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THE ANTIOXIDANT CAPACITY OF MILK – THE APPLICATION OF DIFFERENT METHODS *in vitro* AND *in vivo*

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Abstract

Milk contains a wide array of compounds with established or putative pro- or anti-oxidant function. The functions of these compounds have been intensively studied. This review focusses on some important aspects in this wide field namely the methodology for measurement of the total antioxidant capacity (TAC), the content of TAC and some related compounds in human and animal milks and infant formulas, and the effect of milk intake on antioxidant status in the body and on the activity of dietary flavonoids as studied *in vitro* and *in vivo*. Regarding methodology TAC in milk can be measured by spectrophotometric and electrochemical methods and some of their characteristics are reviewed. Milk, whey, high-molecular-weight and low-molecular-weight (LMW) fractions of whey have all been found to have antioxidant capacity using these techniques. The major antioxidant in the LMW fraction has been identified as urate. An extensive literature survey was made regarding data on the antioxidant capacity and related variables of milk obtained from different sources (human milk, infant formulas and animal milk) and subjected to different treatments. Differences in TAC between milks from different sources have been observed but due to the variety of techniques used no clear pattern is evident at present. Another important aspect is the putative effects of the intake of milk products on the antioxidant status of the consumer. A few studies performed in adults and premature infants are reviewed and it is stated that too little information is available to make any firm conclusions in this regard. Finally, a high interest has been devoted to the possible interference of milk with the antioxidant properties of flavonoid-rich food like tea. Most *in vitro* studies show an inhibition by milk on tea flavonoid activity whereas the results from the corresponding *in vivo* studies are equivocal. Our general conclusion is that several compounds in various milk fractions contribute to the antioxidant capacity of milk and that much further work is needed to unravel the complex interactions among the pro- and antioxidants, and their putative health effects on the consumer.

Key words: Radical scavenging, ferric reduction, cyclic voltammetry, flow injection amperometry, sources and treatment of milk, urate, milk-flavonoid interaction, *in vivo* antioxidant status.

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INTRODUCTION

Much work has been performed to describe the total antioxidant capacity (TAC) of foods and studies with large amounts of data have appeared (11, 26). TAC is an attractive concept to get an overall picture of the antioxidant potential of a material and it also requires much less work and methodological infrastructure than analyzing the often complex composition of individual antioxidants.

Several methods for TAC analysis have been applied to a wide range of foods based on different principles and the most successful results so far have been achieved in the analysis of plant foods. Still many inherent theoretical and practical difficulties apply to the TAC concept and only a limited number of studies have been conducted to compare different methods. Some of the disadvantages and advantages of them have been summarised (7, 23, 50, 54). The application of the TAC concept to milk has presented several problems since milk and related foods are rich in proteins and lipids and have a complex colloidal structure. This review focuses on some important aspects in this wide field. Firstly, the methodology for measurement of the total antioxidant capacity (TAC) is reviewed. Secondly, the available data on the content of TAC and some related compounds in human and animal milks and infant formulas are summarised. Thirdly, investigations of the effects of milk intake on body antioxidant status are reviewed, and fourthly, the effects of milk on the activity of dietary flavonoids as studied *in vitro* and *in vivo*.

METHODS USED FOR THE ASSESSMENT OF TAC IN MILK

Milk contains many compounds which contribute to its prooxidant/antioxidant balance (44) and the measurement of all or only some components is an extensive task. Like for other foods the concept of measuring the TAC or other overall measures is thus an attractive one for many purposes although it by definition does not give any detailed information about individual components (7, 12, 23, 51).

Spectrophotometric and fluorimetric methods

The ABTS method

As summarized elsewhere (12) several methods to measure TAC have been applied to milk (Table 1). Among spectrophotometric methods the 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) method has

been much used for several types of studies. ABTS can donate an electron to generate the cation radical $ABTS^{\bullet+}$ which has a rather long half-life and high extinction coefficients. Different alternative methods for the use of $ABTS^{\bullet+}$ as a reagent were summarised by Rice-Evans and Miller (58) and Chen (12). In one alternative $ABTS^{\bullet+}$ is generated by the addition of potassium persulphate (14, 56) and the quenching of this radical is then measured. The ABTS methods measure the electron- or hydrogen-donating properties of antioxidants, both hydrophilic and lipophilic ones (54). They are widely used due to their simplicity, speed and sensitivity. Moreover, they are not confounded by some factors that contribute to the antioxidant activity in other model systems, such as metal chelation (12).

Table 1. Different methods used to measure TAC in milk and related materials.

Spectrophotometric methods	
	ABTS method
	DPPH method
	FRAP method
	Folin-Ciocalteu
Fluorimetric methods	
	ORAC method
Electrochemical methods	
	Cyclic voltammetry
	Rotating disk electrode voltammetry
	Flow injection amperometry
Other methods	
	Chemiluminescence

The DPPH method

The 1,2-diphenyl-2-picrylhydrazyl radical is stable and can be used in the assay without prior generation (54). Like the ABTS assay the DPPH method is based on both hydrogen atom transfer and single electron transfer mechanisms and its advantages and disadvantages have been reviewed (54). Martysiak- Żurowska and Wenta recently compared the use of the ABTS and DPPH method in human milk samples (48). They found that ABTS was the more appropriate method for measuring TAC in human milk.

The FRAP method

In the ferric reducing antioxidant power (FRAP) method the activity of an antioxidant is measured by following the reduction of ferric to ferrous ions. The latter ion is assayed through chelation to 2,4,6-tri(2'-pyridyl)-1,3,5-s-triazine. Compounds such as polyphenols, ascorbate, tocopherol, Trolox, urate, bilirubin and albumin have been found to reduce ferric ions using this assay. FRAP is a single electron transfer method (54). As discussed elsewhere (8, 12, 51) the FRAP method is suitable for a variety of biological fluids as well as extracts of foods. The reagents are simple to prepare, the results are highly reproducible, and the procedure is straightforward and rapid. As in some other assays of the antioxidant defence, the conditions used in the FRAP reaction are far from physiological, especially the low pH of the reaction mixture. Thus, it should be borne in mind that the hierarchy and activities of the individual antioxidants obtained by this method might not reflect the situation at other pH values.

The ORAC method

The oxygen radical absorbance capacity method is based on hydrogen atom transfer mechanisms and different fluorimetric probes have been used as reviewed elsewhere (50, 54). In the ensuing sections several examples are given concerning TAC data obtained in milk using this method. It has also been used to assess the contribution of different fractions to milk TAC (17) as summarized later.

Electrochemical methods

Cyclic voltammetry (CV)

For mechanistic studies and other purposes electrochemical methods have been used to measure TAC. Among them CV is a faradic detection technique used to determine the redox properties of molecules in solution or attached to the electrode surface (6). The potential of a working electrode is linearly scanned versus a reference electrode from an initial value to a final value and back, while recording the anodic and cathodic currents (Figure 1). As the potential is scanned towards a more positive value (the upper part of the recording), the electrode becomes sufficiently oxidising to give rise to the recorded anodic current, which increases until the concentration of the reducing substances at the anodic surface approaches zero, causing the current to reach a peak and then decline. At +1000mV the scan direction is switched towards negative values (the lower part of the recording) giving rise to an analogous cathodic current, completing the cycle. CV is a method capable of rapidly generating a new species during the forward scan and then monitoring its fate on the reverse scan. CV has been employed to measure the overall antioxidant capacity stemming from the various low molecular weight (LMW) antioxidants without evaluating the specific contribution of each compound (36). The overall reducing power of a biological sample correlates with the overall scavenging capacity of the sample (15). CV traces provide information concerning the types of various antioxidants and their total concentration without quantification of specific compounds, and the CV method is suitable for use with biological fluids and tissue homogenates. The resulting measurements have been correlated with the antioxidant capacity of both hydrophilic and lipophilic free radical scavengers (36).

