Food & Function

Accepted Manuscript

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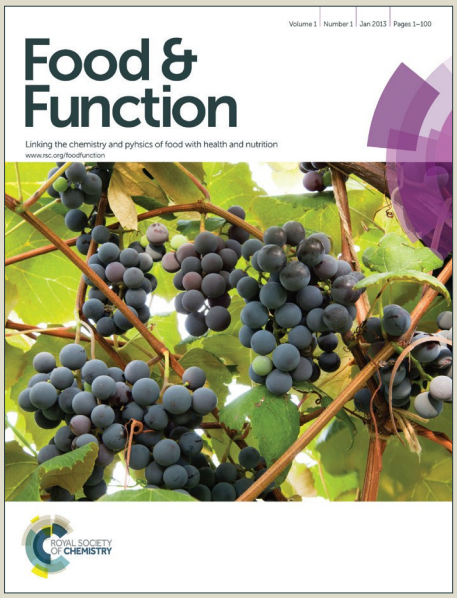

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View Article Online
DOI: 10.1039/C6FO00151C

Encapsulation of ascorbic acid promotes the reduction of Maillard reaction products in UHT milk

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DOI: 10.1039/C6FO00151C

Abstract

The presence of amino groups and carbonyls renders fortified milk with ascorbic acid particularly susceptible to the reduction of available lysine and to the formation of Maillard reaction products (MRPs), as Nε-(carboxyethyl)-L-lysine (CEL), Nε-(carboxymethyl)-L-lysine (CML), Amadori products (APs) and off-flavors. A novel approach was proposed to control the Maillard reaction (MR) in fortified milk: ascorbic acid was encapsulated in a lipid coating and the effects were tested after a lab scale UHT treatment. Encapsulation promoted a delayed release of ascorbic acid and a reduction in the formation of MRPs. Total lysine increased up to 45% in milk with encapsulated ascorbic acid, while reductions in CML, CEL and furosine ranged from 10% to 53% compared with control samples. The effects were also investigated towards the formation of amide-AGEs (advanced glycation end products) by high resolution mass spectrometry (HRMS) revealing that several mechanisms coincide with the MR in the presence of ascorbic acid.

Keywords: Maillard reaction, encapsulation, ascorbic acid, mass spectrometry

Abbreviations: Advanced glycation end products (AGEs); Amadori products (APs), Nε-(carboxyethyl)-L-lysine (CEL), Nε-(carboxymethyl)-L-lysine (CML); ascorbic acid (AA), dehydroascorbic acid (DHAA), Nε-(2-Furoylmethyl)-L-lysine (furosine), high resolution mass spectrometry (HRMS), Maillard reaction (MR), Maillard reaction end products (MRPs), tandem mass spectrometry (MS/MS); Ultra High Temperature (UHT).

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Introduction

The final quality of milk and infant formula is influenced by the Maillard reaction (MR). Thermal treatments including UHT are necessary to reduce the proliferation of food borne pathogens and in the presence of high concentrations of carbonyls and amino groups, thermal processes can promote some of the main concerns for dairy industries such as the loss of nutritive value, the development of off-flavors and the formation of undesired brown pigments. Heat treatments of dairy based products therefore need to be optimized to ensure the maintenance of the beneficial effects while at the same time counteracting the possible undesirable effects.[1]

The MR supervises the dichotomy between undesired and desired molecule formation. It is characterized by a series of consecutive and parallel reactions that lead to the formation of outcomes, such as the development of aroma, flavors, texture, antioxidants, colors, and outcomes such as the loss of certain nutrients, mainly amino acids and proteins, with the consequent formation of off-flavors and potentially toxic molecules.[2] Several pathways can be determined depending on the increase of the temperature. The central hub is represented by the formation of lactose-lysine or fructose-lysine, the Amadori products (APs). Once the APs are formed several reactions take place, leading to the Maillard reaction end products (MRPs) or dietary advanced glycation end products (AGEs).[3, 4]

