Limit of Blank, Limit of Detection

Linearity was evaluated for Trolox diluted at six different concentrations, in a range between 7.30 and 43.60 μmol L^{-1}. In order to perform linearity tests on milk samples, RM, WUM, SUM, WM, and SM were diluted at 2, 3, 4, 5, 6, 7, and 8% in water, and an aliquot of 0.1 mL of each dilution was added to 1 mL of ABTS in 80% acetone. Thus, final concentration of milk samples in cuvette ranged from 0.17% to 0.67%. Total antioxidant activity of each diluted sample was measured as described above.

Limit of blank (LOB) was defined as the highest apparent analyte concentration expected to be found when replicates of a sample containing no analyte are tested, and it was calculated as proposed by Armbruster and Pry (2008) and expressed in raw absorbance units:

$$LOB = mean_{blank} + 1.645 * (SD_{blank}),$$

where SD is the standard deviation.

Limit of detection (LOD) was defined as the lowest analyte concentration reliably distinguished from the LOB, and it was calculated following Armbruster and Pry (2008) and Bonfatti et al. (2008), and expressed in μmol L^{-1} TE:

$$LOD = 10 * (3 * SD_{blank})$$

Statistical Analysis

The normal distribution of milk TAA expressed as absorbance or as TE was checked using Shapiro-Wilk's test. Repeatability of TAA was calculated as the relative standard deviation (RSD_r) of twenty consecutive measures of RM, WUM, SUM, WM, and SM samples within the same day. Similarly, reproducibility of TAA was calculated as the relative standard deviation (RSD_R) of sixty measures obtained across three days of analyses, as proposed by Biswas et al. (2016).

RESULTS AND DISCUSSION

Efficiency of Extraction Solvents

The efficiency of different ES on TAA assays for RM, WUM, SUM, WM, and SM was tested on five consecutive measures and expressed as TE (Fig. 1). The integrity of ABTS radical diluted in all tested ES was checked in blank samples, monitoring absorbance stability for a period of 15 min. Thus we concluded that ABTS deactivation resulting in absorbance decreasing was due to samples antioxidant activity.

Extraction solvents were evaluated and selected on the basis of two fundamental properties: 1) their ability to produce a high absorbance decrease on milk samples compared to the blank solutions, i.e., on their antioxidants extraction efficiency, and 2) their capability of promoting milk protein precipitation during the centrifugation step. In this sense acetone in water (80:20) was the best ES for both properties. This moderately polar solvent was profitably used in other studies for the extraction of a variety of antioxidant molecules (e.g. polyphenol, anthocyanin, β-carotene, thiols), and it is commonly used to promote the precipitation of proteins in biological tissues (Ou et al., 2004; Ajila et al., 2007; Biswas et al., 2011). Ethanol was tested because suggested as ES for food matrices in the review of Huang et al. (2005) who summarized the chemistry behind antioxidant capacity assays: although ethanol efficiency on antioxidant extraction was fairly satisfactory, better results were observed for extraction with 80% acetone. Worst extraction results were obtained for acetonitrile and HCl 1M; they allowed milk precipitation but were not suitable for proper antioxidants extraction. Other solvents such as diethyl ether, methanol, acidified methanol, phosphate buffer and acetate buffer were tested but their use was avoided for turbidity problems due to missing milk precipitation, as reported also by Chen et al. (2003), or because of ABTS radical interactions. Thus, results of the present study are referred to samples extracted in 80% acetone.

Linearity, LOB and LOD

Linearity curve for Trolox was generated by plotting the difference of absorbance between blank sample and samples with increasing Trolox concentration, versus the concentration of the synthetic antioxidant. This curve was successively used to express the difference of absorbance generated by milk samples in terms of TE. Similarly, calibration curves for RM, WUM, SUM, WM, and SM samples were generated by plotting the milk TAA expressed as TE, versus the

percentage of milk (Fig. 2). The equations resulting from linearity test are of the form y = mx + b, where m is the slope of the line and b is the y-intercept, in association with the coefficient of determination (R^2). As reported by Jhang et al. (2004) y-intercept represents the constant systematic error or constant bias, that should be as close as possible to zero, depending on the analyte being evaluated. In the present work, y-intercept of Trolox curve was 0.0121 $OD_{730\ nm}$ and it was lower than 0.02 mM TE in all different types of milk samples. Coefficient of determination was greater than 0.99 for Trolox calibration curve and greater than 0.95 for linearity tests carried out for all types of milks. The addition of increasing amount of milk or further dilution, resulted in slightly bent curves (Chen et al., 2003).

Table 1 reports linearity range, LOB and LOD of TAA calculated for RM, WUM, SUM, WM, and SM. The widest linearity range was observed for WM (19.44 to 39.80 μmol L^{-1} TE) and the most narrow was observed for RM (22.61 to 38.39 μmol L^{-1} TE) and SUM (29.31 to 44.72 μmol L^{-1} TE). The lowest LOD was calculated for WM (0.74 μmol L^{-1} TE) and the greatest was registered for WUM (6.07 μmol L^{-1} TE).

Repeatability and Reproducibility

The precision of the analytical method for the determination of TAA of milk expressed as TE was assessed through repeatability relative standard deviation (RSD_r) and reproducibility relative standard deviation (RSD_R). Values of RSD_r and RSD_R showed good precision for each type of commercial milk, both within and between days (Table 2). Repeatability relative standard deviation ranged from 1.24% (RM in day 2) to 4.04% (SM in day 1), and RSD_R ranged from 2.18% (RM) to 3.52% (WUM). The good precision reached in the present study could be partly due to two analytical precautions adopted during the laboratory analyses: 1) milk TAA was analysed after a dilution step in water (1:20), in order to enhance the homogeneity of samples,

and 2) the best ES was chosen on the basis of the ability to precipitate milk sample and the clearness of reaction solution, thus allowing the proper application of Lambert-Beer law (Hornero-Méndez and Mínguez-Mosquera, 2001). Overall, results of the present study were similar to values reported by Martysiak-Żurowska and Wenta (2012) in a study on total antioxidant capacity of human milk, and even better than findings of other studies on the development of spectrophotometric methods (Biswas et al., 2011). In general RSD_R values for TAA determination were worse than RSD_r values, similarly to findings of Biswas et al. (2011) and by Sturaro et al. (2016).

Effect of Preservative on TAA of RM

Milk TAA was measured once a day for three consecutive days in four individual RM samples, and in the same four samples added with preservative, in order to assess whether or not Bronopol influenced the spectrophotometric assay. Measures of TAA on RM were in good agreement with measures on RM added with preservative ($R^2 = 0.92$) (Fig. 3). Hence, addition of Bronopol as preservative to milk did not interfere with the determination of the TAA of RM, making routine application of TAA assays easier (Dal Zotto et al., 2008). Moreover, the method is fast (20 min for a batch of 10 samples), cheap (1.20 to 1.30 € per milk sample), and it requires a small quantity of milk samples and reagents. Therefore, it is suitable for large-scale studies at cow individual level.

Means and Variation of TAA in Milk

Descriptive statistics of TAA for RM, WUM, SUM, WM, and SM are reported in Table 3. The greatest TAA was measured on SM, which averaged 7521.41 μmol L^{-1} TE, followed by SUM, RM, and WM with means of 7472.97, 7459.09, and 7446.87 μmol L^{-1} TE, respectively.

The lowest TAA was obtained for WUM, which averaged 7106.81 μmol L^{-1} TE. In general, these values are greater than those reported by Chen et al. (2003), probably due to the difference on the solvents used for milk antioxidants extraction.

A greater TAA was found for partially skimmed milk compared with whole milk, both for UHT and pasteurised samples. This finding is apparently in contrast with results of Chen et al. (2003) who observed greater TAA on milk with greater fat content. Nevertheless, the experimental setup in the study of Chen et al. (2003) was different than that of the present work and can only partly be used for comparison; moreover, even if partially skimmed milk is deprived of several lipophilic antioxidants (e.g. retinol, tocopherol, and carotenoids), milk soluble fraction, containing other powerful antioxidant compounds (e.g. ascorbate, thiols, whey proteins), may result more concentrated in a v/v ratio, as a result of the skimming process. Finally, a greater TAA activity was observed for pasteurised compared with UHT milk, both for whole and partially skimmed samples. Indeed, as a consequence of heat treatment at high temperature some vitamins are degraded, most of the serum proteins become insoluble, and casein micelles aggregate and thus are less bioavailable (Walstra et al., 2005).

