Bovine milk antioxidant properties: effect of *in vitro* digestion and identification of antioxidant compounds

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Abstract

Milk proteins contained encrypted in their sequence biologically active components that can be released by enzymatic hydrolysis. Among the biological activities recognised in milk components, the antioxidant activity is of great interest. The objective of the present study was to analyse the antioxidant properties of whole, semi-skimmed and skimmed milk during simulated gastro-intestinal digestion and to identify the compounds responsible for the antioxidant activity. Simulated digestion increased the $ABTS^{\cdot+}$ radical scavenging activity of milk. In digested whole milk, the main contribution to $ABTS^{\cdot+}$ radical scavenging activity was due to high molecular weight fraction (>3 $kg \cdot mol^{-1}$). For semi-skimmed and skimmed milk, the main contribution was due to low molecular weight fraction (<3 $kg \cdot mol^{-1}$). Twelve major peaks were collected from low molecular weight fraction of digested skimmed milk by reversed-phase high-performance liquid chromatography and evaluated for their $ABTS^{\cdot+}$ radical scavenging activity. Among the different fractions, three (F2, F3 and F5) showed high $ABTS^{\cdot+}$ and hydroxyl radical scavenging activity and lipid peroxidation inhibitory capacity. The compounds (free amino acids and peptides) present in these fractions were identified with nanoLC-QTOF MS/MS analysis. The amino acids tryptophan and tyrosine seemed fundamental in the $ABTS^{\cdot+}$ and hydroxyl radical scavenging capacities whereas the amino acids phenylalanine and histidine played an important role in the lipid peroxidation inhibitory activity of the peptides. The results reported in this study suggested that milk proteins could act as a carrier for the delivery of antioxidant compounds in the gastro-intestinal tract possibly protecting the gastro-intestinal tract itself from the oxidative damage.

Keywords: *in vitro* gastro-intestinal digestion; antioxidant activity; mass spectrometry; peptides, aromatic aminoacids.

1. Introduction

Antioxidant compounds are considered important for human health thanks to their ability to scavenge free radicals and contribute to prevent chronic diseases, such as cancers, coronary heart diseases, and neurodegenerative disorders (Del Rio et al. 2013). Dairy product showed antioxidant activity and have to be considered as important dietary components that contribute to the total intake of antioxidants. In milk, proteins (especially caseins) are the most important radical scavenger compounds (Clausen et al. 2009).

Most bovine milk proteins, mainly β-lactoglobulin and αS1-casein, are also potential allergens and cow's milk protein allergy is the most prevalent in infancy, reaching an incidence of about 2% to 7.5% (Bu et al. 2013). Reduction of milk protein allergenicity by technological processing such as heat treatment, lactic fermentation and enzymatic hydrolysis is a topic of major relevance to develop hypoallergenic milk products (Bu et al. 2013).

Enzymatic hydrolysis is an effective means to generate bioactive peptides from intact protein sequences (Pihlanto 2006). The biological activities of these peptides include antimicrobial, anti-hypertensive, antithrombotic and antioxidative activities (Pihlanto 2006; Power-Grant et al. 2013). Antioxidant peptides and amino acids are particularly interesting for their possible contribution to health promotion and disease prevention (Power-Grant et al. 2013).

The composition and the biological properties of the peptide contained in milk hydrolysates depend on the substrate, the proteolytic enzymes, the enzyme to substrate ratio and physicochemical conditions (pH, hydrolysis time and temperature of reaction) (del Mar Contreras et al. 2011). A variety of proteolytic enzymes has been used to generate milk protein hydrolysates with differing degrees of hydrolysis, containing a diverse assortment of peptides and different antioxidant activity. Digestive enzymes and combinations of different proteinases such as alcalase and thermolysin have been utilized to successfully generate antioxidant peptides from various milk proteins (Pihlanto 2006; Power-Grant et al. 2013). For

example, a peptic digest of bovine caseins produced the αS1-casein-derived peptide YFYPEL with a strong superoxide anion scavenging activity (Suetsuna et al. 2000). One potent antioxidant peptide (WYSLAMAASDI) was purified from bovine β-lactoglobulin hydrolysed with Corolase PP (Hernández-Ledesma et al. 2005). Thermolysin was utilized to generate two antioxidant peptides (LQKW and LDTDYKK) from β-lactoglobulin (del Mar Contreras et al. 2011).

Milk proteins are deeply transformed in the human gastro-intestinal tract because of the presence of different proteases. Peptides generated in the gastro-intestinal may have different biological properties such as antimicrobial, antioxidant, antihypertensive, etc. (Boutrou et al. 2015). However, till now, little studies have been carried on the production of antioxidant compounds during the *in vitro* digestion of milk proteins and, above all, the nature of bioactive antioxidant compounds released during digestion has not been revealed. *In vitro* gastro-intestinal digestion enhance the antioxidant activity of a bovine milk-based protein matrix (Power-Grant et al. 2016) as well as of a κ-casein and β-casein preparation (Petrat-Melin et al. 2015; Petrat-Melin et al. 2016). Some antioxidant peptides (such as WSVPQPK and ISELGW) and free amino acids have been generated after *in vitro* gastro-intestinal hydrolysis of human milk or infant formula (Raikos and Diassos 2014).

At present, *in vitro* digestion studies focused on single isolated proteins without considering the influence of other components present in dairy products such as fat. Therefore, our *in vitro* digestion study was targeted on the identification of antioxidant compounds released after the *in vitro* digestion from complex food matrices represented by whole, semi-skimmed and skimmed bovine milk.

2. Materials and methods

2.1. Materials

All electrophoresis, HPLC and MS/MS reagents were from Biorad (Hercules CA, U.S.A.), whereas the remaining chemicals were purchased from Sigma-Aldrich (Milan, Italy) unless otherwise stated. Amicon Ultra-4 regenerated cellulose 3 $kg \cdot mol^{-1}$ were supplied by Millipore (Billerica MA, USA). The homogenized bovine milk (whole, semi-skimmed and skimmed milk), belonging to the same batch of raw milk, were obtained from a local producer. The different types of milk had the same total proteins (3.1 $g \cdot 100mL^{-1}$), caseins (2.6 $g \cdot 100mL^{-1}$), carbohydrates (4.8 $g \cdot 100mL^{-1}$) and calcium (120 $mg \cdot 100mL^{-1}$) content but differ for the fat content (3.60 $g \cdot 100mL^{-1}$, 1.55 $g \cdot 100mL^{-1}$ and 0.05 $g \cdot 100mL^{-1}$ in whole, semi-skimmed and skimmed milk, respectively). The absorbance was read using a Jasco V-550 UV/Vis spectrophotometer (Orlando FL, U.S.A.).

2.2. In vitro gastro-intestinal digestion

The two-stage *in vitro* digestive model was adapted from Helal et al. (2014). Aliquots (50 mL) of milk were mixed with 50 mL of water. The samples were then brought to pH 2.5 with concentrated HCl and the gastric digestion was started by the addition to the 100 mL of overall digestion media of 0.2 g of NaCl and 31500 U of pepsin. The samples were incubated at 37°C in a shaking bath for 2h to simulate the gastric phase of digestion. At the end of the gastric digestion, the pH was brought to 7.5 with $NaHCO_3$, before adding 0.8 $g \cdot L^{-1}$ pancreatin and 5 $mg \cdot mL^{-1}$ bile salts. The solution was then incubated at 37°C in a shaking bath for further 2h to simulate the intestinal phase of digestion. The enzymes were inactivated by heating at 95°C for 15 min, followed by cooling to room temperature. Aliquots of the samples were withdrawn after mixing milk with water (after mixing pH 6.8), after acidification to pH

2.5, at the end of the gastric digestion, after alkalinization to pH 7.5 and at the end of the intestinal digestion. Each sample was digested in triplicate.