Beside the presence of reducing sugars and amino groups some reactants may influence the chemistry of the MR. Certain cations, lipids, polyphenols and vitamins may inadvertently act as triggering agents while on the other hand they may also act as reducing agents by limiting the formation of heat induced toxicants.[5]

Ascorbic acid (AA) plays several roles in fortified milk and infant formula. It acts as an antioxidant in the prevention of oxidation of polyunsaturated fatty acids (PUFA), provides the appropriate dietary reference values and promotes the uptake and bioavailability of dietary iron.[6] Despite its importance, AA also acts as a precursor of several molecules, some of them involved in the pathways of nonenzymic browning both *in vitro* and *in vivo*.[7-9] AA is able to produce furfural and carbon dioxide on its own in aqueous solution above 98 °C leading to browning and also in the presence of an amino group, i.e. glycine, the carbon dioxide comes mainly from AA.[10] AA is one of the main precursors of furan, via 2-furaldehyde formation following two different reaction pathways: (a) electrophilic aromatic substitution with water, forming formic acid as the byproduct and (b) oxidation to 2-furoic acid followed by decarboxylation.[11, 12] According to the reaction conditions (i.e. pH, pressure and temperature), the conversion of AA into dehydroascorbic acid (DHAA) and the reconversion of DHAA into AA can be efficiently described by the Weibull model

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where different sensitivities of the reaction rate constant to the temperature promote sigmoidal kinetics.[13] Smuda and Glomb reported around 75% of the Maillard induced decomposition of AA. The oxidation of AA leads to the formation of DHAA and 2,3-diketogulonic acid (2,3-DKG) that can undergo α and β dicarbonyl fragmentation and oxidative cleavage. Moreover, by using ^{13}C-ascorbic acid isotopomers the formation of carbonyl compounds, carboxylic acids and amide-AGEs, such as glycerinyl-lysine; oxalyl-lysine; xylonyl-lysine, threonyl-lysine and lyxonyl-lysine, was revealed, highlighting the parts of the original backbone of AA incorporated in the products.[14]

Finally, AA is a source of molecules that act as precursors or intermediates for the formation of MRPs. Dunn and co-workers verified that CML is also formed in reactions between ascorbate via its oxidation product, dehydroascorbate, and lysine residues in model systems in vitro. In particular several intermediates can lead to the formation of CML, not only dehydroascorbate, but also L-threose via direct cleavage of threulose-lysine.[15] Hasenkopf and co-workers incubated proteins and poly-lysine in presence of AA, DHAA and glucose in order to evaluate the extent of glycation and ascorbylation. Results highlighted that CML, CEL and oxalic acid mono-Nε-lysinylamide could be simultaneously detected and quantified in glycated and ascorbylated proteins; while Nε-(1-carboxy-3-hydroxypropyl)-L-lysine was identified as a Maillard product of proteins and under the conditions applied it was found only in ascorbylated proteins or poly-L-lysine, but not in glycated proteins.[8, 16] Beside the in vitro ascorbylation, the influence of the AA on the final quality of infant formula was studied by Leclère and co-workers in a lactose-whey protein model system in the presence of iron ascorbate. Results revealed that the accumulation rate of fluorescent MRPs was higher in the presence of iron and/or ascorbate and fluorescence was strongly correlated with the concentration of CML.[17]

The control of the MR is an important factor for ensuring the final quality of foods, particularly in fortified milk and infant formula. Several strategies have been proposed including optimal control of parameters (i.e. time and temperature profile), the use of alternative technologies, the addition of polyphenols and the control of Maillard pathways by multiresponse modeling. Taking into account that the removal of precursors such as sugars and amino groups are inapplicable, recently our group focused on the encapsulation of certain reactants as a main driver for controlling the MR.[5]

Fiore and co-workers verified the ability of three different lipidic coatings for the controlled release of sodium chloride in cookies. Sodium chloride is used for technological and sensorial purposes, but its catalytic activity promotes the pyrolysis of sugars and the consequent formation of higher concentration of HMF. It was demonstrated that the higher the melting point the greater the reduction of HMF, without altering the final taste.[18] A similar approach was used in the control of

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furan formation by using encapsulated AA. In particular, acacia gum and maltodextrin coatings of AA significantly decreased furan formation down to 57% at 120 °C.[19] These previous findings suggest a role for encapsulation as a potential strategy for the control of MRPs and thus warrants further investigation in their effect on functionalized milk, infant formula and on the release of functional molecules upon thermal processing.[20] The present study aimed to examine the effects of encapsulated AA towards the formation of MRPs. According to high potentiality of encapsulation as a strategy for the control of the MR, it was decided to test the effectiveness of AA encapsulation in the tuning of MRPs and amide-AGEs formation.