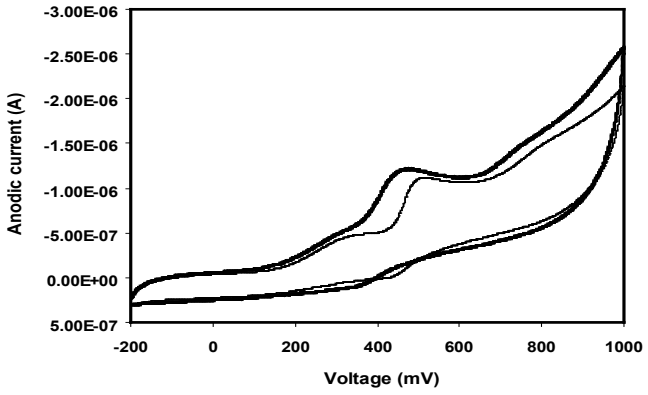

Figure 1. Cyclic voltammograms of fresh milk (medium line, pH 6.7) and whey (heavy line, pH 4.6). Two anodic waves were observed on each curve. The wave at higher potential on each curve represented urate oxidation, while the waves situated at the lower potential was generated by the oxidation of ascorbate (from Chen *et al.*, (12)).

As an example of analysis by CV, Figure 1 shows the analysis of fresh milk and whey. The wave at higher potential on each curve represented urate oxidation, while the waves situated at the lower potential disappeared after ascorbate oxidase treatment, showing that it was generated by the oxidation of ascorbate. This experiment showed the existence of ascorbate in fresh milk and whey, and the possibility of detecting it with electrochemical methods. When stored samples of milk, whey and the LMW fraction of whey were analysed, only the anodic wave corresponding to urate was seen (13).

Rotating disk electrode (RDE) voltammetry

A related technique, RDE voltammetry is based on the same principle as CV and the equipment is essentially the same except that the working electrode is rotated at a constant angular velocity, thus offering hydrodynamic properties to RDE voltammetry. Upon rotation, the solution is drawn up to the electrode by a motion perpendicular to the electrode surface and impinges onto the electrode surface where the dissolved analyte can be oxidised or reduced. Solutions of the dissolved products of the electrode reaction are then spun away from the electrode in radial paths in a plane parallel to the electrode surface. The resulting current from the redox reaction produces a measurable signal which is directly related to the concentration of the analyte. Historically, the technique has been used to study the kinetics of rapid reactions and electron transfer rates to the electrode surface (6).

The use of RDE is a means to establish mass transfer by convection, and as an example it could be used in a set-up to perform linear sweep voltammetry (Figure 2). As shown by curve B, the oxidation of urate in citrate buffer started at +400 mV and at potentials more positive than +700 mV a steady state was reached. When urate was mixed with ascorbate (curve A), ascorbate oxidation started at about +200 mV while urate oxidation started at around +400 mV. When the LMW fraction of whey was mixed with ascorbate (curve C), the oxidation signal started to appear at +200 mV, and at potentials more positive than +800 mV, curve C tended to be parallel with curve A. The RDE voltammograms of untreated and uricase-treated LMW fractions of whey (curve D and E) showed that antioxidant(s) in the untreated LMW sample started to be oxidised at about +500 mV, but after the uricase treatment, this oxidation signal was removed, while ascorbate oxidase treatment of the LMW sample had no significant effect (not shown). This observation confirms that the major antioxidant capacity remaining in the LMW fraction of whey was removable by uricase and that ascorbate made little contribution to it (12, 13).

Flow-injection amperometry (FIAmp)

Another hydrodynamic electrochemical method is the FIAmp technique (68). In the example shown in Figure 3 a system of a three-electrode wall-jet electrochemical cell was used in combination with a flow-injection line (3). The constant flow of solution transports the injected sample along the system and through the wall-jet cell generating mass transfer by convection and the sample plug will be registered as an amperometric response peak. The anodic or cathodic current is generated by oxidation or reduction of redox compounds on the surface of the working electrode at a controlled potential. During transport in the flow system and between the inlet (injection nozzle) and the working electrode surface, the sample is dispersed by the carrier solution (59), which causes a decrease in anodic current. The anodic current is proportional to the concentration of the redox compounds in the sample (12). The FIAmp technique was first employed for TAC assessment in wine by Mannino *et al.* (46). It was also used to determine TAC in olive oil (47) and lipophilic food extracts (9). Some of the TAC values from the FIAmp assay were compared with those from an ABTS method. Good correlation was observed for the olive oil and the lipophilic food extracts, but not for spinach extract (9, 47). The FIAmp method is direct and simple and has good precision for quantitative measurements. It allows high sample throughput and does not require the use of a reactive species in order to determine the antioxidant capacity of samples. The electrochemical ranking of lipophilic compounds determined using the FIAmp method was reported to be in good agreement with the antioxidant activity measured using other methods (9).

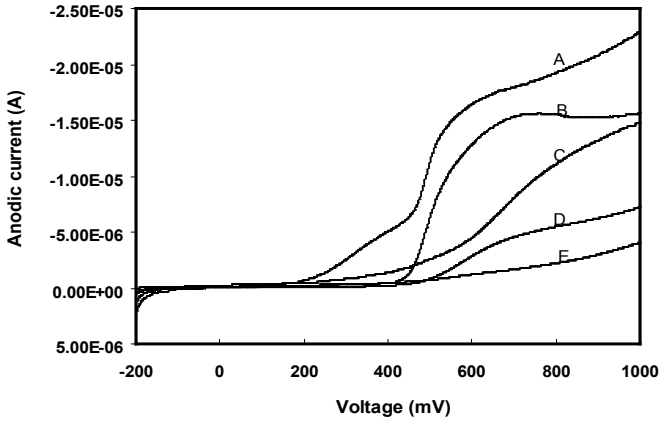

Figure 2. Linear sweep voltammograms using a rotating disk electrode at 100 rpm with the following samples: 200 µmol/L$^-$ urate mixed with 100 µmol/L ascorbate (A), 200 µmol/L urate (B), LMW fraction of whey (pH 4.6) mixed with 100 µmol/L ascorbate (C), LMW (D), and LMW treated by uricase (0.2 U per 10 mL) for 30 min at pH 8.5 at room temperature (E) (from Chen *et al.*, (13)).

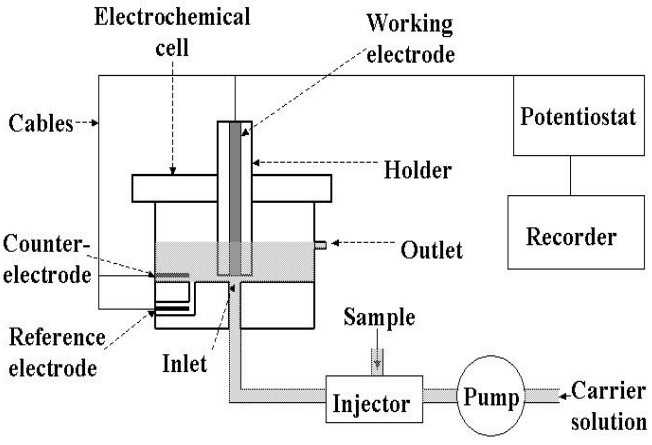

Figure 3. Illustration of a wall-jet cell flow-injection amperometry system (FIAmp) (from Chen, (12)).