2.3. Determination of the degree of hydrolysis

The determination of the degree of hydrolysis of the digested samples was carried out as reported by Adler-Nissen (1979). The hydrolysis degree was calculated as reported in equation (1):

$$\mathbf{DH = (h/h_{tot}) \cdot 100} \quad (1)$$

where **h** is the hydrolysis equivalent, defined as the concentration in milliequivalents$\cdot g^{-1}$ of protein of α-amino groups formed at the different stages of the simulated digestion, and $\mathbf{h_{tot}}$ is the hydrolysis equivalent at complete hydrolysis to amino acids (calculated by summing the contents of the individual amino acids in 1 g of protein and considering caseins as the only proteins in milk). According to Adler-Nissen (1979), the $\mathbf{h_{tot}}$ value was fixed at 8 that is the value calculated for caseins.

2.4. SDS-PAGE Electrophoresis

Samples of different types of un-fractionated milk taken at different times of digestion were subjected to SDS-PAGE electrophoresis using 17% polyacrylamide separating gel as reported in Helal et al. (2014). Three SDS gels were run of each milk sample.

2.5. Fractionation of digested samples

Samples from *in vitro* simulated digestion (4 mL of 5 times water-diluted sample) were subjected to ultrafiltration with Amicon Ultra-4 nominal cutoff 3 kg$\cdot$mol^{-1} (Millipore, Italy), at 7500*g* for 120 min at 4°C. At the end of the separation, two fractions were obtained: the retentate containing mainly high molecular weight compounds (HMW fraction; > 3 kg$\cdot$mol^{-1})

and the permeate containing low molecular weight compounds (LMW fraction; < 3 kg·mol^{-1}). The two fractions were filled up to 4 mL with a solution of HCl 0.01 mol·L^{-1} for the sample collected during the gastric phase of the digestion or potassium phosphate buffer (0.1 mol·L^{-1}; pH 7) for the sample collected during the intestinal phase.

2.6. Reversed-phase high performance liquid chromatography (HPLC) analysis of peptides

HPLC separation of the low molecular weight fractions of digested milk collected at the end of the pancreatic digestion was performed with a Jasco HPLC system equipped with a reversed phase column Hamilton HxSil C18 (Hamilton, Reno, Nevada; 250mm x 4.6mm, 5 µm, 100 Å) as described in Tagliazucchi et al. (2015). The two solvents were: solvent A mixture of water-trifluoroacetic acid (0.037%) and solvent B acetonitrile-trifluoroacetic acid (0.027%). A linear gradient of solvent B in A ranging from 0% to 45% in 115 min with a flow rate of 0.5 mL·min^{-1} was used to separate the peptides contained in the low molecular fractions of digested milk. The photodiode array (PDA) detector was set at 214 nm. Twelve fractions from digested skimmed milk were collected and freeze-dried. These fractions were re-filled to the original volume with a potassium phosphate buffer (0.1 mol·L^{-1}; pH 7) and then analysed for their antioxidant activity.

2.7. $ABTS^{\cdot+}$ radical scavenging activity of digested samples and HPLC fractions

The antioxidant activity of the sample collected during the *in vitro* digestion procedure and from HPLC separation was determined using ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) method as described in Re et al. (1999) both on un-fractionated and fractionated samples. The $ABTS^{\cdot+}$ scavenging capacity was expressed as milligrams of vitamin C per L of milk, by means of a calibration curve obtained with vitamin C (ranging from 1 to 150 mg·L^{-1}), in the same assay conditions.

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147 *2.8 Antioxidant properties of selected HPLC fractions and amino acids*

148 The antioxidant properties of the selected HPLC fractions and amino acids were evaluated
149 using three different assays.

150 The ABTS assay was carried out as described in the paragraph 2.7.

151 The capacity to scavenge hydroxyl radicals was evaluated according to a method reported by
152 Ajibola et al. (2011) with some modifications. The assay consisted of mixing 50 µL of 3
153 mmol·L^{-1} TPTZ (2,4,6-Tri(2-pyridyl)-s-triazine) dissolved in HCl 50 mmol·L^{-1}, 50 µL of 3
154 mmol·L^{-1} $FeSO_4$, 50 µL of sample or vitamin C (at concentration ranging from 1 to 10
155 mmol·L^{-1}), and 50 µL of 0.01% (v/v) hydrogen peroxide, in a clear bottom 96-well plate. The
156 mixture was incubated for 1h at 37°C and the absorbance was measured at 540 nm using a
157 microplate reader.

158 The ABTS and hydroxyl radical scavenging capacities were expressed as milligrams of
159 vitamin C per L of milk or, in the case of the amino acids as mg vitamin C per mmol of amino
160 acid.

161 The ability to inhibit lipid peroxidation was carried out using a linoleic acid emulsion system
162 (Ajibola et al. 2011). For that purpose, 200 µL of sample at concentration of 2 mmol·L^{-1}, 200
163 µL of 99.5% ethanol and 2.6 µL of linoleic acid were mixed and the total volume was
164 adjusted to 500 µL with sodium phosphate buffer, 50 mmol·L^{-1}, and pH 7.0. The mixture was
165 incubated at 40°C in the dark for seven days. The amount of generated lipid hydroperoxide
166 was measured by the FOX assay as reported by Tagliazucchi et al. (2010). The lipid
167 peroxidation inhibitory activity of the samples was expressed as percentage of inhibition
168 respect to a control reaction without the sample.

169

2.9. Peptide profile determination with nanoflow liquid chromatography accurate mass quadrupole time-of-flight mass spectrometry with electrospray ionization (LC-ESI-QTOF MS)

The fractions with the highest antioxidant activity were subjected to QTOF MS/MS analysis for peptide identification. Nano LC/MS and tandem MS experiments were performed on a 1200 Series Liquid Chromatographic two-dimensional system coupled to a 6520 Accurate-Mass Q-TOF LC/MS via a Chip Cube Interface (Agilent Technologies). Chromatographic separation was performed on a ProtID-Chip-43(II) including a 4mm 40 nL enrichment column and a 43 mm × 75 μm analytical column, both packed with a Zorbax 300SB 5 μm C18 phase (Agilent Technologies). The mobile phases composition and the gradient were the same as reported by Tagliazucchi et al. (2015). The mass spectrometer was tuned, calibrated and set with the same parameters as reported by Dei Più et al. (2014).

For peptide identification and sequencing, MS/MS spectra were converted to .mgf and *de novo* peptide sequencing was performed using Pepnovo software (http://proteomics.ucsd.edu/ProteoSAFe/). The following parameters were considered: enzyme, none; peptide mass tolerance, ± 40 ppm; fragment mass tolerance, ± 0.12 Da; variable modification, oxidation (M) and phosphorylation (ST); maximal number of PTMs permitted in a single peptide 3.

A search for the biological activity of peptides identified was carried out through the BIOPEP database (http://www.uwm.edu.pl/biochemia/biopep/start_biopep.php). Confirmation of peptides sequence in bovine milk proteins was performed using Peptide Match (http://research.bioinformatics.udel.edu/peptidematch/index.jsp).

2.10. HPLC analysis of tyrosine and tryptophan

The amount of tyrosine in F2 and tryptophan in F5 was determined according to Frank and Powers (2007). Derivatization was carried out by mixing 50 μL of sample with 50 μL of OPA

(ortho-phthalaldehyde) solution (consisting of 7.45 mmol·L^{-1} of OPA and 11.4 mmol·L^{-1} of 3-mercaptopropionic acid in potassium tetraborate buffer 0.2 mmol·L^{-1} pH 9.5). The HPLC separation of the derivatized amino acids was carried out with the same C18 column as reported in paragraph 2.6 using a binary gradient of mobile phase A (30 mmol·L^{-1} potassium phosphate buffer with 0.4% tetrahydrofuran pH 7.0) and mobile phase B (50% acetonitrile and 50% water). The gradient started at 0% B for 0.5 min then linearly ramped up to 48% B in 22 min. The mobile phase composition was raised up to 60% B in 12 min, then 100% B in 1 min and maintained for 4 min in order to wash the column. Flow rate was 1 mL·min^{-1}. The detection was performed at 340 nm.