CONCLUSIONS

Near infrared spectrophotometry detection and 80% acetone as extraction solvent were successfully used for the development and validation of a simple, robust and fast method for the determination of TAA of different types of milk. The 80% acetone was the most suitable solvent both for antioxidants extraction efficiency and for its ability on promoting milk protein precipitation, thus allowing proper spectrophotometric assays. The coefficient of determination of linearity tests was greater than 0.95 for all types of milk and the limit of detection ranged from 0.74 (WM) to 6.07 μmol L^{-1} TE (WUM). The method was highly repeatable and reproducible.

Preservative added on RM had negligible effects on TAA. Partially skimmed pasteurised milk and WUM showed the greatest and the lowest TAA, respectively. Future researches will focus on the determination of TAA in milk of different species and of different breeds within species as well as on TAA of different dairy products to emphasize the nutraceutical aspects of milk and increase its added value.

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Table 1. Linearity range, limit of blank (LOB) and limit of detection (LOD) of total antioxidant activity of milk (n = 20)

Milk [a]	Linearity range μmol L^{-1} TE [b]	LOB (Absorbance 730 nm)	LOD μmol L^{-1} TE [b]
RM	22.61 – 38.39	1.18	2.63
WUM	24.15 – 43.05	1.11	6.07
SUM	29.31 – 44.72	1.10	1.72
WM	19.44 – 39.80	1.15	0.74
SM	22.65 – 41.43	1.17	4.25

[a] RM = raw milk; WUM = whole UHT milk; SUM = partially skimmed UHT milk; WM = whole pasteurised milk; SM = partially skimmed pasteurised milk.

[b] TE = Trolox equivalents.

Table 2. Repeatability relative standard deviation (RSD_r, n = 20) and reproducibility relative standard deviation (RSD_R, n = 60) for total antioxidant activity of milk

Milk [a]	Day 1		Day 2		Day 3		RSD
	μmol L^{-1} TE [b]	RSD_r	μmol L^{-1} TE [b]	RSD_r	μmol L^{-1} TE [b]	RSD_r	RSD_R
RM	7370.39	2.51	7538.62	1.24	7468.25	2.08	2.18
WUM	7336.66	3.28	7025.61	2.78	6958.15	1.62	3.52
SUM	7342.91	2.47	7345.83	1.82	7730.17	1.96	3.21
WM	7368.73	2.14	7469.91	1.79	7501.98	2.65	2.32
SM	7381.64	4.04	7484.91	2.78	7697.69	1.36	3.35

[a] RM = raw milk; WUM = whole UHT milk; SUM = partially skimmed UHT milk; WM = whole pasteurised milk; SM = partially skimmed pasteurised milk.

[b] TE = Trolox equivalents.

Table 3. Descriptive statistics of total antioxidant activity of milk (μmol L^{-1} of Trolox equivalents; n = 60)

Milk [a]	Mean	SD [b]	Minimum	Maximum
RM	7459.09	162.37	7111.39	7744.33
WUM	7106.81	250.39	6661.67	7777.64
SUM	7472.97	239.52	6936.50	7969.19
WM	7446.87	172.40	7003.12	7810.95
SM	7521.41	252.30	7044.76	7877.58

[a] RM = raw milk; WUM = whole UHT milk; SUM = partially skimmed UHT milk; WM = whole pasteurised milk; SM = partially skimmed pasteurised milk.

[b] SD = standard deviation.

Figure 1. Efficiency (with standard deviation) of different extraction solvents on total antioxidant activity assays for raw milk (RM), whole UHT milk (WUM), partially skimmed UHT milk (SUM), whole pasteurised milk (WM) and partially skimmed pasteurised milk (SM), expressed as μmol L^{-1} of Trolox equivalents. (Number of replications, n = 5)

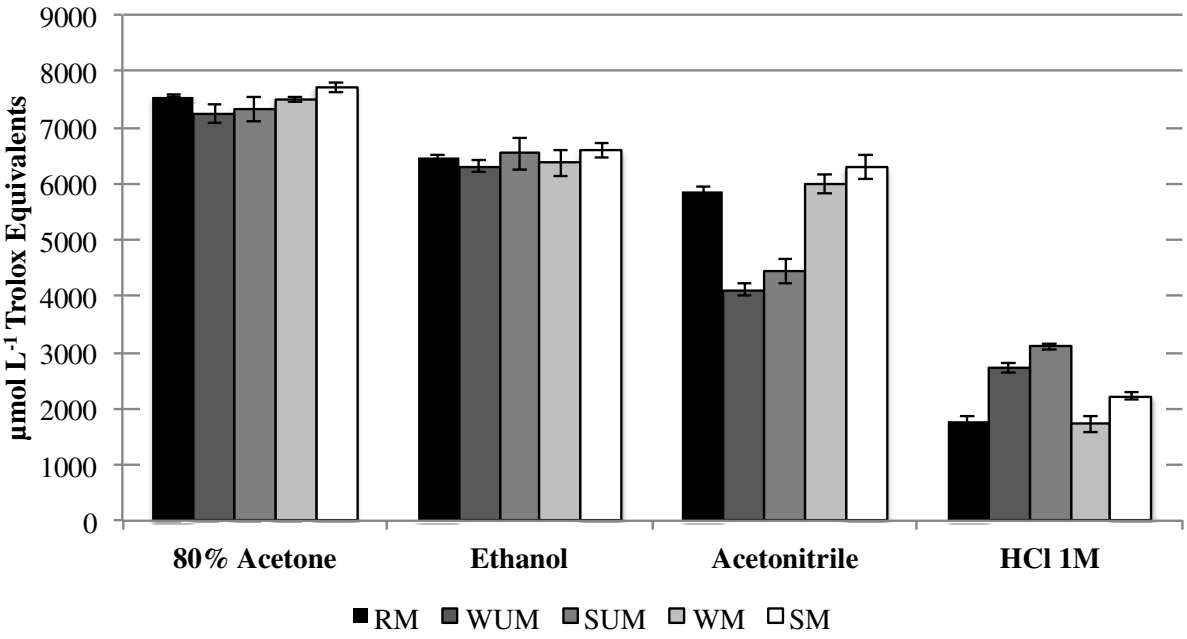


Figure 2. Linearity tests for different concentrations of (A) Trolox standard, (B) raw milk (RM), (C) whole UHT milk (WUM), (D) partially skimmed UHT milk (SUM), (E) whole pasteurised milk (WM) and (F) partially skimmed pasteurised milk (SM)