General remarks on TAC methods

A general comment on the use of all the methods mentioned to assess TAC of milk, is that the ABTS method was found useful in determining the TAC of both the LMW fraction and proteins in milk and whey. Moreover, it was

easy to perform and had a high throughput. The FRAP method has been widely used for measuring the TAC of human plasma, tea, vegetables and fruits, and we also found it suitable for the LMW fraction of whey. However, it showed low reactivity to whey proteins (12). Regarding the electrochemical methods the main advantage is that they directly monitor the electron-donating ability of the compounds, distinguish different reductive compounds according to peak potential, and reveal some reaction details. A limitation is that only low-molecular weight redox substances can be measured, since the current response to proteins would be very low due to the low diffusion coefficient of the macromolecules. In one of our own studies (14) the TAC values of the LMW fraction from whey as measured by three methods (ABTS, FRAP and FIAmp) were highly correlated to each other (Figure 4). It has also been concluded by several research groups that in order to gain a good understanding of the antioxidant properties of milk products, the use of several different methods is necessary (7, 23, 54).

To assess the possible health impact of milk antioxidants it is important to compare milk TAC with that of other foods. A comparison of TAC of bovine milk with that in other foods and biological fluids is shown in Figure 5. Regarding TAC of milk and whey measured by the ABTS method, the TAC of low-fat milk was found to be approximately twice that of orange juice, and also higher than that of white wine. The TAC of whey was approx. 25 % of the values for these plant-based beverages. When values obtained with the FRAP method were considered instead, the TAC of whey was about 40% of those reported for human plasma (12).

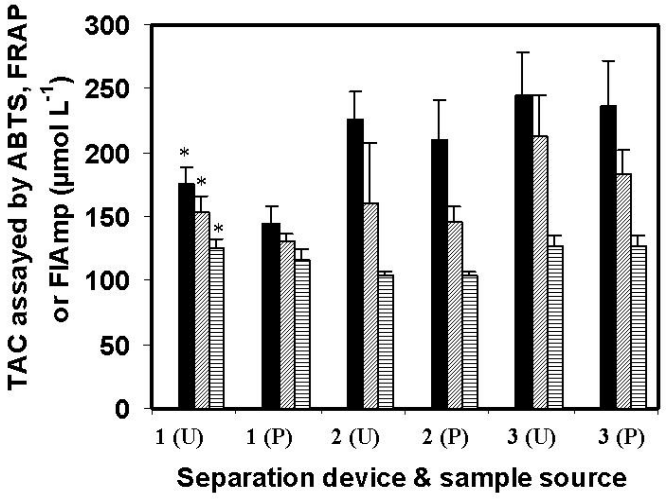

Figure 4. TAC values of LMW fractions as assayed by ABTS (pH 5.0, black), FRAP (diagonal lines) and FIAmp (horizontal lines) methods. Three different ultrafiltration separation devices were used: 1 = Omega cell, 2 = Centriprep concentrator, 3 = Fugisep tube. The sample sources are LMW fractions of whey from unpasteurised milk (denoted U), and LMW fractions of whey from pasteurised milk (denoted P). The mean values of three independent assays and the standard deviations are shown. The main antioxidant in the columns marked by * was urate as indicated by the loss of most of the activity after uricase treatment (Chen *et al.*, (14)).

Other methods

The TRAP, Folin-Ciocalteu, chemiluminescence and other methods have been reviewed by Prior *et al.* (54) and Niki (50).

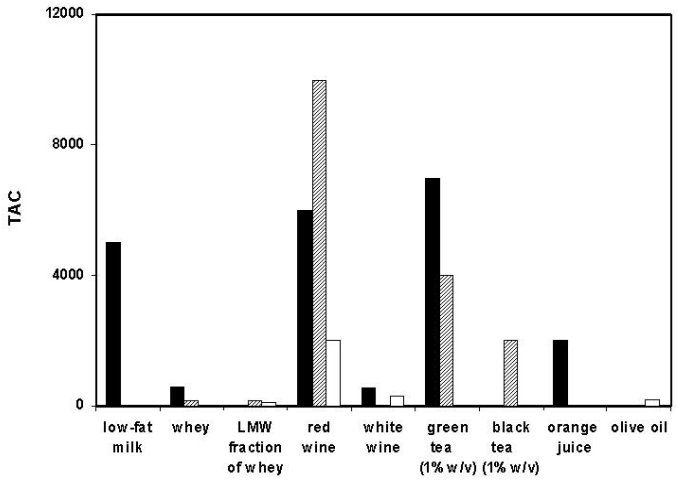

Figure 5. Comparison of TAC values of milk (fractions) with those of other foods. Mean values are shown expressed as µmol/L Trolox equivalent in most cases. From Chen (12) and references for other foods are given there. The methods include ABTS (pH 7.4) (black), FRAP (lines) and FIAmp (open).

TAC IN DIFFERENT MILKS

Human milk has a unique nutritional composition compared to bovine milk. Colostrum which is produced the first days after birth is especially rich in proteins and antibodies. Transitional milk is produced after 3-5 days, and it is a more thin, watery and sweet milk. Mature human milk, which is produced from 2 weeks after birth, contains 0.8-0.9% protein, 3-5% fat, 6.9-7.2 % carbohydrates (lactose), and 0.2% ash (minerals) (33). Human milk also contains numerous bioactive compounds that contribute to antioxidant capacity and functions: antioxidant enzymes like catalase, superoxide dismutase (SOD) and glutathione peroxidase, vitamins E and C, carotenoids and lactoferrin (44). In the following the data on antioxidant capacity of different milk types and milk analogues are summarized together with some data on antioxidant enzymes and lipid peroxidation.

Fresh human milk

Data on TAC and other antioxidant-related variables are available for the three types of fresh human milk (Tables 2-4). The antioxidant status of human milk varied during the different stages of lactation. Zarban *et al.* observed that the TAC values measured by the FRAP assay and DPPH scavenging activity were significantly higher in colostrum compared to in transitional and mature milk (74). A significant decrease of TAC values (ABTS assay) of human milk over time since parturition was also found by Sari *et al.* (61). Another study showed that oxygen radical absorbance capacity (ORAC) values of colostrum and transitional milk were similar but both higher compared to the values of mature milk (2). On the other hand Tijerina-Sáenz *et al.* (69) reported much higher TAC values of mature milk measured by ORAC compared to those found by Alberti-Fidanza *et al.* (2). This could be due to the use of a different fluorescence probe for the ORAC assay. Since many different methods are used to measure antioxidant capacity/activity, it is difficult to compare the data and thus, so far, there is no established reference value for the antioxidant status of human milk available. The collected data indicate that TAC is higher in colostrum and transitional milk than in mature milk (Table 2).

Table 2. Antioxidant activity and related variables (mean (SD)) in fresh human milk using different methods.