2.11. Statistical analysis

All data are presented as mean ± SD for three independent *in vitro* digestion experiments performed on the same milk sample. Two-way univariate analysis of variance (ANOVA) with Tukey post-hoc test was applied to determine significant differences ($P < 0.05$). Correlations between variables were assessed using Pearson's method. All analyses were performed with GraphPad Prism version 6.00 (GraphPad software, San Diego, CA).

3. Results and discussion

3.1 Assessment of protein hydrolysis during simulated digestion of whole, semi-skimmed and skimmed milk

Simulated gastro-intestinal digestion of whole, semi-skimmed and skimmed milk resulted in the partial hydrolysis of the milk proteins with formation of peptides with a molecular weight lower than 10 $kg \cdot mol^{-1}$ as showed by electrophoresis (data not shown). As detailed in **Table 1**, the degree of hydrolysis (DH) of the different types of milk at time 0h was similar and not statistically different (average DH value 2.9 ± 0.3) regardless of milk type. DH increased significantly ($P< 0.05$) during the peptic digestion for all the samples but with some differences. The DH after peptic digestion was higher for samples with low fat content (skimmed>semi-skimmed>whole milk). The pancreatic digestion produced a high and significant ($P< 0.05$) increase in DH for all the digested samples. The degree of protein hydrolysis was different considering the various types of milk and in particular was higher for milk poor in fat respect to the milk rich in fat, despite having the same protein content. Results showed that the presence of fat reduces the proteolysis both at gastric and intestinal level. The exact mechanism is not known and currently under investigation. It could be expected that, since surface plays a very important part in enzyme action, fats may reduce surface tension and so lower surface energy, hence retard protein digestion.

The DH value measured after gastro-intestinal digestion with our model was lower than those determined by Picariello et al. (2015) which found a degree of hydrolysis for skimmed milk between 34.5 and 58 depending on the substrate to digestive enzyme ratio. Simulated digestion of isolated κ- and β-casein resulted in a hydrolysis degree between 40 and 55 (Petrat-Melin et al. 2015; Petrat-Melin et al. 2016).

237 *3.2. $ABTS^{\cdot+}$ radical scavenging activity of digested whole, semi-skimmed and skimmed milk*

238 All three types of milk showed $ABTS^{\cdot+}$ radical scavenging activity before the digestion
239 (**Table 2)**, but with some differences. Skimmed milk had a significant minor radical
240 scavenging activity respect to whole milk ($P< 0.05$). The higher value of $ABTS^{\cdot+}$ radical
241 scavenging activity in samples with more fats can be due to the reactivity of lipid soluble
242 antioxidants, such as α-tocopherol and carotenoids (Re et al., 1999), and fat globule
243 membrane proteins with $ABTS^{\cdot+}$ radical. More than 90% of the antioxidant activity in all the
244 analysed types of milk was in the > 3 $kg \cdot mol^{-1}$ high molecular weight fraction (HMW)
245 underlining the role of protein in the total radical scavenging activity of milk. Clausen et al.
246 (2009) found that caseins are quantitatively the highest radical scavengers in milk whereas the
247 lower contribution of the low molecular weight fraction (LMW) is due to ascorbate and
248 especially urate. Caseins have a high content of antioxidative amino acids such as tyrosine,
249 tryptophan and phosphoserine, and quenching of free radicals by oxidation of these amino
250 acids was proposed as the explanation (Clausen et al. 2009; Cervato et al. 1999).
251 After acidification of the milk samples to pH 2.5 (corresponding to the time zero of the gastric
252 digestion), the $ABTS^{\cdot+}$ radical scavenging activity decreased significantly in all three types of
253 milk. This decrease was caused by a drop in the $ABTS^{\cdot+}$ value of the HMW fraction whereas
254 the value in the LMW fraction was unaffected. The gastric pH value (pH 2.5) is near to the
255 isoelectric point of casein (pI 4.6) and this determines changes in casein aggregation with a
256 masking of antioxidant sequences of amino acids, which can explain the decrease in $ABTS^{\cdot+}$
257 radical scavenging activity after acidification. After 120 min of peptic digestion, the total
258 $ABTS^{\cdot+}$ value increased non-significantly respect to the time zero of gastric digestion in all
259 the milk types. However, the $ABTS^{\cdot+}$ radical scavenging activity was always lower than the
260 original value found in the different types of milk before the digestion except than in the
261 LMW fractions. The main contribution to $ABTS^{\cdot+}$ value, for all the types of milk beverages

after peptic digestion, was due to HMW fraction. The HMW fraction $ABTS^{\cdot+}$ radical scavenging activity decreased, from the milk richest to the milk poorest in fats.

The passage into the alkaline media, from 120 min of the gastric digestion (pH 2.5) to time 0 min of the pancreatic digestion (pH 7.5), led to an increase in the $ABTS^{\cdot+}$ radical scavenging activity in total and LMW and HMW fractions of all the types of milk beverages.

After 120 min of pancreatic digestion there was a high increase in the $ABTS^{\cdot+}$ value, for all the three types of milk beverages. Whole milk showed the highest increase whereas there were no statistically differences between semi-skimmed and skimmed milk.

The distribution of the radical scavenging activity between the LMW and HMW fractions was different considering the diverse types of milk. In digested whole milk, the main contribution to $ABTS^{\cdot+}$ radical scavenging activity was due to HMW fraction. For semi-skimmed and skimmed milk, the main contribution was due to LMW fraction. During *in vitro* gastro-intestinal digestion of bovine milk, protein hydrolysis determines the formation of low molecular weight peptides with $ABTS^{\cdot+}$ radical scavenging activity probably due to unmasking and liberation of some amino acids sequences with antioxidant activities that are buried or inactive in the intact proteins. Hydrolysates obtained after peptic, tryptic and chymotryptic hydrolysis of milk proteins showed radical scavenging activity (Pihlanto 2006; Hernández-Ledesma et al. 2005). There is a clear correlation (Pearson coefficient r=0.928; $P< 0.05$) between the DH and the amount of $ABTS^{\cdot+}$ radical scavenging activity found in LMW fractions. The whole milk sample showed the lowest DH after pancreatic digestion and only 38% of $ABTS^{\cdot+}$ radical scavenging activity was found in the LMW fraction. The percentage of $ABTS^{\cdot+}$ radical scavenging activity in the LMW fraction increased to 79% and 90% in semi-skimmed and skimmed milk samples, respectively, according to the increase in the hydrolysis degree. The fat content negatively influenced the LMW $ABTS^{\cdot+}$ radical scavenging activity at the end of the digestion. Indeed, the presence of fat may lead to

peroxidative phenomena during gastro-intestinal digestion causing depletion of antioxidant compounds. This fact may results in a lower $ABTS^{\cdot+}$ reactivity in the samples richest in fat.

3.3. Antioxidant properties of the HPLC fractions from the permeate of digested skimmed milk

The peptides in the LMW fractions of digested milk were separated in the HPLC C18 column and detected at 214 nm with PDA. As reported in **Figure 1**, the LMW fractions of the three different types of milk showed the same HPLC pattern with the only difference in the intensity of the peaks.

Due to its high $ABTS^{\cdot+}$ radical scavenging activity, the LMW fraction of skimmed milk was selected for MS/MS experiments with the aim to identify the compounds responsible for the activity.