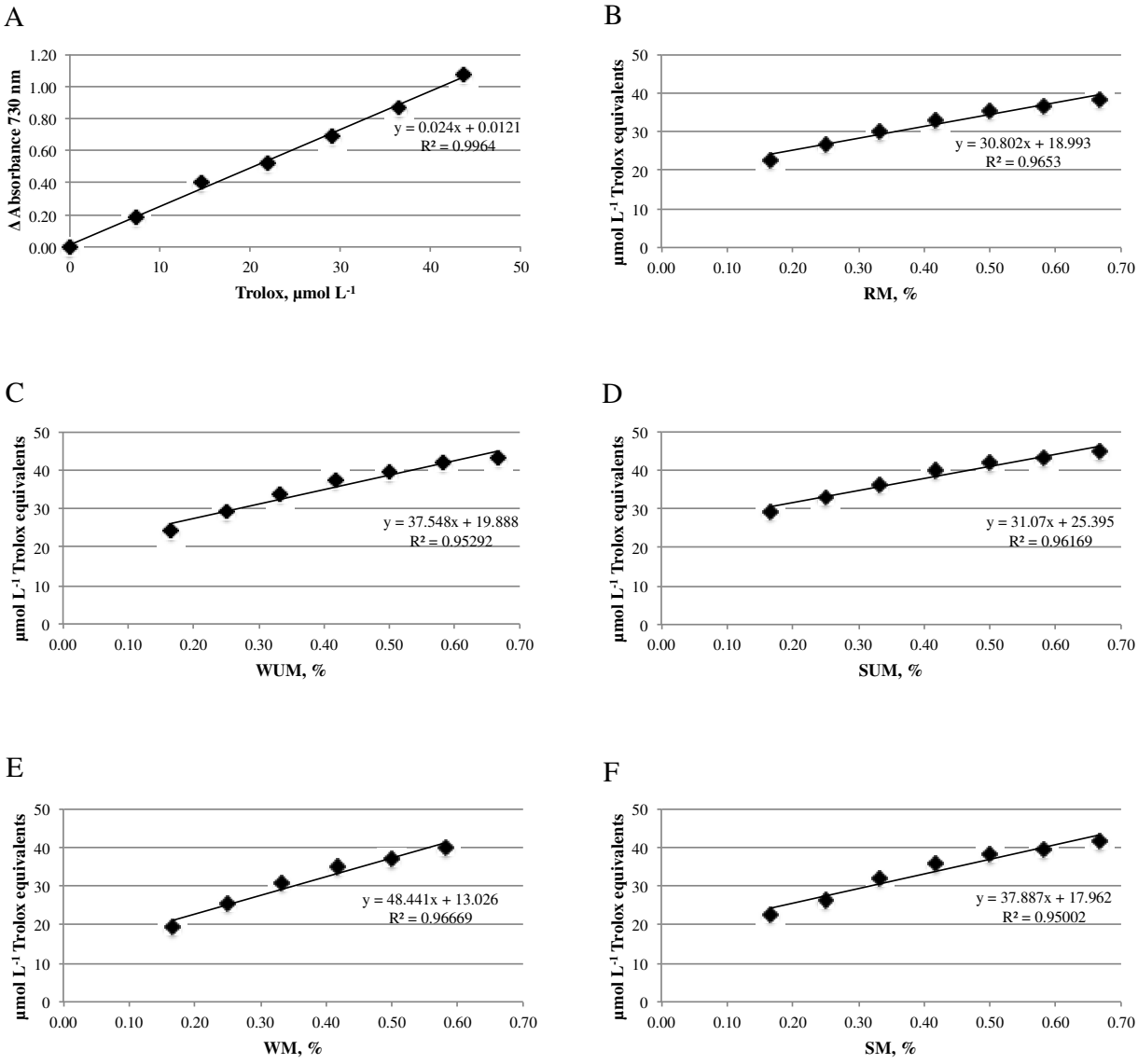
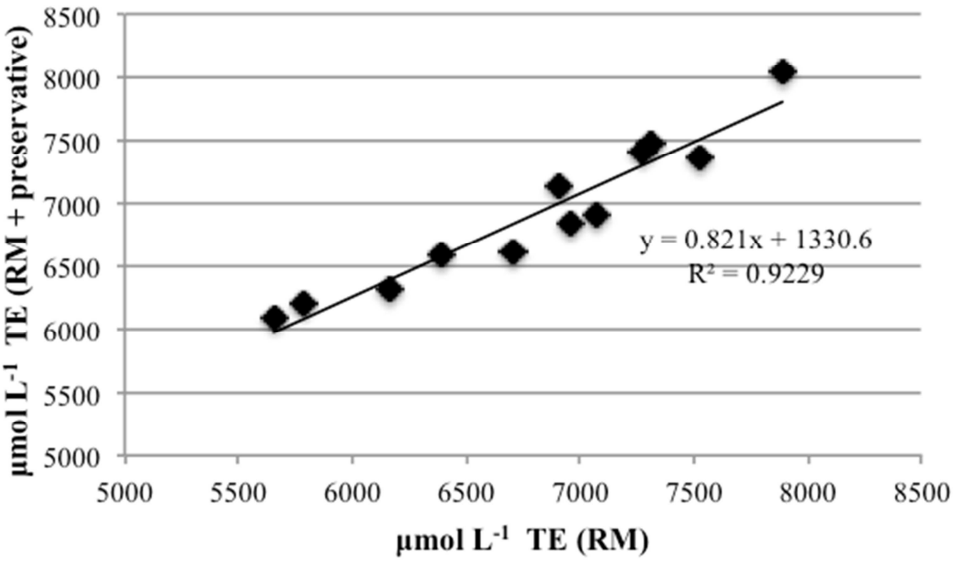


Figure 3. Relationship between total antioxidant activity of raw milk (RM) and RM + preservative (n = 4), expressed as μmol L^{-1} of Trolox equivalents (TE)



6. CHAPTER 4th

Total antioxidant activity of bovine milk: phenotypic variation and predictive ability of mid-infrared spectroscopy

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ABSTRACT

Food antioxidants have been broadly studied for their activity against free radicals and oxidative stress, and their relevance on human health is well documented. Milk contains several antioxidant molecules at low concentration or in traces, and the sum of their activities in neutralising free radicals is defined total antioxidant activity (TAA). The present study aimed to investigate phenotypic variation of milk TAA, and to assess the ability of mid-infrared spectroscopy (MIRS) to predict this novel trait. Total antioxidant activity was measured through the reference spectrophotometric method on 1,249 individual milk samples of Holstein Friesian cows. Sources of variation of milk TAA were investigated using a linear mixed model, which included the fixed effects of days in milk, parity, and calving season, and the random effects of herd-test-date and random error. Mid-infrared spectroscopy prediction models were developed using partial least squares regression approach. Milk TAA averaged 6.93 mmoL/L of Trolox equivalents and exhibited a coefficient of variation of 15%. Total antioxidant activity showed low phenotypic correlations with milk quality traits, ranging from -0.11 with lactose to 0.18 with protein. Values of TAA were lower in early than late lactation. Mid-infrared spectroscopy prediction models reached a coefficient of determination in external validation of 0.41, and a ratio of prediction to deviation of 1.30, suggesting that they are not adequate for analytical purposes.

Key words: antioxidant, cow milk, infrared spectroscopy, nutraceutical value

INTRODUCTION

Milk contains a variety of antioxidant compounds, such as tocopherols (Chauveau-Duriot et al., 2010), retinol and carotenoids (Nozière et al., 2006), ascorbate (Nielsen et al., 2001), phenols (Velàzquez Vàzquez et al., 2015), and low molecular weight thiols (Niero et al., 2015). Also,

peptides derived from casein (**CN**) digestion and lactoferrin have been studied for their antioxidant properties (Pihlanto, 2006; Suetsuna at al., 2000). Food antioxidants play an important role in human health because they are responsible for neutralization and deactivation of free radicals. Indeed, oxidative stress and free radicals cause several cytological injuries, mainly associated with lipids peroxidation, oxidative alteration of proteins, and DNA cleavage (Robbins and Cotran, 2010). This damage at a biochemical level has been associated with clinical pathologies such as cancer, atherosclerosis, rheumatoid arthritis, neurodegeneration, and diabetes (Gilbert, 2000).

Despite the great diversity of antioxidants in milk, the majority of these molecules are present in traces or at low concentration. Moreover, the characterisation of these compounds is expensive, time-consuming, and highly demanding in terms of both milk sample preparation and analytical procedures (Niero et al., 2017). On the other hand, total antioxidant activity (**TAA**) of milk, defined as the sum of antioxidant contribution related to tocopherols, retinol, carotenoids, ascorbate, phenols, low molecular weight thiols, CN and peptides and lactoferrin (Chen et al., 2003), can be easily and cheaply measured using near infrared spectrophotometric method, and might have broader impact on human nutrition and health, compared to that of each single antioxidant molecule (Niero et al., 2017).

Gaining knowledge on phenotypic variation of TAA across different dairy species and breeds, days in milk (**DIM**), parities, and feeding and management systems, and on the relationships of this new phenotype with milk yield and quality traits, is of relevant interest for several reasons: 1) from a physiological point of view, it would allow a better understanding on how and to which extent milk antioxidants are transferred from feed to animal, and from the animal into the milk (Havemose et al., 2006); 2) from a technological point of view, it would allow to examine correlations between milk quality traits and TAA, and to analyse the impact of

different management conditions on milk TAA (De Marchi et al., 2008; Niero et al., 2016b); 3) as regard to human nutrition and health, it would provide added value to milk composition and milk nutraceutical properties (Haug et al., 2007); and 4) the assessment of phenotypic variation for a new trait such as TAA is a prerequisite to develop mid-infrared spectroscopy (**MIRS**) prediction models to record phenotypes at population level (De Marchi et al., 2013, 2014).

Therefore, the objectives of the present study were 1) to assess the associations between milk TAA and traditional milk quality traits, 2) to investigate sources of variation of milk TAA, and 3) to evaluate the feasibility of MIRS to predict TAA of cow milk.

MATERIALS AND METHODS

Milk Sample Collection and Chemical Composition

Individual raw milk samples (n = 1,249) of Holstein Friesian cows from parity 1 to 9 and from 6 to 536 DIM were collected in 17 herds between September 2016 and February 2017. Only one milk sample was collected per cow. Immediately after sampling, preservative (Bronopol, 2-bromo-2-nitropropan-1,3-diol) was added to milk, transferred at 4°C to the laboratory of the Breeders Association of Veneto Region (ARAV, Padova, Italy), and analysed for fat, protein, CN, and lactose content using a MilkoScan FT6000 (Foss Electric A/S, Hillerød, Denmark). Spectra information of analysed samples was stored and used to develop prediction model for milk TAA. Finally, somatic cell count (**SCC**) was determined using a Fossomatic (Foss Electric A/S, Hillerød, Denmark) and values of SCC were transformed to somatic cell score (**SCS**) to achieve normality and homogeneity of variances using the formula of Wiggans and Shook (1987): $SCS = 3 + \log_2(SCC/100{,}000)$.