Type of milk (postpartum)	Method	Antioxidant capacity value	Reference
Antioxidant capacity			
Colostrum	TAC (ORAC assay)	1.17 (0.50) mmol/L	*2*
Transitional	TAC (ORAC assay)	1.18 (0.38) mmol/L	
Mature	TAC (ORAC assay)	1.01 (0.37) mmol/L	
Colostrum	TAC (ABTS assay)	1.66 (0.06) TE	27
Colostrum	TAC (ABTS assay)	3.37 (0.58) mmol/L	70
Mature	TAC (using kit)	0.24 (0.16) mMeq uric acid	65
Mature (1 month)	TAC (ORAC assay)	3.41 (0.07) mmol/L	69
Colostrum (2 days)	TAC (FRAP assay)	1061.6 (500.6) µmol/L	74
	DPPH-scav activity (%)	50.4 (19.7) µmol/L	
Transitional (7 days)	TAC (FRAP assay)	915.3 (511.4) µmol/L	
	DPPH-scav activity (%)	40.8 (20.0) µmol/L	
Transitional (30 days)	TAC (FRAP assay)	816.3 (379.4) µmol/L	
	DPPH-scav activity (%)	41.9 (19.4) µmol/L	
Mature (3months)	TAC (FRAP assay)	862.7 (457.7) µmol/L	
	DPPH-scav activity (%)	44.6 (18.5) µmol/L	
Mature (6 months)	TAC (FRAP assay)	724.7 (302.4) µmol/L	
	DPPH-scav activity (%)	38.2 (17.3) µmol/L	
Colostrum	TAC (ABTS assay)	4.1 (2.9-5.4) mmol/L	61
Transitional	TAC (ABTS assay)	3.5 (2.2-4.3) mmol/L	
Mature	TAC (ABTS assay)	1.6 (0.1-3.7) mmol/L	
Other antioxidant variables			
Mature	MDA concentration	0.03-2.53 µmol/L	49
	GPx activity	15.4-51.4 mmol/L x min	
Colostrum	Conjugated dienes	24.33 (9.62) µmol/L	70
	TBARS	3.17 (0.77) µmol/L	
	Lipid peroxidation	6.15 (1.75) µmol/L	
Colostrum (3-5days)	MnSOD	0.32 (0.05) U/mg protein	34
	CuZnSOD	1.47 (0.25) U/mg protein	
Transitional (3 weeks)	MnSOD	0.94 (0.17) U/mg protein	
	CuZnSOD	3.07 (0.30) U/mg protein	
Mature (4 months)	MnSOD	0.13 (0.04) U/mg protein	
	CuZnSOD	0.83 (0.25) U/mg protein	
Mature (7 months)	MnSOD	0.32 (0.08) U/mg protein	
	CuZnSOD	1.44 (0.44) U/mg protein	
Mature	MDA concentration	0.9 (0.5) µmol/L	65
	GSH concentration	7.74 (5.53) µmol/L	
	GPx activity	17.7 (5.0) mmol/L x min	
Mature	MDA concentration	0.7 (0.4) µmol/L	66
	GPx activity	18.2 (9.5) mmol/L x min	

ABTS, 2-2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) radical method; DPPH, 1,1-diphenyl-2-picrylhydrazyl radical method; FRAP, ferric reducing antioxidant power assay; GPx, glutathione peroxidase; GSH, glutathione; MDA, malondialdehyde; ORAC, oxygen radical absorbance capacity; TBARS, thiobarbituric acid reactive compounds; TE, Trolox equivalent.

Regarding other antioxidant variables only few data were collected (Table 2). Kasapović *et al.* found that milk samples collected in the third week after birth had a significant higher SOD activity compared to colostrum (34). The content of MDA and the activity of glutathione peroxidase obtained in different studies were in the same range but also showed some variation.

Treated and stored human milk

Human milk from donors is often treated by heating to inactivate pathogenic bacteria and stored for administration to newborns. One study investigated the effect of two kinds of pasteurization on the TAC and antioxidant enzyme activities of human milk (65) (Table 3). Warming of the milk to 63°C for 30 min significantly decreased the TAC value compared to that of fresh milk, whereas pas-

teurization by warming milk to 75°C for 15 sec had no effect on the TAC value. In the same study glutathione concentration and glutathione peroxidase activity were both decreased in thermally treated milk samples compared to fresh milk samples, but the concentration of malondialdehyde (MDA) in milk was not affected by pasteurization. High temperature pasteurization treatment might thus be a preferable thermal treatment as regards antioxidant properties.

Storage of human milk may decrease the nutritional value and, therefore, it is important to evaluate the nutritional and antioxidant values of human milk stored at different temperatures and time periods (Table 3). In one study, TAC as measured by ABTS assay showed the highest values for fresh milk (27). Freezing of milk samples at -20°C resulted in significantly decreased TAC values compared to that of fresh or refrigerated samples. TAC values were also lower after 7 days of storage compared to at 48 hr both for refrigerated and frozen samples. In another study the TAC values were measured by the ABTS assay in human fresh colostrum and samples from a hospital milk bank stored at -20°C for 2 months (70). These scientists found no significant differences between TAC values of fresh and stored samples but observed that the data for stored samples showed a higher variation. Very recently, Sari *et al.* showed that TAC values, as measured by the ABTS assay, of transitional or mature milk samples were significantly decreased when the samples have been stored at -80°C for 2 months (61). In contrast, TAC values of colostral milk were not significantly affected by freezing.

Regarding enzyme activity, it has been shown that glutathione peroxidase activity was significantly decreased in refrigerated (4°C for 24hr) and frozen (-20°C for 10 days) milk samples compared to fresh milk (49). The concentration of MDA was increased in refrigerated and frozen samples, although the MDA concentration in refrigerated milk samples was only significantly different from that of fresh milk samples (49). These results indicate that freezing is a better storage method compared to refrigeration. In contrast, in another study it was concluded that human colostral milk is best stored at refrigerator temperature (27). In still another study (70), lipid peroxidation products like thiobarbituric acid reactive compounds (TBARS) and conjugated dienes remained unaffected after different types of storage whereas the concentration of lipid peroxides after storage (-20°C for 2 months) was significantly higher than in fresh milk. This might be due to the occurrence of lipoprotein lipase activity at -20°C.

In several additional studies the effects of storage on antioxidant enzyme activities have been reported. Silvestre *et al.* compared the antioxidant enzyme activities of mature milk stored at -20°C and -80°C for 2 months (66). They observed that glutathione peroxidase activity was significantly decreased after storage at -20°C for 15 days, 1 and 2 months compared to that of control samples. Storage at -80°C for 30 days resulted in a non-significant decrease in glutathione peroxidase activity compared to fresh samples, whereas after storage for 2 months the decrease was significant compared to fresh samples. It was concluded that the extent of decrease in glutathione peroxidase activity was dependent on the donor and the storage conditions. Freezing at -80°C preserved better the glutathione peroxidase activity than freezing at -20°C for a period of 30 days or less. This might be explained by a higher stability of the lipid fraction at lower temperatures. The MDA concentration of mature milk samples during the first 30 days of storage at -20°C or -80°C were not significantly different compared to control samples. However, after 2 months of storage the MDA concentration was significantly increased. This indicates that lipid peroxidation products in mature milk are stable for at least 30 days both at -20°C and -80°C.

The data of TAC values and other antioxidant enzyme activities of stored human milk are thus heterogeneous and there is so far no optimal storage condition described from this point of view. It is also not clear in detail which components of milk are affected by storage.

Infant formulas

Much effort has been made to develop infant formulas simulating the nutritional composition and properties of human milk. Regarding TAC values some variation has been observed in infant formulas of five different brands (data obtained using ABTS assay vary from 2.7 (2.3) to 4.7 (0.7) mmol/L) as well as in the levels of lipid peroxides (from 0.8 (1.3) to 2.2 (3.5) µmol/L) (70). The nutritional composition in the different infant formulas was however similar. Remarkably, no significant difference was observed between TAC values of human milk and infant formulas (70). This could be explained by the fact that TAC takes into account many antioxidants and that infant formulas usually contain higher amount of vitamins A, E and C compared to human milk. Hanna *et al.* observed no significant differences in TAC, measured by ABTS assay, of five infant formulas but found significant lower TAC values in infant formulas compared to that of fresh colostrum (27). Moreover, higher TAC values were also found in plasma of infants given breast milk compared to plasma samples from infants given formula-based milk (5). In contrast, one study suggested that infant formulas have a higher TAC value than human milk using ORAC assay (2).