Twelve fractions from skimmed milk permeate (**Figure 1C**) were collected, freeze dried and evaluated for their $ABTS^{\cdot+}$ radical scavenging activity. Only seven fractions were found to have a considerable $ABTS^{\cdot+}$ radical scavenging activity (**Figure 2**).

Three fractions (F2, F3 and F5) were found to be the major contributors on the $ABTS^{\cdot+}$ radical scavenging activity of the skimmed milk LMW fraction. These three fractions were further characterized for their ability to scavenge hydroxyl radical and to inhibit lipid peroxidation. All of the three fractions exhibited a certain degree of hydroxyl scavenging activity. Fraction F2 was the most active against hydroxyl radical whereas fraction F3 showed the highest lipid peroxidation inhibitory activity (**Table 3**).

The compounds responsible for the antioxidant activity of these three fractions were tentatively identified with mass spectrometry.

3.4. NanoLC-ESI-QTOF-MS/MS analysis of the HPLC collected fractions

Figure 3 shows the full MS spectra of the fractions F2, F3 and F5. Each peak was selected for peptide identification by MS/MS ion scan using de novo sequencing software. Results from peptide identification were subjected to a manual evaluation, and the validated peptide sequences explained most of the major peaks in the MS spectra.

In the lowest part of the MS spectra of fraction F2 (**Figure 3A**) the most intense signals were identified as the amino acids (iso)leucine (Lx; m/z=132.1037) and tyrosine (Y; m/z=182.0851), and the dipeptide GP (m/z=173.0840). Additional intense signals were identified as the dipeptide GLx (m/z=189.1246) and the tripeptides VVD (m/z=332.1821) and LSH (m/z= 356.1945). The list of compounds identified in fraction F2 is shown in **Table 4** together with the MS data, the protein precursor and the potential bioactivity.

In the lowest part of the MS spectra of fraction F3 (**Figure 3B**) the most intense signals were identified as the amino acid phenylalanine (F; m/z=166.1055) and the dipeptide GP (m/z=173.0819). An additional signal at m/z of 120.0859 was assigned to the amino acid threonine. In the peptidic part of the spectra the most intense signals corresponded to the dipeptides VLx (m/z 231.1736), ALx (m/z= 203.1415) and QLx (m/z=260.1639) and the tripeptide SLxT (m/z= 320.1849). The list of compounds identified in fraction F3 is shown in **Table 4** together with the MS data, the protein precursor and the potential bioactivity.

An additional free aromatic amino acid, tryptophan (W; m/z=205.2208), gave an intense signal in the fraction F5 (**Figure 3C**). The most intense signals in the peptidic part of the MS spectra of fraction F5 were identified as the tetrapeptide SAPL (m/z=387.2272) from β-lactoglobulin (f36-39) and the αS1-casein-derived (f8-13) peptide HQGLPQ (m/z=340.1831; double-charged ion). Additional high signals were attributed to the peptide TKIPA from β-lactoglobulin (f76-80) present both as double-charged (m/z=265.1741) and mono-charged (m/z=529.3435) ions and the αS2-casein-derived peptides ITVDDK (f71-76) as double-charged ion (m/z=345.6897) and FPQ (f92-94) with m/z value of 391.2055. The peptide

AMEDIK (αS1-casein f53-58) was present in the spectra both in reduced and oxidized (at methionine level) forms with m/z values of 353.6766 and 361.6770, respectively (**Table 5**).

3.5. Identification of antioxidant compounds in HPLC fractions F2, F3 and F5

To identify the amino acids and peptides with the most potential antioxidant activity, the antioxidant properties of the peptides constitutive amino acids was determined (**Table 6**). Tryptophan was the amino acid with the highest $ABTS^{\cdot+}$ value followed by cysteine and tyrosine. The rest of the amino acids analysed did not exhibit antioxidant activity with this method at 2 $mmol \cdot L^{-1}$ concentration. Tyrosine and cysteine were also the amino acids with the highest hydroxyl radical scavenging activity followed by methionine and tryptophan. The amino acids phenylalanine and histidine showed the highest ability to inhibit lipid peroxidation. The amino acid tryptophan appeared to be the most effective as antioxidant since it showed high activity in all the assays.

Therefore, only the peptides containing the amino acids with antioxidant properties as well as the corresponding free amino acids were considered as potential radical scavengers.

Among the different compounds identified in fraction F2, tyrosine seemed fundamental in the $ABTS^{\cdot+}$ and hydroxyl radical scavenging capacities of the fraction, and to play a role in the inhibition of formation of lipid hydroperoxide. Tyrosine was further quantified in the fraction resulting in a concentration of 2.2 ± 0.1 $mmol \cdot L^{-1}$ of milk. The free tyrosine standard showed an $ABTS^{\cdot+}$ radical scavenging activity of 124.7 ± 12.3 mg of vitamin $C \cdot mmol^{-1}$ of amino acid, which resulted in an $ABTS^{\cdot+}$ value of 274.3 mg of vitamin C per 2.2 mmol of tyrosine. Considering that the $ABTS^{\cdot+}$ radical scavenging activity of the fraction F2 was 303.1 mg of vitamin $C \cdot L^{-1}$ of milk, we concluded that the 90% of the $ABTS^{\cdot+}$ radical scavenging activity of this fraction is due to the presence of free tyrosine. Free tyrosine also accounted for the 36.5% of the total hydroxyl radical scavenging activity of this fraction. Tyrosine is an

362 aromatic amino acid, which is known for its antioxidant activity. The antioxidant properties of
363 tyrosine is due to the presence of the phenolic moiety (aromatic ring with a hydroxyl group),
364 which makes tyrosine a good scavenger of free radicals and metal chelator (Pihlanto 2006).
365 Two additional peptides (LSH and GP), which contained amino acids able to scavenge
366 hydroxyl radical may account for the remaining scavenging capacity and lipid peroxidation
367 inhibitory activity of the fraction F2. The peptide LSH contained the amino acids serine and
368 histidine, which displayed hydroxyl radical scavenging capacity and strong lipid peroxidation
369 inhibitory activity (**Table 6**). The dipeptide GP is of particular interest because it displayed
370 multifunctional properties (**Table 4**).
371 Fraction F3 contained some peptides with previously demonstrated radical scavenging
372 activity, which can explain the high value of $ABTS^{\cdot+}$ and hydroxyl radical scavenging activity
373 found in this fraction. The αS1-casein-derived peptide YPEL (146-149) demonstrated radical
374 scavenger activity against DPPH, superoxide anion and hydroxyl radicals (Suetsuna et al.
375 2000). The presence of the tyrosine residue seems to be very important for the antioxidant
376 properties of the peptide YPEL since its deletion from the sequence halves the radical
377 scavenging activity (Suetsuna et al. 2000). This peptide also gave an intense signal in the MS
378 spectra suggesting that it may be present in high amounts in the fraction F3. The tetrapeptide
379 VRYL (αS2-casein 205-208) forms part of the antioxidant peptide PYVRYL, derived from
380 ovine casein hydrolysate (López-Expósito et al. 2007). The sequence RYL played an
381 important role in the activity since it still showed antioxidant activity (De Gobba et al. 2014a).
382 The peptide AVPYPQ (β-casein 177-182) is a precursor of two well-known antioxidant
383 peptides, namely VPYPQ and PYPQ, identified in human milk submitted to gastro-intestinal
384 digestion (Raikos and Dassios 2014; Hernández-Ledesma et al. 2007). The domain PYPQ is
385 primary in determining their antioxidant properties. However, the peptide VPYPQ showed a
386 higher antioxidant activity than the peptide PYPQ.

This fraction also showed the best lipid peroxidation inhibitory activity. It contained the free amino acid phenylalanine, which had strong inhibitory activity towards lipid peroxidation (**Table 6**). This compound is therefore expected to be the primary contributor to the lipid peroxidation inhibitory activity of fraction F3.