Material and methods

Chemical and reagents

Acetonitrile, water, methanol and acetic acid for liquid chromatography tandem mass spectrometry (LC-MS/MS) and liquid chromatography high resolution mass spectrometry (LC-HRMS) analyses were obtained from Merck (Darmstadt, Germany). The ion-pairing agent perfluoropentanoic acid (NFPA), ascorbic acid (AA), dehydroascorbic acid (DHAA), ethylenediaminetetraacetic acid (EDTA) and the analytical standards [4,4,5,5-*d4*]-L-lysine hydrochloride (*d4*-Lys) and lysine were purchased from Sigma-Aldrich (Saint-Louis, MO), while hydrochloric acid (37%) was purchased from Carlo Erba (Milano, Italy). Analytical standards Nε-(2-furoylmethyl)-L-lysine (furosine), Nε-(carboxymethyl)-L-lysine (CML) and its respective deuterated standard Nε-(carboxy[$^2H_2$]methyl)-L-lysine (*d2*-CML) and ε-N-(2-furoyl)-methyl-L-[4,4,5,5-$^2H_4$]Lysine HCl salt (*d4*-furosine) were obtained from Polypeptide laboratories (Strasbourg, France), Nε-(carboxyethyl)-L-lysine and its internal standard Nε-(carboxy[$^2H_4$]ethyl)-L-lysine (*d4*-CEL) were purchased from TRC-Chemicals (North York, Canada). The calibration solution (see "AA and DHAA quantitation" section) was obtained from Thermo Fisher Scientific (Bremen, Germany). Encapsulated AA (50% in palmitic acid blend) was obtained from TasteTech (Bristol, UK).

Milk model systems preparation

Milk model systems were prepared by first dissolving water soluble ingredients: 1.2% (w/w) skimmed milk powder, 2.5% (w/w) whey protein (Prolacta, Lactalis, France) and 5% (w/w) lactose monohydrate in 87.9% (w/w) water along with the micronutrients listed in **Table 1**. The lipid mixture was prepared separately by melting 3.3% (w/w) of a mixture of lipids (palm oil, tripalmitin, triolein 1:1:1) and 0.1% (w/w) of sucrose esters (HLB 16, Sisterna, Roosendaal, the Netherlands) at 45 °C for 20 min. The lipid mixture was added to the water soluble mixture and continuously stirred at 45 °C. This mixture was primarily homogenized 3 times, 30 s, by using a digital Ultraturrax T25

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(IKA, Stockholm, Sweden) working at 22000 rpm. The coarse milk was homogenized under pressure (160 bar, 3 passes) in a bench-top homogenizer (GEA-Niro Soavi, Parma, Italy). The four model systems were prepared prior the homogenization procedure by adding the quantities of Free AA (FAA), encapsulated AA (EAA) and coating only (COA) reported in **Table 1.** The three samples along with control milk (CTL milk) were aliquoted in headspace vials (10 mL) with a crimp seal with PTFE/silicone septa (Phenomenex, Torrance, CA) and processed for 2, 4, 6 and 8 min, while control sample was aliquoted prior the thermal treatment. The four milk samples were thermally treated at 140 °C by using the same procedure, system and thermal profile previously described.[21]