The differences between the TAC data described above might be due to the use of different methods for the determination of antioxidant properties which are based on different mechanisms and thus do not measure exactly the same analytes. Furthermore, infant formulas are often enriched with vitamins A, E and C while human milk may contain more of other compounds with antioxidant properties than infant formulas. Additional studies are needed to elucidate similarities and differences between human milk and infant formulas and they might contribute to the further development of optimal infant formulas.

Animal milks

Cow milk

Several studies have been performed to determine the TAC and antioxidant enzyme activities in milk from e.g. cow, sheep, goat and donkey (Table 4). Cow milk contains 3.2% protein, 3.2% fat, 5% carbohydrates (lactose) and 0.7% ash. Cow colostrum contains much more of proteins than mature milk (19). The TAC values of commercial ultra-high temperature (UHT) treated cow milk samples, as measured by ORAC assay, ranged from 11995 (456) to 14518 (356) µmol/L, and TAC values of commercial pasteurized cow milk samples were between 13624 (715) and 14216 (1051) µmol/L (75). No significant differences were found between the mean TAC value of UHT-treated and pasteurized milk samples. The milk fat content was not re-

Table 3. Antioxidant activity and related variables (mean (SD)) in treated and stored human milk.

Type of milk and storage conditions	Method	Antioxidant capacity value	Reference
Antioxidant activity			
Colostrum, fresh	TAC (ABTS assay)	1.66 (0.06) TE	27
Colostrum, 4°C, 48hr	TAC (ABTS assay)	1.58 (0.06) TE	
Colostrum, 4°C, 7 days	TAC (ABTS assay)	1.48 (0.05) TE	
Colostrum, -20°C, 48hr	TAC (ABTS assay)	1.45 (0.05) TE	
Colostrum, -20°C, 7 days	TAC (ABTS assay)	1.34 (0.04) TE	
Colostrum, fresh	TAC (ABTS assay)	3.37 (0.58) mmol/L	70
Colostrum, -20°C, 2 months	TAC (ABTS assay)	3.1 (1.22) mmol/L	
pasteurization (63°C, 30min)	TAC (using kit)	0.08 (0.06) mMeq uric acid	65
pasteurization (75°C, 15 sec)	TAC (using kit)	0.24 (0.14) mMeq uric acid	
Colostrum, fresh	TAC (ABTS assay)	4.1 (2.9-5.4) mmol/L	61
Colostrum, -80°C, 2 months	TAC (ABTS assay)	3.7 (2.1-4.9) mmol/L	
Transitional, fresh	TAC (ABTS assay)	3.5 (2.2-4.3) mmol/L	
Transitional, -80°C, 2 months	TAC (ABTS assay)	1.3 (0.1-2.3) mmol/L	
Mature, fresh	TAC (ABTS assay)	1.6 (0.1-3.7) mmol/L	
Mature, -80°C, 2 months	TAC (ABTS assay)	0.7 (0.5-1.2) mmol/L	
Other antioxidant variables			
Mature, fresh	MDA concentration	Range 0.03- 2.53 µmol/L	49
	GPx activity	Range 15.4-51.4 mmol/L x min	
Mature, 4-6°C, 48hr	MDA concentration	Range 0.20-4.05 µmol/L	
	GPx activity	Range 6.0-43.4 mmol/L x min	
Mature, -20°C, 10 days	MDA concentration	Range 0.37-5.06 µmol/L	
	GPx activity	Range 12.2-45.4 mmol/L x min	
Colostrum, fresh	Conjugated dienes	24.33 (9.62) µmol/L	70
	TBARS	3.17 (0.77) µmol/L	
	Lipid peroxidation	6.15 (1.75) µmol/L	
Colostrum, -20°C, 2 months	Conjugated dienes	25.02 (8.42) µmol/L	
	TBARS	3.67 (1.27) µmol/L	
	Lipid peroxidation	14.95 (12.05) µmol/L	
pasteurization (63°C, 30min)	MDA concentration	0.8 (0.6) µmol/L	65
	GSH concentration	4.17 (3.24) µmol/L	
	GPx activity	6.6 (5.4) mmol/L x min	
pasteurization (75°C, 15 sec)	MDA concentration	0.8 (0.5) µmol/L	
	GSH concentration	5.78 (3.24) µmol/L	
	GPx activity	6.7 (4.3) mmol/L x min	
Mature, fresh	MDA concentration	0.7 (0.4) µmol/L	66
	GPx activity	18.2 (9.5) mmol/L x min	
Mature, -20°C, 15 days	MDA concentration	0.8 (0.2) µmol/L	
	GPx activity	11.7 (6.8) mmol/L x min	
Mature, -20°C, 30 days	MDA concentration	1.0 (0.3) µmol/L	
	GPx activity	7.2 (2.9) mmol/L x min	
Mature, -20°C, 60 days	MDA concentration	1.1 (0.3) µmol/L	
	GPx activity	6.2 (2.0) mmol/L x min	
Mature, -80°C, 15 days	MDA concentration	0.8 (0.3) µmol	
	GPx activity	14.7 (8.1) mmol/L x min	
Mature, -80°C, 30 days	MDA concentration	0.7 (0.3) µmol/L	
	GPx activity	14.9 (8.7) mmol/L x min	
Mature, -80°C, 60 days	MDA concentration	1.4 (0.6) µmol/L	
	GPx activity	10.3 (4.0) mmol/L x min	

ABTS, 2-2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) radical method; GPx, glutathione peroxidase; GSH, glutathione; MDA, malondialdehyde; TBARS, thiobarbituric acid reactive compounds; TE, Trolox equivalent.

Table 4. Antioxidant activity and related variables (mean (SD)) in animal milks.

Animal source and breed, type of milk and storage conditions	Method	Antioxidant capacity value	Reference
Antioxidant capacity			
Commercial (pasteurized) cow milk 0.1% fat, pH 5.0	TAC (ABTS assay)	1246 µmol/L	14
Commercial (pasteurized) cow milk 0.1% fat, pH 7.4	TAC (ABTS assay)	4560 µmol/L	
Commercial (pasteurized) cow milk 0.5% fat, pH 5.0	TAC (ABTS assay)	1561 µmol/L	
Commercial (pasteurized) cow milk 1.5% fat, pH 5.0	TAC (ABTS assay)	1852 µmol/L	
Commercial (pasteurized) cow milk 3% fat, pH 5.0	TAC (ABTS assay)	2241 µmol/L	
Commercial (pasteurized and defatted) cow milk 4% fat, pH 5.0	TAC (ABTS assay)	755 µmol/L	
Commercial (pasteurized and defatted) cow milk 4% fat, pH 7.4	TAC (ABTS assay)	3777 µmol/L	
Unpasteurized cow milk 0.1% fat, pH 5.0	TAC (ABTS assay)	1249 µmol/L	
Commercial UHT cow milk	TAC (ORAC assay)	Range: 11995 (456) to 14518 (356) µmol/L	75
Commercial pasteurized cow milk	TAC (ORAC assay)	Range: 13624 (715) to 14216 (1051) µmol/L	
Ewe (Berrichon du Cher, n=15):			45
Colostrum (0 min)	TAC (FRAP assay)	4.74 (1.60) µmol/g protein	
Colostrum (12 hr)	TAC (FRAP assay)	7.73 (2.76) µmol/g protein	
Colostrum (24 hr)	TAC (FRAP assay)	9.28 (2.06) µmol/g protein	
Colostrum (48hr)	TAC (FRAP assay)	10.56 (3.0) µmol/g protein	
Mature (5 days)	TAC (FRAP assay)	9.73 (4.5) µmol/g protein	
Ewe (Uhruska, n=13):			45
Colostrum (0 min)	TAC (FRAP assay)	4.78 (1.70) µmol/g protein	
Colostrum (12 hr)	TAC (FRAP assay)	6.63 (2.6) µmol/g protein	
Colostrum (24 hr)	TAC (FRAP assay)	9.81 (2.42) µmol/g protein	
Colostrum (48hr)	TAC (FRAP assay)	7.72 (2.53) µmol/g protein	
Mature (5 days)	TAC (FRAP assay)	6.69 (2.85) µmol/g protein	
Cow (Holstein-Friesian, n=25):			1
Colostrum (0 min)	TAC (FRAP assay)	3.92 (1.27) µmol/g protein	
Colostrum (24hr)	TAC (FRAP assay)	6.81 (1.34) µmol/g protein	
Colostrum (48hr)	TAC (FRAP assay)	9.37 (2.25) µmol/g protein	
Mature (6 days)	TAC (FRAP assay)	13.41 (2.69) µmol/g protein	
Mature (12 days)	TAC (FRAP assay)	11.96 (3.50) µmol/g protein	
Cow milk (commercial, pasteurized whole fat)	TAC (Blue CrO5)	42.29 (2.10) mmol/L α tocopherol eq.	67
Goat milk (Prisca, Greece)	TAC (Blue CrO5)	66.7 (2.30) mmol/L α-tocopherol eq.	
Goat milk (Saanen, Italy)	TAC (Blue CrO5)	35.8 (2.70) mmol/L α-tocopherol eq.	
Goat milk (Ionica, Italy)	TAC (Blue CrO5)	33.6 (3.20) mmol/L α-tocopherol eq.	
Donkey milk (Martina Franca, Italy)	TAC (Blue CrO5)	31.2 (2.90) mmol/L α-tocopherol eq.	