Tryptophan is a potent radical scavenger, which contains an indole group that is involved in the stabilization of the tryptophan radical through resonance or delocalization of the unpaired electron (Pihlanto 2006). Tryptophan in fraction F5 was quantified resulting in a value of 1.3 ± 0.1 mmol·L^{-1} of milk. The free tryptophan standard showed an $ABTS^{\cdot+}$ radical scavenging activity of 219.2 ± 16.1 mg of vitamin C·$mmol^{-1}$ of amino acid, which corresponded to a value of 285 mg vitamin C per 1.3 mmol of tryptophan. Considering that, the $ABTS^{\cdot+}$ radical scavenging activity of the fraction F5 was 450 mg of vitamin C·L^{-1} of milk, tryptophan accounted for the 63.3% of the $ABTS^{\cdot+}$ radical scavenging activity in this fraction. Based on the data in **Table 3** and **6** free tryptophan also accounted for the 59.9% of the hydroxyl radical scavenging activity of fraction F5. Additional peptides with potential radical scavenging activity were found in the fraction F5. For example the peptides DAYPSGA (αS1-casein 157-163) and DAYPS (αS1-casein 157-163) are precursors of the antioxidant peptide AYPS (De Gobba et al. 2014b). Interestingly, this last peptide was identified, after casein hydrolysis, in a fraction with high antioxidant activity together with the peptides RYPS and SRYPS, suggesting that the sequence YPS could be primary for the antioxidant properties of these peptides. This peptides (DAYPSGA and DAYPS) contained the amino acid tyrosine which displayed strong ABTS and hydroxyl radical scavenging capacities and the amino acids proline and serine active against the hydroxyl radical (**Table 6**). Several antioxidative peptides contain the sequence YL or YI in their structure such as the tripeptides YYL, YLY, YYI, YIY (Saito et al. 2003) and RYL (De Gobba et al., 2014a) as well as the longer peptides YIPIQY, FALPQYLK, GYLEQ, YLKT and PYVRYL (De Gobba et al., 2014b; López-

412 Expósito et al. 2007). The amino acids phenylalanine and histidine played an important role in
413 the lipid peroxidation inhibitory activity of the peptides (De Gobba et al. 2014a). Therefore,
414 the peptides present in fraction F5 containing these amino acids could be considered the major
415 contributor to the lipid peroxidation inhibitory activity of this fraction. The peptides FPQ
416 (αS1-casein 92-94) and HQGLPQ (αS1-casein 8-13) also gave very intense peak in the MS
417 spectra (**Figure 3**), suggesting that they could be present at high concentration in fraction F5.
418
419 *3.6. Milk proteins as a carrier for the delivery of antioxidant compounds in the gastro-*
420 *intestinal tract*
421 Various evidence suggests that oxidative stress is closely associated with the onset and
422 progression of several chronic diseases (Willcox et al. 2004). Therefore, it is generally
423 speculated that antioxidants in the diet can be helpful in counteracting the onset of these
424 diseases. However, the link between *in vitro* and *in vivo* antioxidant capacities has not been
425 clearly established. With regard to this, despite the large number of *in vitro* studies reporting
426 the antioxidant activity of bioactive peptides, the *in vivo* effect of milk-derived antioxidant
427 peptides on human health remains unclear (Power-Grant et al., 2013).
428 The gastrointestinal tract is constantly exposed to reactive oxygen species, from the diet or
429 generated in the gastro-intestinal tract itself. Reactive radical species can derive from dietary
430 iron, which in the gastric environment (i.e. in presence of oxygen, acidic pH and H_2O_2) may
431 promote Fenton reaction generating superoxide anion and hydroxyl radicals (Halliwell et al.
432 2000). Reactive oxygen species in the gut can initiate, in presence of transition metals, the
433 lipid peroxidation of dietary poly-unsatured fatty acids, resulting in the production of lipid
434 hydroperoxydes and advanced lipoxidation end products, which can be further absorbed and
435 involved in the pathogenesis of some cardiovascular diseases (Tagliazucchi et al. 2010). In
436 addition, dietary heme proteins are powerful pro-oxidant which can initiate gastric lipid

peroxidation (Tagliazucchi et al., 2010). Indeed diet can also be a source of lipid hydroperoxide, lipo-oxidation end-products and hydrogen peroxide (Halliwell et al. 2000). An additional source of free radical rise from the activation of immune cells naturally present in the gastro-intestinal tract by diet-derived bacteria and toxins (Halliwell et al. 2000). Severe oxidative stress in the gastrointestinal tract has been involved in the pathogenesis of colorectal cancer and in inflammation-based gastro-intestinal tract diseases (Kim et al. 2012). Bioactive peptides might exert direct protective effects in the gastro-intestinal tract by scavenging reactive oxygen species and reducing the oxidative stress. The gastrointestinal tract is in contact with digested food proteins and therefore, with a significant amount of food derived peptides. With this view, milk proteins can be considered as a carrier for the delivery of antioxidant compounds in the gastro-intestinal tract. In milk, antioxidant amino acids and peptides are preserved from oxidation and degradation since they are encrypted in the protein sequences. The simultaneous action of intestinal proteases determines a slow and continuous release of antioxidant peptides and amino acids from the parent proteins protecting the gastro-intestinal tract itself from the oxidative damage and the onset of oxidative diseases. The low bioavailability of protein-derived bioactive peptides supports this hypothesis, suggesting that, at least in part, the physiological effect of bioactive peptides on the organism could derive from a biological effect in the gastro-intestinal tract.

4. Conclusion

Our results indicate that the amino acids tyrosine and tryptophan, released during *in vitro* gastro-intestinal digestion and some identified tyrosine-containing peptides were the major responsible for the radical scavenging activity of digested milk, whereas phenylalanine and histidine-containing peptides played a crucial role in the lipid peroxidation inhibitory capacity of digested milk . Many previous studies were carried out with the aim to identify antioxidant peptides released from bovine milk after proteases treatment. Despite numerous antioxidant peptides having been identified, it is likely that they lack a real physiological systemic effect because they can be further degraded by membrane-bound amino-peptidase in the intestine or they can be poorly absorbed due to their size and thus possibly are no longer available to elicit a biological response. However, we propose that the biological activity of these antioxidant compounds can be relevant for the gastro-intestinal tract. In our view, antioxidant compounds can be slowly and continuously released from milk proteins protecting the gastro-intestinal tract itself from oxidative damage.

Further studies should be carried out to elucidate the *in vivo* contribution of these antioxidant compounds to the antioxidant status of the gastro-intestinal tract after milk consumption.

Disclosure of Conflict of interest

Davide Tagliazucchi, Ahmed Helal, Elena Verzelloni, and Angela Conte declare that they have no conflict of interest

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Figure captions

Fig. 1 UV-chromatograms of the low molecular weight fractions (< 3 kg·mol^{-1}) obtained from the whole (A), semi-skimmed (B) and skimmed (C) bovine milk after *in vitro* gastro-intestinal digestion. Twelve fractions (from F1 to F12) were collected from the low molecular weight fraction of skimmed milk (see panel C). Detection was achieved at 214 nm. The showed chromatograms are representative of three independent experiments.

Fig. 2 Antioxidant activity of the high-performance liquid chromatography-collected fractions from permeate (< 3 kg·mol^{-1}) obtained from skimmed bovine milk after *in vitro* gastro-intestinal digestion. Bars with different letters are different from one another ($P < 0.05$) based on two-way ANOVA analysis of variance and subsequent Tukey's *post hoc* test.

Fig 3 Mass spectrum of high-performance liquid chromatography fraction F2 (**A**), F3 (**B**) and F5 (**C**) from nanoLC-qTOF MS/MS analysis of the permeate (< 3 kg·mol^{-1}) obtained from skimmed bovine milk after *in vitro* gastro-intestinal digestion. Identified compounds are reported in Tables 4 and 5. The showed mass spectra are representative of three independent experiments.