Analysis of Milk TAA

An aliquot of each individual milk sample was transferred to the laboratory of the Department of Agronomy, Food, Natural Resources, Animals and Environment of the University of Padova (Legnaro, Italy) for the determination of milk TAA according to the method developed and validated by Niero et al. (2017). Briefly, before milk TAA assay, 2,20-Azino-Bis[3-Ethilbenzotiazolina-6 Sulfonic Acid] Diammonium Salt (**ABTS**) 14 mM water solution, was added with $K_2S_2O_8$ 4.9 mM water solution (1:1). The resulting mixture was stored in the dark for 12 h at room temperature, in order to activate the ABTS radical. Acetone 80% was added with activated ABTS radical solution, until reaching an absorbance of 1.10 ± 0.05 $OD_{730\ nm}$, and used as milk antioxidants extraction solvent. Subsequently, 1 mL of extraction solvent was added with 0.1 mL of milk diluted in water (1:20). Immediately after milk addition, samples were vortexed to promote antioxidant extraction, incubated at room temperature for 10 min, and centrifuged at 18,000 g for 5 min to promote milk protein precipitation. Finally, absorbance of surnatant was read at 730 nm and subtracted to the absorbance of the blank. This difference, directly proportional to milk TAA, was expressed in mmol/L of Trolox equivalents (**TE**).

Statistical Analysis

Shapiro-Wilk's test and visual inspection of data distribution highlighted that milk TAA was normally distributed (Figure 1). Pearson correlations of TAA with traditional milk quality features were assessed through the CORR procedure of the SAS software (ver. 9.4; SAS Institute Inc., Cary, NC) and sources of variation of TAA were investigated using the MIXED procedure of SAS, according to the following linear mixed model:

$$y_{ijkl} = \mu + DIM_i + P_j + C_k + HTD_l + \varepsilon_{ijkl},$$

where y_{ijkl} is milk TAA; μ is the overall intercept of the model; DIM_i is the fixed effect of the *i*th class of stage of lactation of the cow (*i* = 1 to 9, with the first being a class from 6 to 40 d, followed by 7 classes of 40 d each, and the last being a class > 320 d); P_j is the fixed effect of the *j*th parity of the cow (*j* = first, second, third, and fourth and later parities); C_k is the fixed effect of the *k*th calving season (*k* = spring, summer, autumn, and winter); HTD_l is the random effect of the *l*th herd-test-date (*l* = 1 to 17) ~ $N(0, \sigma^2_{HTD})$; and ε is the random residual ~ $N(0, \sigma^2_{\varepsilon})$. In the model, the herd and test-day effects were confounded because cows in each herd were sampled only once, all on the same test-day. Multiple comparisons of means was performed for DIM, parity, and calving season effects. Significance was set at $P < 0.05$.

Development of MIRS Prediction Model for TAA

Prediction model for TAA was developed using the SAS software. Spectral data were converted to absorbance by taking the log10 of the reciprocal of the transmittance. Spectral regions between 1,580 and 1,710 cm^{-1}, and 2,990 and 3,690 cm^{-1} were discarded as characterized by low signal-to-noise ratio. Principal component analysis was carried out on edited spectral matrix to identify similarities and differences between milk spectra. The 98.6% of the total spectral variability was represented by the first 5 principal components. In descending order, the first 5 principal components explained 70.51, 12.36, 10.73, 3.98 and 1.38% of the total variation. Based on the Mahalanobis distance, a total of 17 milk samples were identified as outliers and discarded from the dataset. The prediction model was developed using partial least squares regression, including the vector of milk TAA as dependent variable, and the matrix of spectral wavenumbers as predictor. The dataset was firstly sorted by milk TAA, and subsequently divided in two different subsets, namely a calibration dataset (75% of the total observations) and a validation dataset (the remaining 25% of the total observations). The calibration dataset was used

to develop the prediction model and the validation dataset was used to assess the predicting ability of the developed model, as the samples included in this last dataset were not used to generate the MIRS model. This process was repeated four times; in the first round the first observation every four was excluded from the calibration dataset, the second round the second observation every four was excluded, and similarly for the third and fourth round. For each iteration, one-at-a-time cross validation was performed on the calibration dataset. Mathematical pre-treatments (Savitzky-Golay first and second derivatives) were applied to the raw spectra but no improvement on the prediction models accuracy was detected; therefore, the untreated spectra were used to generate the models. The optimal number of model terms was the least number of extracted factors whose residuals were not significantly greater than those of the model with minimum error (van der Voet, 1994). Variable importance in the projection (**VIP**) scores were calculated accordingly to Wold (1994).

Goodness-of-fit statistics considered in the present study were the coefficients of determination in cross- and external validation ($\mathbf{R^2_{CV}}$ and $\mathbf{R^2_V}$, respectively) and the standard errors of prediction in cross- and external validation ($\mathbf{SE_{CV}}$ and $\mathbf{SE_V}$, respectively). The ratio of prediction to deviation (**RPD**) was calculated as the SD of the trait divided by the SE_V. Bias was calculated as the mean difference between the reference TAA and the respective MIRS-predicted TAA, and a *t*-test was carried out in order to determine if the bias was statistically different from zero. The predicted values were also linearly regressed on the respective reference values and *t*-test was performed to verify if the coefficient of regression (slope) differed statistically from one.

RESULTS AND DISCUSSION

Mean and Variation of Milk TAA

Milk TAA averaged 6.93 mmol/L TE, with values ranging from 3.71 to 10.18 mmol/L TE (Table 1). The coefficient of variation (**CV**) of milk TAA (15%) suggested that exploitable phenotypic variability exists for this trait, being similar to that of protein and CN percentages, and not far from that of fat percentage. Average milk TAA of the present study was slightly lower than values of Niero et al. (2017) for several types of commercial milks (from 7.11 to 7.52 mmol/L TE for whole UHT milk and partially skimmed pasteurised milk, respectively), but considerably greater than that (2.24 mmol/L TE) reported by Chen et al. (2003), probably due to differences in milk antioxidants extraction procedures. The comparisons with other studies (e.g. Zulueta et al., 2009) is difficult due to different units of measure to express TAA and different assays involved in the quantification of antioxidant capacity. In terms of variability, the CV reported in our study (15%) was much greater than those (2.18 to 3.52% for raw milk and whole UHT milk, respectively) obtained by Niero et al. (2017). Two reasons can be argued to explain this huge difference: first, milk analysed by Niero et al. (2017) was purchased in commercial stores and thus it was bulk milk, whereas we analysed individual milks, which allow to catch more variability of the trait of interest; and second, the sample size of Niero et al. (2017) was much lower than that of the present work.

Relationships of Milk TAA with Milk Yield and Quality Traits

Phenotypic correlations of milk TAA with milk yield and quality traits were weak (Table 2). Overall, the complex pattern and variety of milk antioxidants, their low concentration distributed among different milk constituents (including fat, protein, and CN), and thus their small individual contribution to milk TAA, are possible reasons to explain the weak relationships between TAA

and traditional milk quality traits observed in the present study. Milk TAA was unfavourably correlated with milk yield (-0.22; $P < 0.001$), meaning that high-producing cows yielded milk with lower TAA compared with milk of low-producing animals; a dilution effect can be hypothesized, similarly to what happens for other milk constituents such as protein and fat (Ng-Kwai-Hang et al., 1982; Niero et al., 2016a). Among composition traits, CN and protein percentages had the most favorable correlation with TAA (0.15 and 0.18, respectively; $P < 0.001$); this was somewhat expected and is in agreement with findings from other studies. Indeed, Zulueta et al. (2009) reported that CN is one of the main compounds contributing to milk TAA, due to its high content of potentially antioxidant amino acids (**AA**) (Rival et al., 2001). Furthermore, in a review of Lindmark-Månsson and Åkesson (2000) about antioxidant factors in milk, it has been reported that CN is in complex with glutathione peroxidase enzyme, responsible of glutathione antioxidant capacity. Regarding protein percentage, the favorable association with TAA is due to the previously discussed CN antioxidant properties and to the whey protein contribution to milk TAA. Whey proteins have been studied for their antioxidant properties, in particular lactoferrin which is able to bind iron and to block its pro-oxidant action (Cichosz et al., 2017). Also β-lactoglobulin and derivate peptides have antioxidant effects, firstly preserving retinol and α-tocopherol from oxidation along digestive trait (Liang et al., 2011), and secondly deactivating free radicals through Trp, Tyr, and Met AA residuals (Cichosz et al., 2017). A favorable but weak correlation (0.13; $P < 0.001$) was observed between milk TAA and fat percentage (Table 2), similarly to findings of Chen et al. (2003). Milk fat contains several liposoluble vitamins and antioxidant compounds, which contribute to milk TAA. Small amounts of retinol, α- and γ-tocopherol, and β-carotene in milk fat have been observed by Calderón et al. (2007), Chauveau-Duriot et al. (2010), and Ramalho et al. (2012). The correlations of TAA with fat and protein of the present work were greater than those reported by Ramalho et al. (2012).