Other antioxidant variables			
Ewe (Berrichon du Cher, n=15):			45
Colostrum (0 min)	GPx	23.62 (5.7) pkat/mg protein	
	GTr	93.0 (26.0) pkat/mg protein	
Colostrum (12 hr)	GPx	37.54 (9.1) pkat/mg protein	
	GTr	71.0 (23.0) pkat/mg protein	
Colostrum (24 hr)	GPx	45.8 (7.8) pkat/mg protein	
	GTr	71.0 (19.0) pkat/mg protein	
Colostrum (48hr)	GPx	60.0 (17.0) pkat/mg protein	
	GTr	58.0 (17.0) pkat/mg protein	
Mature (5 days)	GPx	51.8 (16.0) pkat/mg protein	
	GTr	50.0 (17.0) pkat/mg protein	
Ewe (Uhruska, n=13):			
Colostrum (0 min)	GPx	29.63 (5.3) pkat/mg protein	
	GTr	37.0 (12.0) pkat/mg protein	
Colostrum (12 hr)	GPx	38.87 (8.8) pkat/mg protein	
	GTr	57.0 (13.0) pkat/mg protein	
Colostrum (24 hr)	GPx	49.0 (8.1) pkat/mg protein	
	GTr	69.0 (20.0) pkat/mg protein	
Colostrum (48hr)	GPx	57.0 (19.0) pkat/mg protein	
	GTr	61.0 (16.0) pkat/mg protein	
Mature (5 days)	GPx	49.3 (17.0) pkat/mg protein	
	GTr	63.0 (13.0) pkat/mg protein	
Cow (Holstein-Friesian, n=25):			1
Colostrum (0 min)	GPx	3.81 (1.34) pkat/mg protein	
	Lipid peroxidation	0.087 (0.009) mmol/g protein	
	Protein peroxidation	3.8 (0.8) mmol/g protein	
Colostrum (24hr)	GPx	7.23 (1.62) pkat/mg protein	
	Lipid peroxidation	0.124 (0.030) mmol/g protein	
	Protein peroxidation	6.3 (2.0) mmol/g protein	
Colostrum (48hr)	GPx	11.59 (2.16) pkat/mg protein	
	Lipid peroxidation	0.112(0.017) mmol/g protein	
	Protein peroxidation	4.6 (2.0) mmol/g protein	
Mature (6 days)	GPx	10.87 (2.30) pkat/mg protein	
	Lipid peroxidation	0.102 (0.023) mmol/g protein	
	Protein peroxidation	5.3 (1.0) mmol/g protein	
Mature (12 days)	GPx	17.31 (3.74) pkat/mg protein	
	Lipid peroxidation	0.126 (0.030) mmol/g protein	
	Protein peroxidation	7.3 (3.0) mmol/g protein	

ABTS, 2-2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) radical method; FRAP, ferric reducing antioxidant power assay; GPx, glutathione peroxidase; GTr, glutathione transferase; ORAC, oxygen radical absorbance capacity.

lated to TAC in UHT-treated milk samples, whereas TAC values of pasteurized low fat milk samples were significantly lower compared to those of samples with a higher fat content. The kind of thermal treatment might thus affect TAC and it has been reported that more severe heat treatments increase TAC which might be due to the formation of brown melanoidins (10). Chen *et al.* also observed that TAC values, as measured using the ABTS assay, in commercial cow milk (3% fat) were significantly higher than in cow milk with lower fat percentages (0.5-1.5%) and defatted milk (14). Therefore, lipid interference and the reactivity of fat-soluble antioxidants might influence TAC. In that study, heating of the milk samples to 63°C for 1 hr did not affect TAC values (14).

Albera and Kankofer (1) compared colostrum and milk samples from Holstein-Friesian cows regarding TAC, glutathione peroxidase and indices of lipid and protein peroxidation (1). The TAC and glutathione peroxidase values increased significantly over the examined time period (from 0 min to 12 days post-partum), whereas the concentration of SH groups (index of protein peroxidation) was highest at 24 hr and then decreased over the time period. The index of lipid peroxidation was also highest at 24 hr but fluctuated between 48 hr and 12th day post-partum.

Sheep milk

Similar to findings in cow colostrum (1), TAC values in sheep colostrum were lowest in samples taken immedia-

tely after parturition and increased with time (45). TAC values increased until 24 hr for the sheep breed Uhruska (Poland), whereas the highest TAC values for the sheep breed Berrichon du Cher (France) were found at 48 hr after parturition. The results showed that TAC values of milk varied at the different stages of lactation, particularly within the 24-48 hr period (1, 45). The changes might be linked to temporal imbalances between the production and neutralization of reactive oxygen species (45). The TAC values of milk taken within a 24 hr period after parturition from the ewes of the two different breeds were similar. However, samples taken at 48 hr and 5 days after parturition were significantly different between the breeds. An explanation for the different TAC values by differences in feed intake can be excluded in this study since both breeds were given the same feed. The observed TAC values in cow and sheep milk were within the same range whereas glutathione peroxidase values were remarkably higher in sheep colostrum/milk compared to those found in cow colostrum/milk (1, 45).

Goat milk

The composition of goat milk is different from that of milk from cow and other animal species and can exert several health-promoting properties including an improved fat digestibility and digestion, reduced cholesterol concentrations and a lower allergenic potential (25). Goat milk from Prisca breed (Greece) showed a significantly higher TAC value compared to that from cow and donkey milk (67). This could be explained by its high content of antioxidants (16) or to the particular combination of compounds. Other factors such as natural pasture, feeding and season probably influence nutritional composition of milks from several species and it needs to be investigated to which extent they affect TAC properties.