Table 1

Degree of hydrolysis (DH) of bovine milk proteins before and after *in vitro* gastro-intestinal digestion. Data are means ± SD; n=3

Sample	Whole milk	Semi-skimmed milk	Skimmed milk
Before digestion	3.1 ± 0.5^{a}	2.8 ± 0.5^{a}	3.1 ± 0.2^{a}
After peptic digestion	7.2 ± 0.6^{b}	7.4 ± 0.2^{b}	8.8 ± 0.7^{c}
After pancreatic digestion	20.8 ± 0.4^{d}	24.3 ± 0.3^{e}	30.7 ± 0.8^{f}

[a–f] significant differences are shown by different letters (Tukey's test, $P < 0.05$).

Table 2.

Changes in antioxidant activity determined with ABTS assay on the different types of milk during digestion (LMW: low molecular weight fraction, HMW: high molecular weight fraction). Results are expressed as mg of vitamin C·L^{-1} of milk. Data are means ± SD; n=3

Sample	Before digestion	Gastric digestion		Pancreatic digestion	
	After mixing pH 6.8	Time 0 min pH 2.5	Time 120 min pH 2.5	Time 0 min pH 7.5	Time 120 min pH 7.5
Whole milk					
Total	622.3 ± 44.5	282.4 ± 19.3[a]	415.2 ± 9.1[a]	881.0 ± 39.9[a,b,c]	3374.3 ± 104.6[a,b,c,d]
LMW (< 3 kg·mol^{-1})	27.6 ± 4.7	13.6 ± 4.0	80.5 ± 9.5	180.6 ± 16.7[a,b,c]	1267.7 ± 100.2[a,b,c,d]
HMW (> 3 kg·mol^{-1})	597.2 ± 24.9	280.0 ± 21.8[a]	367.7 ± 30.5[a]	719.9 ± 22.4[b,c]	2044.6 ± 183.1[a,b,c,d]
Semi-skimmed milk					
Total	571.4 ± 22.4	221.7 ± 24.6[a,e]	268.6 ± 3.6[a,e]	642.7 ± 18.5[a,b,c,e]	2657.1 ± 39.6[a,b,c,d,e]
LMW (< 3 kg·mol^{-1})	39.4 ± 6.2	16.6 ± 3.2	62.5 ± 5.1	169.1 ± 14.2[a,b,c]	2104.6 ± 80.6[a,b,c,d,e]
HMW (> 3 kg·mol^{-1})	527.0 ± 38.1	203.1 ± 16.4[a,e]	198.1 ± 12.9[a,e]	484.8 ± 27.0[a,b,c,e]	489.6 ± 21.1[a,b,c,e]
Skimmed milk					
Total	515.3 ± 15.2[e]	176.5 ± 27.1[a,e]	229.6 ± 8.0[a,e,f]	592.7 ± 23.4[a,b,c,e]	2751.2 ± 46.9[a,b,c,d,e]
LMW (< 3 kg·mol^{-1})	42.5 ± 10.8	14.7 ± 5.2	76.3 ± 9.1	146.9 ± 12.1[a,b,c]	2481.4 ± 110.1[a,b,c,d,e]
HMW (> 3 kg·mol^{-1})	462.8 ± 34.7[e]	146.9 ± 23.4[a,e,f]	143.4 ± 11.6[a,e,f]	420.1 ± 19.6[b,c,e,f]	351.8 ± 38.6[a,b,c,e]

[a] indicates significantly different respect to the same sample before the digestion (Tukey's test, $P < 0.05$).
[b] indicates significantly different respect to the time 0 of gastric digestion (Tukey's test, $P < 0.05$).
[c] indicates significantly different respect to the time 120 of gastric digestion (Tukey's test, $P < 0.05$).
[d] indicates significantly different respect to the time 0 of pancreatic digestion (Tukey's test, $P < 0.05$).
[e] indicates significantly different respect to whole milk at the same time and pH (Tukey's test, $P < 0.05$).
[f] indicates significantly different respect to semi-skimmed milk at the same time and pH (Tukey's test, $P < 0.05$).

Table 3. Radical scavenging properties and lipid peroxidation inhibitory activity of the reversed phase-high performance liquid chromatography fractions F2, F3 and F5 of < 3 kg·mol^{-1} permeate obtained from skimmed bovine milk after *in vitro* gastro-intestinal digestion.

	ABTS radical scavenging	*Hydroxyl radical scavenging*	*Inhibition of lipid peroxidation*
	mg vitamin C·L^{-1}		*% of inhibition*
F2	303.1 ± 12.8^{a}	4643.1 ± 153.6^{a}	21.0 ± 3.6^{a}
F3	476.0 ± 27.7^{b}	1084.4 ± 61.9^{b}	97.4 ± 4.7^{b}
F5	450.0 ± 25.3^{b}	457.8 ± 28.1^{c}	58.9 ± 7.3^{c}

Data are means ± SD (n = 3). Values in the same columns with different lowercase letter are significantly different (Tukey's test; P< 0.05).

Table 3. Compounds identified in the reversed phase-high performance liquid chromatography fractions F2 and F3 of < 3 kg·mol^{-1} permeate obtained from skimmed bovine milk after *in vitro* gastro-intestinal digestion

Fraction	*Observed mass (m/z)*	*Calculated mass*[a]	*Peptide sequence*[b]	*Protein precursor*	*Bioactivity*[c]
F2					
	132.1037	132.1025	Lx	various proteins	/
	182.0851	182.0812	Y	various proteins	Antioxidant
	173.0840	173.0921	GP	various proteins	Peptide regulating the stomach mucosal membrane activity; DPP IV inhibitor; ACE inhibitor; PEP inhibitor
	189.1246	189.1234	GLx	various proteins	DPP IV inhibitor (GI); ACE inhibitor (GL/GI)
	332.1821	332.1816	VVD	Digestive enzymes (α-amylase, lipase)	/
	356.1945	356.1928	LSH	various proteins	/
F3					
	120.0859	120.0655	T	various proteins	/
	166.1055	166.0863	F	various proteins	/
	173.0819	173.0921	GP	various proteins	Peptide regulating the stomach mucosal membrane activity; DPP IV inhibitor; ACE inhibitor; PEP inhibitor
	203.1415	203.1309	ALx	various proteins	DPP IV inhibitor (AL); ACE inhibitor (AI)
	219.1533	219.1339	SLx	various proteins	DPP IV inhibitor (SL/SI)
	231.1736	231.1703	VLx	various proteins	Glucose uptake stimulating peptide (VL); DPP IV inhibitor (VL/VI)
	260.1639	260.1605	QLx	various proteins	DPP IV inhibitor (QL/QI)
	275.6693	550.3348	VRYL	αS2-casein f (205-208)	ACE inhibitor; Fragment and precursor of

				antioxidant peptide
320.1849	320.1816	SLxT	β-casein (various fragments)	/
334.1987	334.1973	TLT	β-casein f (126-128)	/
485.3159	485.3082	IQPK	αS2-casein f (194-197)	/
521.2642	521.2606	YPEL	αS1-casein f (146-149)	Antioxidant
674.3555	674.3508	AVPYPQ	β-casein f (177-182)	Precursor of antioxidant peptide

[a]Monoisotopic mass
[b]Lx indicates leucine or isoleucine
[c]Potential bioactivities were achieved from the BIOPEP database; ACE: Angiotensin Converting Enzyme; DPP IV: Dipeptidyl peptidase IV; PEP: Prolyl endopeptidase

Table 5. Compounds identified in the reversed phase-high performance liquid chromatography fraction F5 of < 3 kg·mol^{-1} permeate obtained from skimmed bovine milk after *in vitro* gastro-intestinal digestion