High milk SCS is one of the main indicators of mastitis, defined as inflammation of udder tissues as a consequence of pathogens infection (Pyörälä, 2003). This altered physiological status is associated with high free radicals development (Atakisi et al., 2010), thus resulting in amplified antioxidant response (Conner and Grisham, 1996), and explaining the unfavorable relationship between TAA and SCS of the present study. Additionally, the negative correlation between TAA and lactose (Table 2) confirms this hypothesis, since milk lactose content significantly decreases in presence of high SCS and mastitis (Miglior et al., 2007).

Effects of Lactation Stage, Parity, and Calving Season on Milk TAA

The fixed effect of classes of DIM was significant in explaining the variation of milk TAA ($P < 0.01$). Total antioxidant activity decreased slightly from 6 to 40 DIM and increased thereafter (Figure 2), exhibiting a trend that was opposite to that of milk yield (results not shown). This is in agreement with the previously discussed unfavourable correlation between milk TAA and milk yield (Table 2). Fat, protein, and casein percentages resembled the trend of milk TAA, i.e., they were lower in the first part of the lactation and increased thereafter, corroborating the positive correlations assessed between milk TAA and the aforementioned quality traits (Table 2).

Parity and calving season did not significantly contribute to explain the variation of milk TAA. Overall, TAA was almost stable across classes of parity and calving season. This equilibrium may be due to the complex biological systems designed to maintain cellular redox status, that is crucial not only to maintain normal cellular function, but also to prevent mediated oxidative stresses (Ray et al., 2012).

Accuracy of Prediction Model for Milk TAA

Figure 3 depicts the scatter plot between milk TAA measured by spectrophotometric reference method and milk TAA predicted by MIRS. Mid-infrared spectroscopy prediction model was developed using 17 factors, and it showed R^2_{CV} and R^2_V of 0.46 and 0.41, respectively, and both SE_{CV} and SE_V of 0.72 mmol/L TE. Results indicate that prediction models are not adequate for analytical purposes. To our knowledge no studies have attempted to investigate the potential of MIRS to predict milk TAA, and thus the comparison of our results with the scientific literature can be made only with regard to other innovative and trace milk quality traits. For example, R^2_V observed in the present study is close to that reported by Visentin et al. (2016) on MIRS-predicted milk coagulation properties, but lower than R^2 reported by Niero et al. (2016b) on detailed milk protein composition and Visentin et al. (2016) on major milk mineral composition. The RPD value in external validation was 1.30, and the mean bias of prediction (i.e., the average of the difference between the gold standard and predicted values for each individual sample) was not significantly different from zero in external validation. The slope of the linear regression of the reference on the predicted values was 0.44 (SE = 0.02). Again, due to the low RPD, prediction model can not be considered adequate for analytical purposes because a prediction model can be considered useful for analytical purposes when RPD is greater than 2 (Williams, 2007).

According to VIP scores and regression coefficients, the prediction of milk TAA showed specific absorption peaks, as previously reported by De Marchi et al. (2009, 2011): 1,550 to 1,570 cm^{-1} related to protein absorption and 2,800 to 2,959 cm^{-1} related to lipid absorption (Figure 4). This result corroborate the previously discussed positive correlations of milk TAA with protein and fat content.

CONCLUSIONS

The present study is the first contribution to the phenotypic characterization of TAA of bovine milk. This new phenotype exhibited an interesting and exploitable variability (CV of 15%), similar to that of other quality traits. Favorable weak phenotypic correlations of TAA with fat, protein, and CN percentages were observed, as well as with SCS. Total antioxidant activity of milk increased across lactation. Mid-infrared prediction models developed to predict milk TAA were not enough accurate for analytical purposes. Feeding and genetic aspects need to be investigated in order to account for other possible sources of variation influencing milk TAA.

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Table 1. Descriptive statistics of milk total antioxidant activity, production related traits, and milk quality traits

Trait[1]	n	Mean	Minimum	Maximum	CV, %
TAA, mmol/L TE	1,239	6.93	3.71	10.18	15
Production related traits					
Milk yield, kg/d	1,245	29.8	4.0	59.9	32
DIM, d	1,249	174	6	536	69
Parity, n	1,249	2.14	1.00	9.00	60
Milk quality traits					
Fat, %	1,240	3.95	1.30	6.80	22
Protein, %	1,240	3.36	2.40	4.69	12
CN, %	1,239	2.63	1.80	3.70	13
Lactose, %	1,226	4.86	4.10	5.57	4
SCS, units	1,249	3.43	-1.64	9.73	60

[1]TAA = total antioxidant activity, expressed as mmol/L of Trolox equivalents (TE).

Table 2. Pearson correlations of milk total antioxidant activity with milk yield and quality traits

Trait	Total antioxidant activity
Milk yield	-0.22***
Fat	0.13***
Protein	0.18***
CN	0.15***
Lactose	-0.11***
SCS	0.13***

***$P < 0.001$.

Figure 1. Distribution of measured milk total antioxidant activity (TAA), expressed as mmol/L of Trolox equivalents (n = 1,239).

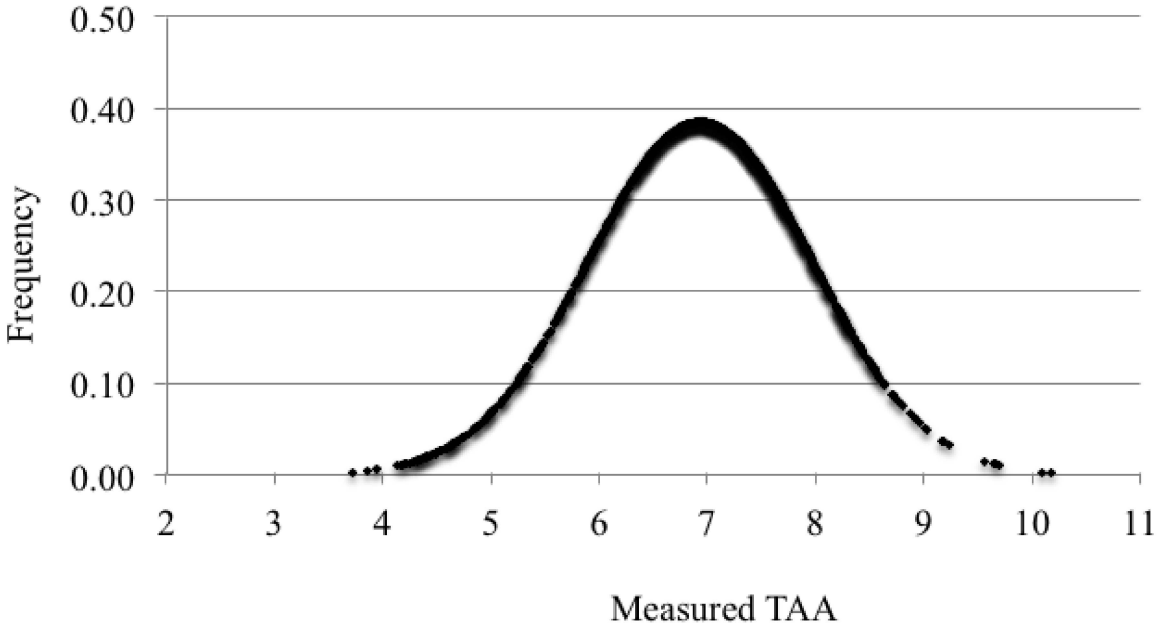


Figure 2. Least squares means (with SE) of total antioxidant activity (TAA) of milk, expressed as mmol/L of Trolox equivalents, across lactation. Least squares means with different letters differ significantly ($P < 0.05$).