EFFECTS OF INTAKE OF MILK ON ANTIOXIDANT PARAMETERS IN HUMANS

Studies in adults

Although *in vitro* studies of TAC are important, it is crucial that putative health effects of milk antioxidants also can be documented in vivo. Many dietary intervention studies have been made on the effect of milk products in the diet, but only few of them have focussed on the effect of milk on the antioxidant systems of the consumer. Some examples will be given in the following on the different experimental designs that have been used and the results obtained. Regarding the effects of milk products in adults, a study was performed on female volunteers given two different yoghurts for four weeks. There were some changes in the concentrations in plasma of individual antioxidants during the study period and the TAC in plasma decreased to some extent mainly due to a decrease in the concentration of urate (22). Thus no certain positive effects of yoghurts on antioxidant status could be demonstrated. In another study the effect of a 4-week daily consumption of 0.75-1 L of cow milk, oat milk and soy milk was compared. Although significant effects on blood lipids levels were observed, no effects on plasma TAC (ABTS method) were found (52).

Selenium (Se) is one of the nutrients raising most attention as having both antioxidant and other health effects and milk is a food rich in Se. To study the effects of Se in milk further Ravn-Haren *et al.* performed a 4 x 1 week randomised, double-blind cross-over study in which healthy young men supplemented their usual diet with either Se-enriched milk, Se-enriched yeast, selenate or placebo (55). All types of Se increased plasma Se levels after supplementation for 1 week. The effect of the two organic forms did not differ significantly and both increased plasma Se more than selenate. Conversely, thrombocyte glutathione peroxidase was increased in the periods where subjects were supplemented with selenate but not in those where they were given Se-enriched yeast or Se-enriched milk. No effects were found on plasma lipid resistance to oxidation, or the activities of glutathione peroxidase activity, glutathione reductase and glutathione S-transferase as measured in erythrocytes. Several other variables measured in plasma or leucocytes were also unaffected. No specific effects of Se-enriched milk could be demonstrated compared to Se-enriched yeast. The milk had been prepared by giving cows a high dose of selenomethionine (30) and most of the Se in milk was bound in proteins.

Studies in infants

Since milk or its substitutes is the sole food of infants and since there is evidence indicating that premature infants are more exposed to oxidative stress than term infants, several feeding studies with milk have been performed in premature infants. In one study, 65 premature infants were fed with formula or human milk with different amounts of Human Milk Fortifier (HMF) and it was found that the use of a low percentage of HMF (and thus a high proportion of human milk) decreased the excretion of F2-isoprostanes in urine (an indicator of lipid peroxidation) whereas after a high percentage of HMF this excretion was higher than in formula-fed infants (24). No effect of the different feedings on plasma TAC (ORAC method) was found, however. In another study of preterm infants two groups were given either human milk or preterm formula (42). In the formula group the excretion of oxidised DNA bases and oxidative derivatives of phenylalanine was higher than in the human milk group. The excretion of these indicators of oxidative stress was also higher than that in a control group of normal term infants. The authors concluded that human milk might be partially protective against the oxidative stress occurring in premature infants. Also in a study of term infants feeding with human milk compared to formula was found to result in a higher TAC and lower concentration of peroxides in plasma (5).

We conclude that milk may affect the antioxidant status of the consumer in many ways and much more remains to be learnt on how the antioxidant properties of milk affect the human consumer using different assessment methods. Recent reviews have indicated that much of the existing evidence for positive health effects of dietary antioxidants in general remains equivocal and that much more elaborated methodology for the demonstration of such effects in human intervention studies are needed (32, 35).

EFFECT OF MILK ON THE ANTIOXIDANT ACTION OF DIETARY FLAVONOIDS

Another issue in this field to which much interest and many studies have been devoted is the interaction of milk with tea and this is reviewed in the following. Flavonoids have antioxidant capacity and they can inhibit lipid peroxi-

dation and scavenge free radicals. The intake of dietary flavonoids (e.g. in fruits, vegetables, tea, wine) is potentially beneficial for health and epidemiological studies have shown that flavonoid consumption is associated with a reduced risk of coronary heart disease, stroke and cancer (73). However, there is only limited evidence showing that different polyphenol-rich products modify biomarkers of oxidative damage and TAC in humans (32). Furthermore, Hertog *et al.* reported that the intake of tea flavonols (e.g. catechin, theaflavins and thearubigins) was not inversely associated with ischemic heart disease risk in the United Kingdom (29). A possible explanation is that the addition of milk to tea, a common habit in the United Kingdom, reduces the absorption of flavonols and thereby abolishes the antioxidant effect.

In vitro studies

In many *in vitro* studies a reduced antioxidant capacity of tea flavonoids has been noticed when milk was added to tea. For example, in one study the effect of the addition of 5% skim milk (2% fat) to Darjeeling, English breakfast and green tea on the antioxidant capacity of tea flavonols was investigated using three complementary methods (18). Using the ABTS assay, the antioxidant capacity of Darjeeling, English breakfast and green tea was significantly decreased by -8.3%, -19.6% and -6.0%, respectively, after the addition of milk. Voltammetry also showed significant decreased antioxidant capacity of tea when milk was added (Darjeeling -27.7%, English breakfast -76.5% and green tea -36%) and the TAC of English breakfast tea was thus considerably decreased by the addition of milk. It was also shown that these three kinds of teas have different total polyphenol contents and that tea polyphenols found in English breakfast tea might show a stronger affinity to milk components than those from the other types of tea. A third method to evaluate the antioxidant capacity was the measurement of inhibition of lipid peroxidation. Remarkably, a positive synergistic effect of milk and tea polyphenols (Darjeeling +9.1%, English breakfast +11.0% and green tea +15.8%) was seen which showed that tea with milk added better protected lipid micelles from peroxidation than tea without milk. The values of TAC of tea after the addition of milk were thus dependent of the method used and Dubeau *et al.* concluded that milk has a dual effect on TAC of tea, i.e. an inhibitory effect for reactions occurring in solution or at a solid-liquid interface and an enhancing effect for those in oil-in-water emulsion (18).

In another study it was reported that the addition of cow's milk diminished the antioxidant effect of black tea preparations by 12-28% as measured by the FRAP assay (40). A similar result was observed for soy milk, indicating that the presence of milk proteins was not the only possible determinant of the reduced antioxidant effect. Interactions between flavonoids and proteins have also been studied by Arts *et al.* (4), who reported that TAC *in vitro* was masked when proteins were added (e.g. albumin, β-casein) to flavonoids. Thus, proteins might play an important role as a modifier of TAC.

In the study by Langley-Evans the reduction in antioxidant capacity of tea was more pronounced after the addition of whole milk (-28%) compared to skimmed cow milk (-12%) (40). This suggests that also the fat content of milk plays a role for the antioxidant capacity of tea flavonoids and that also milk lipids might inhibit the antioxidant activity. Catechins are fat-soluble and it is possible that an interaction between flavonoid compounds and milk lipids prevents interactions of the antioxidant with the reactive oxygen species. In contrast, in another study it was recently shown that the addition of whole milk decreased the antioxidant capacity of tea to a lesser extent compared to semi-skimmed and skimmed milk (60). The role of milk lipids for the antioxidant activity of tea thus needs to be further studied. Concerning the interaction of different polyphenols with milk proteins as detailed study was made by Xiao *et al.* (72). Methylation, hydroxylation, glycosylation and hydrogenation of polyphenols significantly affected their binding to bovine milk proteins. A marked effect was found by galloylation of catechins which improved the binding affinities by about 100-1000-fold.

The antioxidant capacity of green and black tea has also been studied using an *in vitro* gastrointestinal model (38). The model simulated the *in vivo* absorption from the gastrointestinal lumen into the blood and the antioxidants in the fluid collected represented the amount of antioxidants available for absorption. Addition of whole milk, semi-skimmed or skimmed milk to black tea reduced the antioxidant capacity in the simulated jejunal dialysates by 18, 40 and 46%, respectively (38).