Fraction	*Observed mass (m/z)*	*Calculated mass*[a]	*Peptide sequence*[b]	*Protein precursor*	*Bioactivity*[c]
F5					
	205.2208	205.2262	W	various proteins	Antioxidant
	231.1734	231.1703	LxV	various proteins	Glucose uptake stimulat peptide (LV/IV); DPP IV inhibitor (LV)
	231.1734	231.1703	VLx	various proteins	Glucose uptake stimulat peptide (VL); DPP IV inhibitor (VL/V
	265.1741	529.3344	TKIPA	β-lactoglobulin f (76-80)	/
	295.1655	295.1652	YLxx	various proteins	DPP IV inhibitor (YL/Y ACE inhibitor (YL) Fragment of antioxidant peptides
	317.6827	634.3923	YKVPK	αS1-casein f (104-108)	/
	340.1831	679.3522	HQGLPQ	αS1-casein f (8-13)	/
	345.6897	690.3668	ITVDDK	αS2-casein f (71-76)	/
	353.6766	706.3440	AMEDIK	αS1-casein f (53-58)	/
	387.2272	387.2165	SAPL	β-lactoglobulin f (36-39)	/
	391.2055	391.1976	FPQ	αS2-casein f (92-94)	/
	446.5737	1337.6808	HIQKEDVPSER	αS1-casein f (80-90)	/
	552.2430	552.2227	DAYPS	αS1-casein f (157-161)	Precursor of antioxidant peptides
	680.3035	680.2886	DAYPSGA	αS1-casein f (157-163)	Precursor of antioxidant peptides

[a]Monoisotopic mass
[b]Lx indicates leucine or isoleucine
[c]Potential bioactivities were achieved from the BIOPEP database; ACE: Angiotensin Converting Enzyme; DPP IV: Dipeptidyl peptidase IV

Table 6. Antioxidant properties of pure amino acids and the dipeptide glycine-proline.

Amino acids[a]	*ABTS radical scavenging*	*Hydroxyl radical scavenging*	*Inhibition of lipid peroxidation*
	mg vitamin C·mmol^{-1} amino acid		*% of inhibition*[b]
Trp	219.2 ± 5.9[a]	211.1 ± 3.1[a]	31.7 ± 1.1[a]
Tyr	124.7 ± 3.8[b]	769.7 ± 9.5[b]	4.9 ± 0.2[b]
Cys	162.5 ± 4.4[c]	766.4 ± 8.7[b]	n.d.
Met	n.d.	450.9 ± 6.4[c]	11.3 ± 0.8[c]
Thr	n.d.	64.1 ± 2.1[d]	n.d.
Pro	n.d.	59.1 ± 3.4[d]	n.d.
His	n.d.	55.3 ± 2.8[d,e]	80.3 ± 2.3[d]
Arg	n.d.	39.4 ± 1.4[e]	13.8 ± 1.1[c]
Lys	n.d.	n.d.	n.d.
Asp	n.d.	n.d.	n.d.
Glu	n.d.	n.d.	n.d.
Phe	n.d.	165.6 ± 8.3[f]	91.4 ± 5.6[e]
Leu	n.d.	n.d.	n.d.
Ile	n.d.	n.d.	n.d.
Gly	n.d.	n.d.	n.d.
Val	n.d.	n.d.	n.d.
Ala	n.d.	n.d.	n.d.
Ser	n.d.	188.1 ± 7.3[g]	50.3 ± 1.7[f]

[a] Three letters code
[b] % of inhibition referred to a control reaction without amino acids (set as 100% of peroxidation). Amino acids were tested at concentration of 2 mmol L^{-1}
n.d. not detected activity
Data are means ± SD (n = 3). Values in the same columns with different lowercase letter are significantly different (Tukey's test; $P < 0.05$).

Figure 1

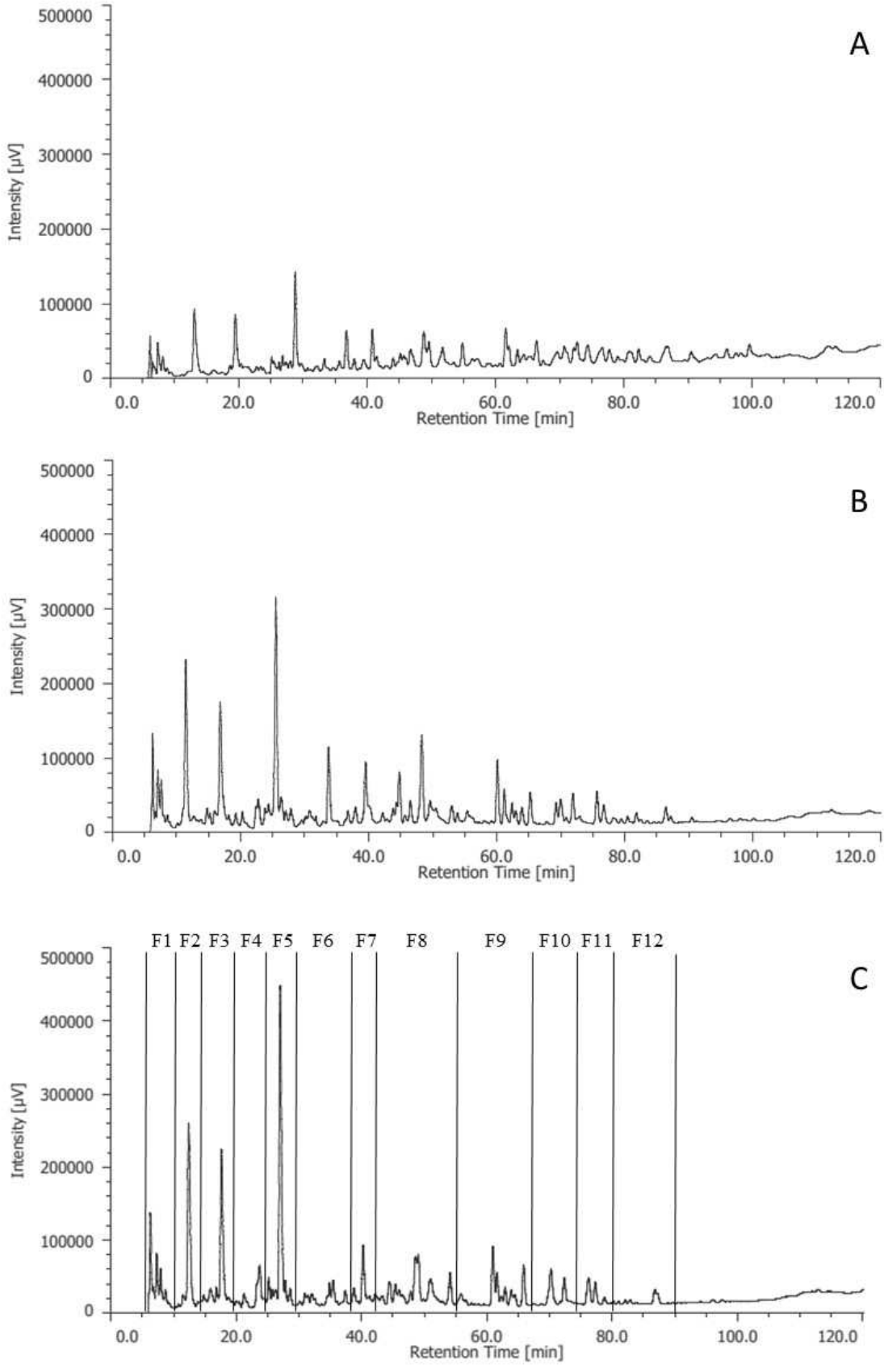
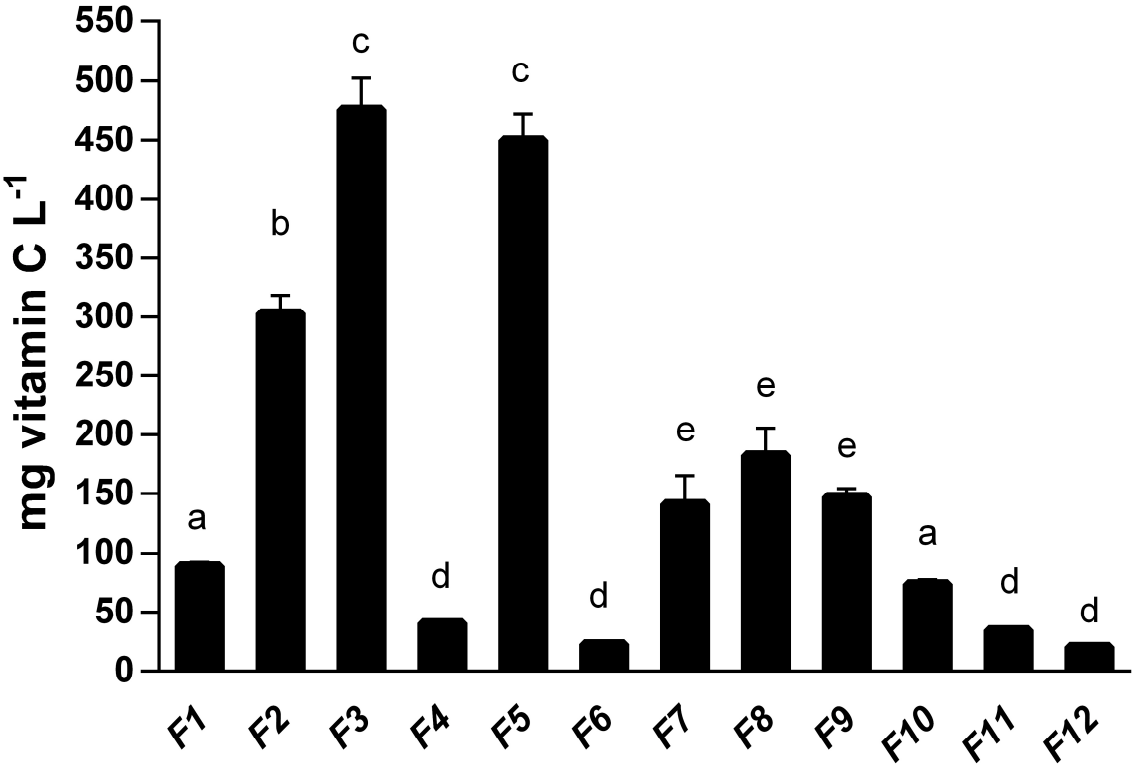