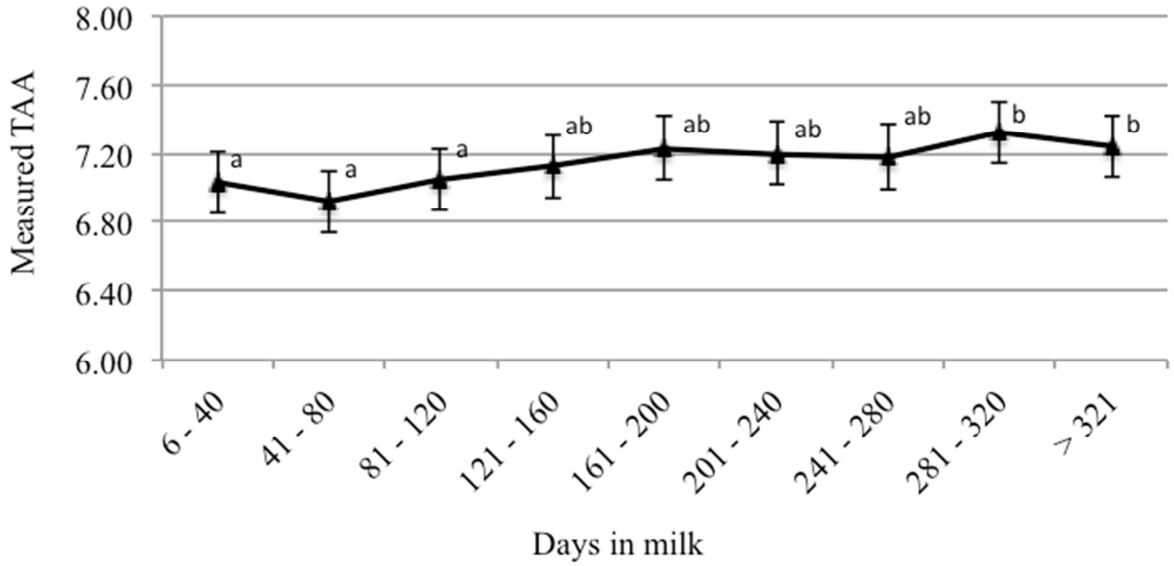


Figure 3. Scatter plot of predicted total antioxidant activity (TAA) (y-axis) versus measured TAA (x-axis) of milk, expressed as mmol/L of Trolox equivalents.

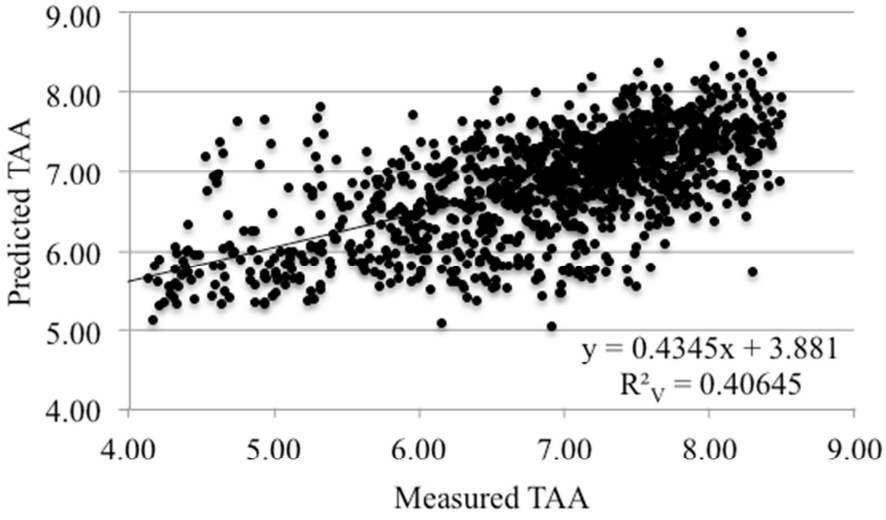
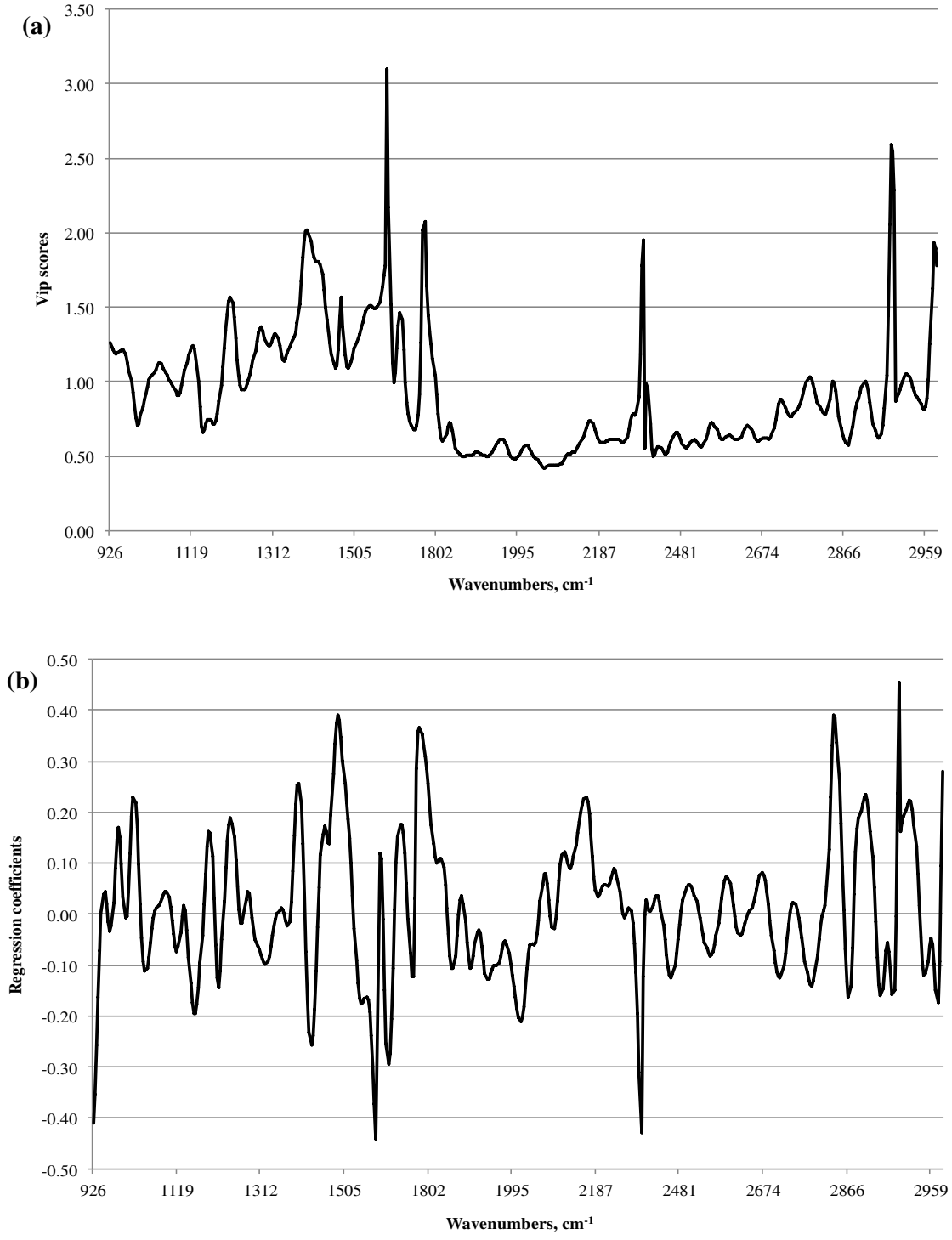


Figure 4. Variable importance in the projection (VIP) scores (a) and coefficient of regression (b).



7. CHAPTER 5th

Short communication: **Phenotypic characterisation of total antioxidant activity of buffalo, goat, and sheep milk**

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ABSTRACT

Free radicals are reactive and unstable waste scrap molecules produced by cells, responsible of damages and alteration on DNA, proteins, and fat. The daily intake of antioxidant compounds, acting against free radicals and their detrimental effects, is essential for human health. Milk contains several compounds with antioxidant activity, and the sum of their reducing potential blocking free radicals development is defined total antioxidant activity (TAA). This novel trait has been described in literature both in individual and bulk cow milk, while there are no reports from other dairy species. Therefore, the present study aimed to investigate phenotypic variation of TAA in individual samples of buffalo (n = 105), goat (n = 112), and sheep (n = 198) milk. Total antioxidant activity was measured through a reference spectrophotometric method, and expressed as mmol/L of Trolox equivalents (TE). The greatest TAA was observed in sheep milk, averaging 7.78 mmol/L TE and showing also the broadest phenotypic variation expressed as coefficient of variation (13.98%). Significantly lower TAA values were observed for buffalo (7.35 mmol/L TE) and goat (6.80 mmol/L TE) milk, with coefficient of variation of 8.18 and 8.47%, respectively. Total antioxidant activity exhibited weak correlations with milk yield and chemical composition. Phenotypic values of TAA presented in this study will be used to assess the ability of mid-infrared spectroscopy to predict this new trait and thus to collect data at population level.