AA and DHAA quantitation

The extraction of AA and DHAA from milk model systems was performed according to Fenoll and coworkers with slight modifications.[17] Briefly, 100 µL of milk were diluted 50 times in 0.05% (w/v) EDTA in a volumetric flask, and centrifuged at 4000 rpm for 15 min at 4 °C. The supernatants were collected and 1000 µL was filtered through a 0.45 µm nylon filter (Phenomenex, Torrance, CA). Finally, 10 µL was injected into the LC-HRMS system. AA and DHAA separation was performed on a U-HPLC Accela system 1250 (Thermo Fisher Scientific, Bremen, Germany) consisting of a degasser, a quaternary pump, a thermostated autosampler (5 °C) and a column oven set at 30 °C. Mobile phase A was 0.1% formic acid, and mobile phase B was 0.1% formic acid in methanol and separation was achieved by using a Synergi-Hydro column (150 x 2.0 mm, 4.0 µm; Phenomenex, Torrance, CA) and the following gradient of solvent B (min/%B): (0/2), (4/2), (9/70), (12/70) was used at a flow rate of 200 µL/min. The autosampler needle was rinsed with 800 µL of methanol before each injection. To set up the optimal condition, an aqueous solution of AA and DHAA (10 µg/mL) was infused directly into the Exactive Orbitrap HRMS system (Thermo Fisher Scientific, Bremen, Germany) equipped with a heated electrospray interface operating in the positive and negative mode and scanning the ions in the *m/z* range of 50−550. The resolving power was set to 75000 full width at half-maximum (FWHM, *m/z* 200), resulting in a scan time of 1 s. The automatic gain control was used to fill the C-trap and the maximum injection time was 50 ms. The interface parameters were as follows: spray voltage, 3.0 kV (-3.0 kV for negative ion mode); capillary temperature 320 °C and heather temperature at 250 °C, capillary voltage, 48.5 V (-48.5 V, for negative ion) ; skimmer voltage, 14 V (-12 V for negative ion) ; sheath gas flow, 30 (arbitrary units); and auxiliary gas flow, 6 (arbitrary units). Before the AA and DHAA determination, the instrument was externally calibrated by infusion with a positive ions solution that consisted of caffeine, Met-Arg-Phe-Ala (MRFA), Ultramark 1621, and acetic acid in a mixture of acetonitrile/methanol/water (2:1:1, v/v/v), then with a negative ions solutions that consisted of

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sodium dodecyl sulfate, sodium taurocholate, Ultramark 1621, and acetic acid in a mixture methanol/water (1:1, v/v). Reference mass (lock mass) of diisooctyl phthalate ($[M + H]^+$, exact mass = 391.28429) was used as recalibrating agent for positive ion detection. To optimize the HRMS conditions and the mass accuracy, the instrument was calibrated each day both in positive and negative mode. A stock solution of AA and DHAA was prepared by dissolving 10 mg of standard in 1 mL of mass spectrometry grade water. This solution was diluted and stored at −20 °C until use. The range of the calibration curve was between 10−1000 ng/mL according to the limit of detection (LOD) and the limit of quantitation (LOQ). Three replicates of 1 ng/mL solutions were injected into the U-HPLC-HRMS system to verify the lowest concentration for which the signal-to-noise ratio was >3. Concentrations of 1 ng/mL resulted in no signal. The LOQ was 10 ng/mL for the standard solution, and the r^2 value was >0.99 in the above-mentioned range. Reproducibility of the method was evaluated through the intraday and interday assay. The slope among the three subsequent calibration curves showed a % RSD of < 10%. CTL milk samples were spiked with three different concentrations of AA and DHAA (50 ng/mL, 1000 ng/mL and 5000 ng/mL) and the recovery was calculated according to the following formula:

$$(1)\ R = \left(\frac{C_a}{C_s}\right) \times 100$$

Where R is the recovery; C_a is the concentration of the spiked analytes in the samples, C_s is the concentration of the standard in water. Analytical performances are summarized in **Table 2.** The analytical setup for the detection of AA and DHAA was also used for the identification of amide-AGEs derivatives in aqueous model systems and in milk.