A
B
C
Intensity [µV]
Retention Time [min]
0
100000
200000
300000
400000
500000
0.0
20.0
40.0
60.0
80.0
100.0
120.0
F1 F2 F3 F4 F5 F6 F7 F8 F9 F10 F11 F12

Figure 2


mg vitamin C L-1
550
500
450
400
350
300
250
200
150
100
50
0
a
b
c
d
c
d
e
e
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a
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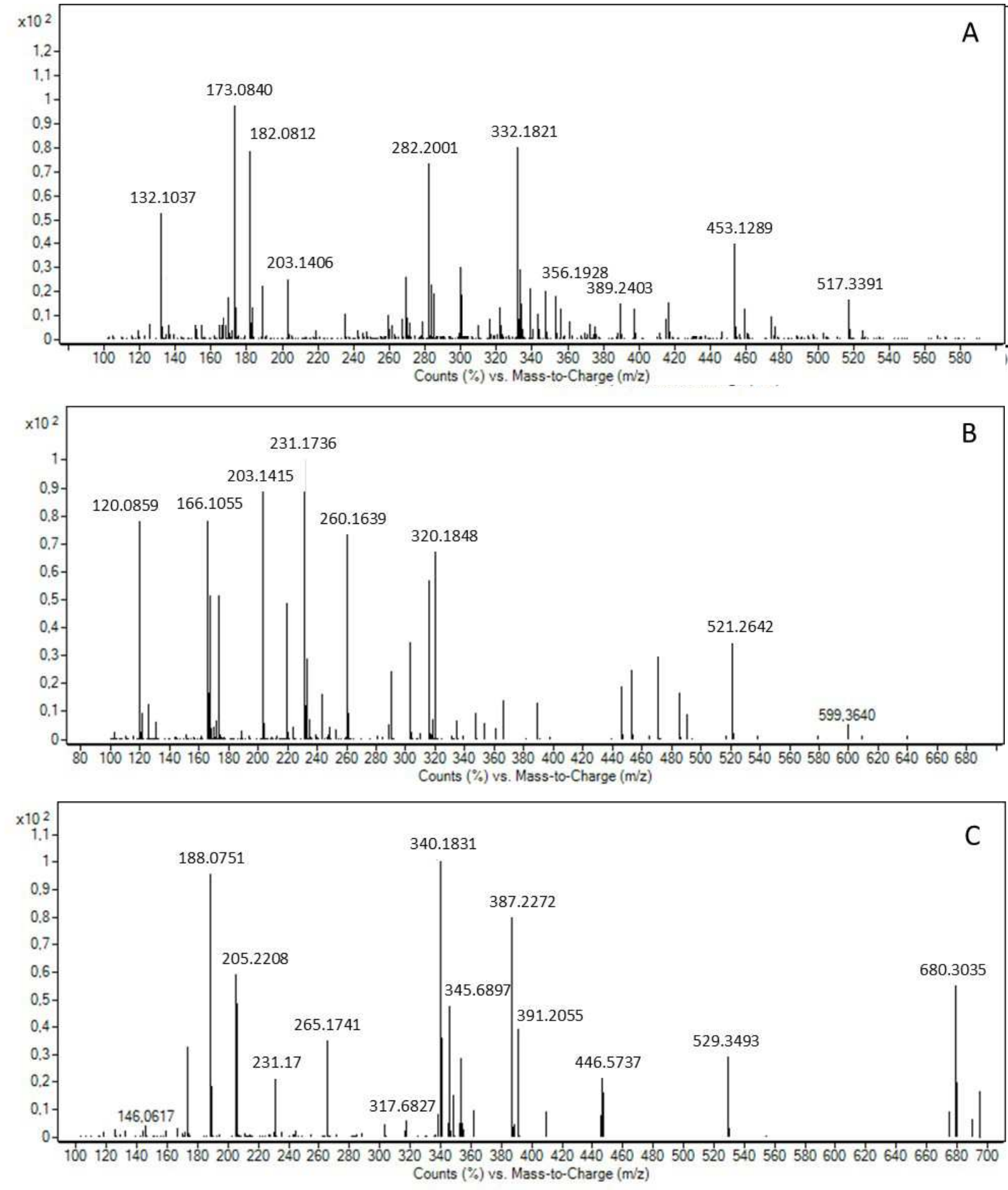
F6
F7
F8
F9
F10
F11
F12

Figure 3


A
x10 2
173.0840
182.0812
282.2001
332.1821
132.1037
453.1289
203.1406
356.1928
389.2403
517.3391
Counts (%) vs. Mass-to-Charge (m/z)
B
x10 2
231.1736
203.1415
120.0859
166.1055
260.1639
320.1848
521.2642
599.3640
Counts (%) vs. Mass-to-Charge (m/z)
C
x10 2
340.1831
188.0751
387.2272
205.2208
345.6897
680.3035
391.2055
265.1741
529.3493
231.17
446.5737
317.6827
146.0617
Counts (%) vs. Mass-to-Charge (m/z)