Key words: milk total antioxidant activity, buffalo, goat, sheep

SHORT COMMUNICATION

Free radicals are reactive oxygen derived molecules, produced as a consequence of space radiations (Kovalev, 1983), or as by-product during mitochondrial phosphorylative oxidation in animal cells (Dröge, 2002). These molecules are responsible for oxidative alteration of lipids,

proteins, and DNA (Robbins and Cotran, 2010), and their activity has been associated with clinical diseases such as cancer, atherosclerosis, and neurodegeneration (Gilbert, 2000). In this scenario, food antioxidants have been broadly studied for their positive effects on human health, mainly related to the neutralization of free radicals and prevention of oxidative stress (Halliwell and Gutteridge, 2015). Milk has been investigated as source of lipophilic antioxidants such as tocopherols, retinol, and carotenoids (Nozière et al., 2006; Chauveau-Duriot et al., 2010), hydrophilic antioxidants such as ascorbate, phenols, and low molecular weight thiols (Nielsen et al., 2001; Niero et al., 2014, 2015; Velàzquez Vàzquez et al., 2015), and antioxidants derived from casein and whey proteins (Suetsuna at al., 2000; Pihlanto, 2006). The sum of antioxidant activities related to these molecules was recently defined as total antioxidant activity (**TAA**; Chen et al., 2003; Niero et al., 2017b). Total antioxidant activity is a novel phenotypic trait, gaining increasing attention in the dairy sector for its potential role in human nutrition and health. Niero et al. (2017a) investigated phenotypic variation of TAA in milk from Holstein-Friesian cows, and Revilla et al. (2016) studied TAA on cheeses manufactured using different mixtures of milk from cows, ewes, and goats, over 6 mo of ripening. Recently, infrared spectroscopy has been used as alternative and cost-effective tools for the determination of TAA in milk and cheese (Revilla et al., 2016; Niero et al., 2017a). This technique has been proved to be beneficial for the collection of phenotypic data at population level (De Marchi et al., 2014). There is a lack of studies that have investigated TAA of milk from dairy species other than cattle. Therefore, the present study aimed to describe the phenotypic variation of TAA of buffalo, goat, and sheep milk, and to assess correlations between TAA and milk yield and quality traits.

Individual raw milk samples of buffalo (n = 105), goat (n = 112), and sheep (n = 198) were collected in 4, 7, and 10 herds, respectively, from January to April 2017. Animals were from parity 1 to 10 for buffalo and goat, and 1 to 6 for sheep, and from 6 to 307 DIM, 6 to 125 DIM,

and 6 to 197 DIM for buffalo, goat, and sheep, respectively. Immediately after sampling, milk was transferred at 4°C to the laboratory of the Istituto Zooprofilattico Sperimentale del Lazio e della Toscana "Mariano Aleandri" (Rome, Italy), and analysed for fat, protein, casein, and lactose percentages using a MilkoScan FT6000 (Foss, Hillerød, Denmark). Somatic cell count was determined using a Fossomatic (Foss, Hillerød, Denmark) and values of SCC were transformed to SCS by taking the $\log_{10}$ of SCC.

An aliquot of each sample was transferred to the laboratory of the Department of Agronomy, Food, Natural Resources, Animals and Environment of the University of Padova (Legnaro, Italy) for milk TAA assessment. Accordingly to the method proposed by Niero et al. (2017b), milk TAA was measured monitoring a colorimetric reaction, through Biospectrometer Kinetic 1.3.6.0 spectrophotometric device (Eppendorf, Hamburg, Germany). A mixture of 2,20-Azino-Bis [3-Ethilbenzotiazolina-6 Sulfonic Acid] Diammonium Salt (**ABTS**) 14 mM water solution, and $K_2S_2O_8$ 4.9 mM water solution (1:1) was stored in the dark for 12 h at room temperature, in order to achieve ABTS radical activation. Activated ABTS radical solution was added with 80% acetone, until 1.10 ± 0.05 $OD_{730\ nm}$ absorbance score, and used as milk antioxidants extraction solvent. Successively, 0.1 mL of milk in water (1:20) was added with 1 mL of extraction solvent. Samples were vortexed, incubated at room temperature for 10 min to promote antioxidant extraction, and centrifuged at 18,000 *g* for 5 min to accelerate milk protein precipitation. Absorbance of surnatant was read at 730 nm and subtracted to the absorbance of the blank. This difference, directly proportional to milk TAA, was expressed in mmol/L of Trolox equivalents (**TE**). Shapiro-Wilk's test, and visual inspection of data distribution highlighted that TAA values of buffalo, sheep, and goat milk were normally distributed (Figure 1). Comparisons between means of milk TAA from the three species were performed through paired *t* test in SAS software (ver. 9.4; SAS Institute Inc., Cary, NC), including Satterthwaite's test in case of unequal

variances (Ruxton, 2006). Pearson correlations of TAA with milk yield and traditional quality traits were estimated within species through the CORR procedure of the SAS software.

Buffalo milk showed similar composition to that reported by Cecchinato et al. (2012) and Manuelian et al. (2017), except for the notably greater fat percentage of the present study (Table 1). Average fat percentage of goat milk was greater and lactose, protein, and casein percentages were almost comparable to values of Park et al. (2007). Similarly, fat, lactose, protein, and casein percentages of sheep milk were close to findings of Park et al. (2007). Sheep milk showed the largest range of variation (5.61 mmol/L TE), as well as the lowest and the greatest milk TAA values observed in the three species, with a minimum of 4.92 mmol/L TE and a maximum of 10.53 mmol/L TE. Similar distribution was observed for TAA of Holstein-Friesian cow milk, ranging from 3.71 to 10.18 mmol/L TE (Niero et al, 2017a). Instead, buffalo and goat showed lower and similar TAA ranges of variation, scoring 2.45 and 2.41 mmol/L TE, respectively. Total antioxidant activity of buffalo, goat, and sheep milk averaged 7.35, 6.80, and 7.78 mmol/L TE, respectively (Table 1), and paired *t* test showed that mean TAA differed for all comparisons ($P < 0.001$). Total antioxidant activity of goat milk in the present study was close to the average value reported by Niero et al. (2017a) in milk of Holstein-Friesian cows, whereas TAA of sheep and buffalo milk were greater, when compared to the same work.

It is well established that milk fat fraction contains several fat soluble antioxidants (e.g., tocopherols, retinol, and carotenoids) (Nozière et al., 2006; Chauveau-Duriot et al., 2010), and that casein and whey proteins have antioxidant properties (Suetsuna at al., 2000; Pihlanto, 2006). Therefore, the observed differences in TAA could be related to the species-specific milk chemical composition, with particular regard to fat, protein, and casein percentage of cow, buffalo, goat, and sheep milk. According to this hypothesis, goat milk TAA exhibited similar values to that of cow milk, having almost comparable chemical composition, while considerably

greater TAA was observed in sheep and buffalo milk, having also greater fat, protein, and casein percentages. Phenotypic variation of milk TAA expressed as coefficient of variation (**CV**), mirrored the distributions of milk TAA across the three species (Figure 1). The greatest CV was observed for TAA of sheep milk (13.98%), with goat (8.47%) and buffalo (8.18%) exhibiting the same variability. Coefficients of variation reported in the present study were slightly lower than CV reported by Niero et al. (2017a) for milk TAA of Holstein-Friesian cows, partly because of the lower sample size of the three species considered in the current paper. Conversely, CV were greater than those obtained by Niero et al. (2017b) in commercial milk samples, ranging from 2.18 to 3.52%. Nevertheless, a comparison between the two studies is difficult, due to different research aims: Niero et al. (2017b) calculated CV as repeatability and reproducibility relative standard deviations to describe the accuracy of a near infrared spectrophotometric method for milk TAA assessment, while CV of the present study are calculated on a larger array of individual milk samples, thus allowing to catch the variability of the trait of interest.

Phenotypic correlations of milk TAA with milk yield and quality traits were weak within species (Table 2). Milk TAA was unfavourably correlated with milk yield, but the relationship was significant only for buffalo (-0.18; $P < 0.05$). Therefore, high-producing animals yielded milk with slightly lower TAA compared with milk of low-producing animals, meaning that a dilution effect can be hypothesized, similarly to what happens for protein and fat percentage in cow milk (Ng-Kwai-Hang et al., 1982; Niero et al., 2016). Casein percentage was positively correlated with TAA of goat milk (0.20; $P < 0.05$), in agreement with previous studies reporting that casein is the major antioxidant compound in milk (Zulueta et al., 2009). This might be due to its high content of AA with antioxidant effect (Rival et al., 2001), to the association complex between casein and glutathione peroxidase enzyme which is responsible of glutathione antioxidant capacity (Lindmark-Månsson and Åkesson, 2000), and to the antioxidant activity of

casein derived fragments and peptides observed in goat milk (Li et al., 2013). In particular, phospho-peptide derived from the tryptic hydrolysis of casein, exhibited both primary and secondary antioxidant activity towards transition ferrous ion sequestering and direct free radical quenching, respectively (Kitts, 2005). Similarly, protein percentage showed positive correlation with TAA of goat milk (0.17; $P < 0.05$). This favourable association was due to the previously discussed casein antioxidant properties, as well as to whey protein contribution to milk TAA. In particular, among whey proteins, lactoferrin is able to bind iron thus preventing its pro-oxidant activity (Cichosz et al., 2017), while β-LG and derivate peptides have antioxidant effects, firstly preserving retinol and α-tocopherol from oxidation along digestive trait (Liang et al., 2011), and secondly deactivating free radicals through Trp, Tyr, and Met AA residuals (Cichosz et al., 2017).