Formation of amide-AGEs

In order to investigate the presence of amide-AGEs, an aqueous model system was prepared. Briefly, equal amounts of AA and lysine (200 μg/mL) were mixed and sealed in a screw capped flask saturated with nitrogen. The mixtures were incubated at 140 °C for 2, 4, 6 and 8 min, following the same thermal treatment profile used for the milk samples. After each step of the thermal treatment, samples were diluted 20 times in water and injected into LC-HRMS system by following the procedures described for AA and DHAA. Precursors, reaction intermediates and end products such as carboxylic acids amides and amide-AGEs were investigated by using an in-house database developed according to the degradation pathways reported in **Figure 1**. Specific molecular formulas and their respective *m/z* ratios were included in Exact Finder (Thermo Fisher Scientific, Bremen, Germany) and the following parameters were selected: isotopic pattern and retention time for the identification, signal to noise ratio higher than 5. The procedure used for the lysine/ascorbic acid model system was also applied for milk samples.

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Maillard reaction end products (MRPs) quantification

Typical markers of the MR, CML, CEL and furosine, as well as total lysine in milk were monitored according to Troise et al.[22] Briefly, 100 µL of milk was mixed along with 4 mL of HCl 6 M. The mixture was saturated by nitrogen and hydrolyzed in an air forced circulating oven (Memmert, Schwabach, Germany) for 20 h at 110 °C. The mixture was filtrated by polyvinylidene fluoride filters (PVDF, 0.22 Millipore, Billerica, MA) and 200 µL was dried under nitrogen flow in order to prevent the oxidation of the constituents. The samples were reconstituted in 190 µL of water and 10 µL of internal standard mix was added in order to obtain a final concentration of 200 ng/mg of samples for each standard (*d4*-Lysine, *d2*-CML and *d4*-CEL and *d4*-furosine). Samples were loaded onto equilibrated Oasis HLB 30 mg cartridges (Waters, Wexford, Ireland) and eluted according to the method previously described; then 5 µL was injected onto the LC-MS/MS system. Separation of furosine, CML, CEL, lysine and their respective internal standards was achieved on a reversed-phase core shell column Kinetex C18 2.6 µm, 2.1 mm x 100 mm (Phenomenex, Torrance, CA) using the following mobile phases: A, 5 mM perfluoropentanoic acid and B, acetonitrile 5 mM perfluoropentanoic acid. The compounds were eluted at 200 µL/min through the following gradient of solvent B (t in [min]/[%B]): (0/10), (2/10), (5/70), (7/70), (9/90), (10/90). Positive electrospray ionization was used for detection and the source parameters were selected as follows: spray voltage 5.0 kV; capillary temperature 350 °C, dwell time 100 ms, cad gas and curtain gas were set to 45 and 5 (arbitrary units). The chromatographic profile was recorded in MRM mode and the characteristic transitions were monitored by using an API 3000 triple quadrupole (ABSciex, Carlsbad, CA). The mass spectrometry parameters were optimized according to Troise et al.[22]

Statistical analysis

Each sample was analyzed in duplicate from two independent thermal treatment sets and injected twice. The results were reported as g/100 g of protein for lysine, mg/100 g of protein for furosine, CEL and CML, while AA and DHAA were reported as mg/L of milk. Amide-AGEs including glycerinyl-lysine; oxalyl-lysine; xylonyl-lysine, threonyl-lysine e lyxonyl-lysine were compared using the area counts. Evolution of the markers was recorded by using Prism (GraphPad Software, La Jolla, CA), while the Tukey test ($\alpha = 0.05$) for bound MRPs and for the AA and DHAA were performed by using XLStat 4.6 (Addinsoft, New York, NY). In particular, for bound MRPs the test was independently performed for each marker and different letters correspond to significative differences within each group.

Results and discussion

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AA is widely used in food and pharmaceutical industries as an additive; in particular its protective and functional effects are required in infant formula preparations. Despite its antioxidant properties, AA is highly unstable and during thermal treatments or storage it can be degraded, leading to the loss of nutritive values, loss of color or color formation via nonenzymic browning and volatile formation.[8] As a consequence, the effects of AA on the formation of MRPs were investigated in a milk model system and the encapsulation of AA was evaluated as a potential control strategy for the formation of undesired compounds that contribute to the loss of available lysine, such as lysine Amadori product, CML and CEL, as well as amide-AGEs.