In vivo studies

Several *in vivo* studies have also been performed on the role of milk intake for the effects of dietary flavonoids. In a meal study the intake of 6 cups of black tea (3.25 g of tea solids/cup) at hourly intervals by healthy volunteers resulted in significantly increased plasma FRAP values after 3 hr and 6 hr compared to baseline values (41). This effect seemed to be abolished when milk was added to the tea, although the response of subjects to tea with added milk was highly variable. Plasma FRAP values of four subjects were increased, in two subjects plasma FRAP levels were similar, whereas plasma FRAP values in two other subjects showed decreased levels after the intake of tea with milk.

On the other hand, in several other *in vivo* studies no effects were reported on antioxidant capacity when milk was added to tea. Van het Hof *et al.* (71) found that after the ingestion of one cup of black or green tea (3 g of tea solids), blood levels of catechins were rapidly increased and were not significantly different from catechin blood levels obtained after ingestion of black tea with semi-skimmed milk. Similar results were found by Leenen *et al.* who reported a significantly increased level of plasma total catechins and antioxidant capacity (FRAP assay) after a single consumption of black and green tea (2 g of tea solids) with or without whole milk by 21 healthy subjects (43).Increased plasma antioxidant capacity and concentrations of catechins after black tea consumption (3 g of tea solids) were also seen by Kyle *et al.* (39). They also measured plasma flavonols (quercetin and kaempferol) and observed increased concentrations also of these compounds after tea ingestion. The addition of semi-skimmed milk did neither influence the antioxidant capacity nor the concentrations of catechins and flavonols compared to drinking tea without milk. An increased absorption of flavonols (quercetin and kaempferol) was observed in healthy adult males after ingestion of black tea and this increase was not significantly different from that after the ingestion of black tea with milk (31). In another *in vivo* study it was reported that the area under curve of plasma catechins was lower

after black tea consumption (7 g of tea solids) with milk compared to black tea alone (57). However, this did not affect the TAC values in plasma (FRAP assay).

Thus, in most of the *in vivo* studies no influence of the simultaneous intake of milk on the antioxidant activity of tea was shown, whereas in most *in vitro* studies mainly an inhibitory effect of milk on antioxidant capacity of tea flavonoids was demonstrated. Therefore, the hypothesis that milk impairs the antioxidant capacity of flavonoids remains equivocal. Other factors that might influence the extent of antioxidant capacity of tea flavonoids as pointed out different studies include the amount of added milk, time of infusion, water temperature, stirring, squeezing of the tea bag, and interaction with the tea bag material.

Furthermore, the effect of milk on the antioxidant capacity of phenolic compounds in other foods than tea has been investigated in a few investigations. Consumption of blueberries together with water by healthy subjects significantly increased the concentration of plasma markers of TAC, whereas consumption of blueberries together with whole milk had no effects on plasma antioxidant capacity and reduced the absorption of caffeic acid (64). These results indicated that blueberry consumption together with milk impairs the *in vivo* antioxidant capacity of blueberries. In contrast Hassimoto *et al.* found no significant effects on plasma TAC of an intake of blackberry juice either with or without milk (28) although several interesting correlations among antioxidant variables were observed. Consumption of dark chocolate, a flavonoid-rich food, significantly increased *in vivo* plasma antioxidant levels, as measured using FRAP assay (63). In contrast, consumption of dark chocolate with milk or milk chocolate did not affect plasma antioxidant activity. This might indicate that the inhibition of antioxidant activity might be due to the formation of secondary bonds between chocolate flavonoids and milk proteins, which reduce the absorption of flavonoids. Schroeter *et al*. however, did not find a difference in plasma TAC (total antioxidant potential assay) after the intake of a cocoa-containing milk beverage or a cocoa-containing water beverage (62). The presence of milk in cocoa products might thus not counteract the absorption and bioavailability of flavonoids. Moreover, it was suggested that the difference observed in the study of Serafini *et al.* might be due to a delayed absorption of the antioxidants and that the structure of the food matrix is an important factor when measuring *in vivo* antioxidant capacity (63).

In summary, the discrepancy among the results obtained *in vivo* is considerable and there was a lack of effect in half of the studies and an inhibitory effect of milk on the antioxidant capacity of the dietary flavonoid in the other half of studies. The study design, the method measuring the antioxidant activity and the food matrix used might play a major role for the differences observed. Further research is warranted to elucidate the potential inhibitory effect of milk on dietary flavonoid antioxidant actions.

ANTIOXIDANT MECHANISMS IN MILK

Regarding the question which are the most active antioxidants in milk, the work performed by our group (13, 14) indicated that urate is an important low-molecular weight antioxidant in agreement with the data of Østdahl *et al.* (53). Urate was detected as the major antioxidant in the LMW fraction and its TAC as assayed by FIAmp was in the range of 100–150 µmol/L (13). Our studies showed that urate had both reducing and radical-scavenging capacities. These properties may enable urate to protect against oxidation damage to milk components. In analogy with these results, in human plasma the concentration of urate was found to be strongly correlated with the FRAP value, and it was estimated to contribute around 60% of the total ferric-reducing ability (8).

A very interesting study concerning the antioxidant role of different milk components was recently performed by Clausen *et al.* (17). They compared the radical scavenging activities in milk, milk serum and whey after subjecting the samples to size-exclusion chromatography. Caseins contributed most of the activity both in the ABTS and ORAC assays (89%) in agreement with the much higher ABTS scavenging activity found in defatted milk compared to the LMW fraction by Chen *et al.* (14). Beta-lactoglobulin and alpha-lactalbumin contributed much less to TAC and in the LMW fraction urate and ascorbate were identified as antioxidants. Clausen *et al.* also found that the ORAC assay measuring the scavenging of peroxyl radicals was more sensitive toward whey proteins than the ABTS radical scavenging assay (17). Further experiments indicated that the antioxidant activity of caseins was mediated by its constituent amino acids whereas the activity in whey was largely dependent on the free thiol group of beta-lactoglobulin. Clausen *et al.* also concluded that the number of significant contributors to milk radical scavenging activity is relatively low (17). In this context it is important to note the many possible antioxidant actions of food proteins and peptides e.g. from milk and this was reviewed by Elias *et al*. (20). It is an interesting strategy if hydrolysed proteins can be used as antioxidants for different purposes.

Using another approach in a study of human milk Tijerina-Sáenz *et al.* found that the antioxidant capacity was correlated to the content of alpha-tocopherol but not to that of vitamin A and polyunsaturated fatty acids (69). In still another approach Kristensen *et al.* in studying two buttermilks with different content of polyunsaturated fatty acids found that the antioxidant capacity in the serum phase was similar for the two samples although buttermilk made from more unsaturated milk was less oxidatively stable (37). Elisia and Kitts studied how hexanal in human milk as an index of lipid peroxidation was related to TAC (21). They found that the hexanal level was inversely correlated to those of α–tocopherol and ascorbic acid but not to TAC as measured by the ORAC method.

CONCLUDING REMARKS

Much interest is devoted to the antioxidant capacity of plant foods but this review shows that also milk has considerable antioxidant capacity as demonstrated using several methods. Milk as a matrix presents several methodological challenges due to its high content of protein and lipids which has to be considered when choosing the suitable methods. Recent research indicates that the major fractions with antioxidant capacity in milk are relatively few but their role for the oxidative stability of milk and possible influence on the antioxidant status of the consumer needs much more documentation. Milk proteins are an interesting starting material for the preparation of peptides with putative antioxidant capacity. In addition to its inherent antioxidant capacity milk has also been studied for its

possible inhibitory or stimulatory influence on the antioxidant activity of flavonoids. In vitro a clear inhibition is seen but any such effects on the consumer after ingestion are more difficult to substantiate. Regarding the health effects of dietary antioxidants of milk and in foods in general much more refined methods are now developing and hopefully more substantial evidence will emerge in the future.

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