The present study is the first work dealing with the phenotypic characterization of TAA of buffalo, goat, and sheep milk. Total antioxidant activity is a new phenotypic trait, with potential positive outcomes for human health. Among the three considered species, sheep milk showed the greatest TAA. This is probably due to its relatively high content in fat, protein, and casein percentages, that are known as compounds contributing to milk antioxidant capacity. Accordingly, buffalo and goat milk had lower TAA as well as lower fat, protein and casein percentages. Milk TAA was unfavourably correlated with milk yield, but the relationship was significant only for buffalo. Protein and casein percentages were positively correlated with TAA of goat milk. Values of TAA presented in the present study will be used as reference data to build mid-infrared spectroscopy models for the prediction of this new phenotype on a large scale.

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M. De Marchi and M. Cassandro coordinated the project. S. Currò performed statistical analyses and together with A. Costa performed laboratory analyses. G. Niero and M. Penasa wrote the first draft of the manuscript. All authors contributed to the discussion of the results, commented on the manuscript, reviewed the paper, and approved the final version of the work.

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Table 1. Descriptive statistics of total antioxidant activity, yield, and quality traits of buffalo, goat, and sheep milk

	Buffalo (n = 105)		Goat (n = 112)		Sheep (n = 198)	
Trait[1]	Mean	CV, %	Mean	CV, %	Mean	CV, %
TAA, mmol/L TE	7.35	8.18	6.80	8.47	7.78	13.98
Milk yield, kg/d	7.68	44.46	1.68	42.85	1.09	76.06
Fat, %	8.67	30.54	4.96	32.03	7.34	27.40
Protein, %	4.70	9.80	3.22	18.36	6.10	13.66
Casein, %	3.72	14.13	2.18	21.60	4.55	14.68
Lactose, %	4.61	8.08	4.42	6.23	4.62	8.17
pH	6.74	3.26	6.65	1.34	6.63	1.94
TA, °SH/100mL	6.96	17.10	6.56	8.12	7.51	11.60
SCS, units	5.05	9.99	5.74	9.25	5.18	10.13

[1]TAA = total antioxidant activity, expressed as mmol/L of Trolox equivalents (TE); TA = titratable acidity, expressed as Soxhlet-Henkel degrees (°SH) in 100 mL.

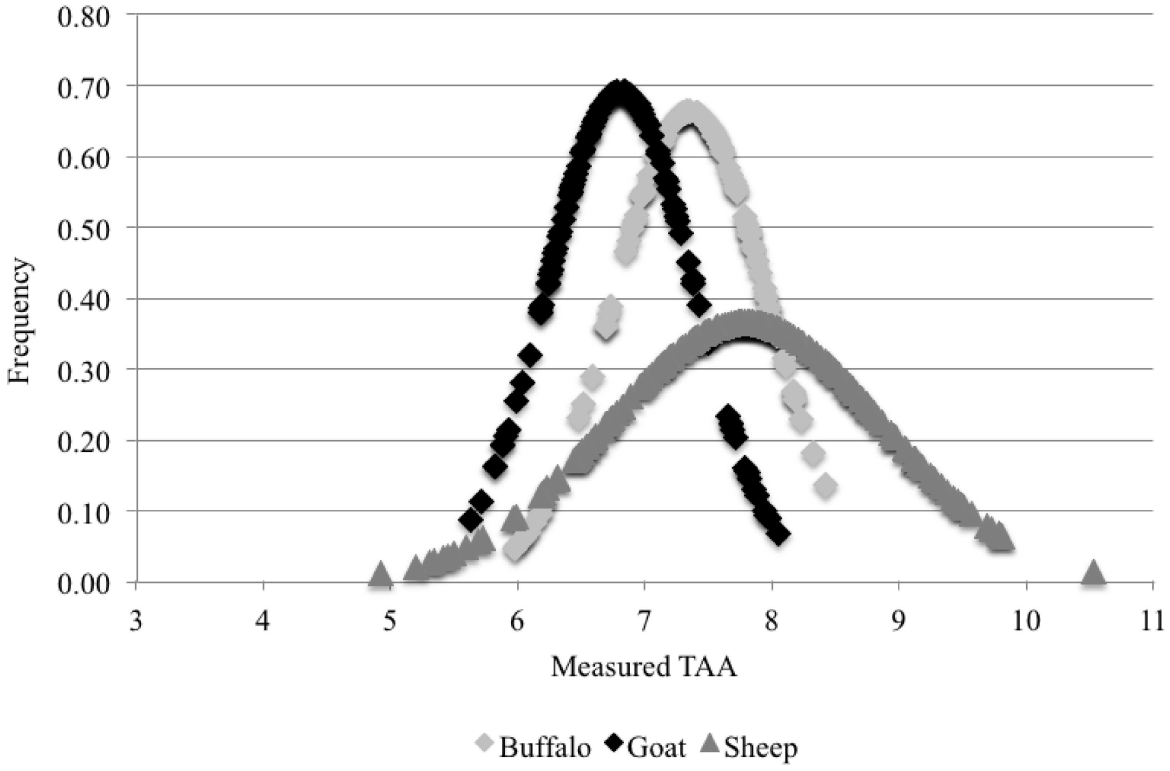
Table 2. Pearson correlations of milk total antioxidant activity with milk yield and quality traits in buffalo, goat, and sheep

Trait[1]	Milk total antioxidant activity		
	Buffalo	Goat	Sheep
Milk yield	-0.18*	-0.05	-0.02
Fat	-0.17	-0.04	-0.07
Protein	0.07	0.17*	0.08
Casein	-0.05	0.20*	0.05
Lactose	-0.06	0.29*	-0.06
pH	0.13	-0.12	-0.04
TA	-0.14	0.10	-0.22*
SCS	0.12	-0.17	0.10

[1]TA = Titratable acidity.

*$P < 0.05$.

Figure 1. Distribution of total antioxidant activity (TAA) in buffalo (n = 105), goat (n = 112), and sheep (n = 198) milk, expressed as mmol/L of Trolox equivalents



8. GENERAL CONCLUSIONS

Basing on the three years of research and on results obtained in the different experiments, the following main conclusions can be drawn:

i. An HPLC method based on methanol elution and fluorescence detection was successfully validated for the quantification of α-T and γ-T in RM, WUM, SUM, WM, and SM. α-tocopherol was the major tocopherol in all milk samples. Adverse effects of skimming and UHT treatments on α-T and γ-T were detected;

ii. Six antioxidant thiol species were found in individual milk samples of different cow breeds. Two of them, GSH and Cys-Gly, were identified and quantified. Milk from Simmental and Alpine Grey was richer in thiols than milk from Holstein-Friesian and Brown Swiss.

iii. A robust and fast spectrophotometric method was developed for the determination of TAA in RM, WUM, SUM, WM, and SM. The method was linear, repeatable, and reproducible. The greatest TAA was measured on SM, followed by SUM, RM, WM, and WUM.

iv. Milk TAA exhibited an exploitable phenotypic variability in milk of Holstein-Friesian cows. Favourable phenotypic correlations of TAA with fat, protein, casein, and SCS were observed. Total antioxidant activity of milk increased across lactation. Mid-infrared prediction models were not enough accurate for analytical purposes.

v. Among buffalo, goat, and sheep milk, sheep milk showed the greatest TAA, probably due to its relatively high content in fat, protein, and casein percentages. Milk TAA was unfavourably correlated with milk yield, whereas protein and casein percentages were positively correlated with TAA of goat milk.

vi. Further investigations are required to assess feeding and genetic aspects, as possible sources of variation influencing milk antioxidants content and milk TAA; phenotypic variation of tocopherols in cow milk and in milk of other dairy species should be evaluated.

9. LIST OF PUBLICATIONS

Journal Publications

Niero, G., M. De Marchi, A. Masi, M. Penasa, and M. Cassandro. 2015. Characterization of soluble thiols in bovine milk. J. Dairy Sci. 98:6014-6017.

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