Model systems were prepared according to **Table 1** and the concentration of AA and DHAA, the formation of amide-AGEs were monitored in the two of four recipes of the model system: milk with free AA (FAA), milk with encapsulated AA (EAA), control without AA and with lipidic coating (COA) and a control without AA and lipidic coating (CTL) were only subjected to the analysis of MRPs and total lysine.

The first step was characterized by the set-up of a valid LC-HRMS procedure able to quantify AA and DHAA in milk samples. Moreover, it was possible to develop a robust method to simultaneously quantify AA and DHAA in milk samples without any derivatization procedure. The use of the exact mass up to the fifth decimal digit along with a mass tolerance up to 3 ppm limited the matrix effects and controlled the interference due to the first eluting impurities. The analytical performances are summarized in **Table 2** and they are of the same order of magnitude towards other MS procedures reported previously, in particular the recovery was higher than 91% for DHAA and AA.[17, 23]

In **Figure 2 A and B** the concentration of AA and DHAA in milk samples FAA and EAA were reported. According to the thermal loading, the concentration of AA slightly decreased up to 6 min, and then rapidly reached its lowest value at the end of the thermal treatment: 299.5 ± 1.5 mg/L. As expected the concentration of AA in EAA milk was 27% lower than FAA milk and the initial concentration was 270 ± 6.9 mg/L. Interestingly, the concentration of AA rapidly increased after 2 min, reaching its maximum after 4 min: 313.3 ± 1.5 mg/L, then it decreased down to 267.5 ± 0.5 mg/L after 8 min. In EAA milk, the initial concentration of DHAA was 12.3 ± 0.4 mg/L and differed from FAA milk. The concentration decreased up to 2 min, then it rapidly increased reaching its maximum at the end of thermal treatment 12.4 ± 0.5 mg/L. The evolution of AA and DHAA during the thermal treatment was not surprising. Firstly, it should be considered that around 20% of AA was not inside the capsules, but linked to the external part of the lipidic wall; secondly the homogenization procedure (160 bar 3 passes) promoted the desegregation of around 50% of

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capsules and made AA available and free to react in the first stages of the incubation (data not shown). In this respect, the use of lipidic coating guaranteed a slower release ratio than other hydrophilic coatings.[20, 24] In FAA and EAA milk the kinetic profiles of AA and DHAA were consistent with the reaction mechanism: in the first stages the concentration of DHAA rapidly increased following a first order kinetic, then at the end of the thermal process it rapidly decreased turning the chemical pathways to the formation of 2,3-diketogulonic acid and other degradation products according to the β-dicarbonyl fragmentation and the oxidative α-dicarbonyl cleavage route.[14, 25] The evolution of DHAA in EAA milk demonstrated the effectiveness of the capsules in sequestrating the AA: the slight increase after 2 min was in line with the increase of AA during the first stages of the thermal treatment in FAA milk, while the reduction during the first two minutes is a direct consequence of the initial concentration of AA released from the capsules.[26]

The impact of free and encapsulated AA on furosine, CML, CEL and total lysine was evaluated with different ingredient composition of the four recipes over 8 min in a lab scale UHT system and the results were reported in **Figure 3** and **table 3** (Tukey test, $\alpha = 0.05$). The concentration of furosine in CTL milk ranged from 227.5 ± 11.3 to 465 ± 22.0 mg/100 g of protein after 6 min when the compound reached its maximum concentration. The same trend was obtained for the other milk systems: after 6 min the concentration of furosine was 446 ± 10.0, 520 ± 5.0 and 429.7 ± 10.0 mg/100 g of protein for FAA, COA and EAA milk, respectively. During the last stage the concentration of furosine decreased in all the samples: 386.7 ± 14.9, 380.6 ± 8.14, 388.9 ± 5.3, 450.5 ± 11.6 mg/100 g of protein for EAA, CTL, FAA and COA, respectively. As expected, CML increased over the storage in the three samples: in the presence of AA (free and encapsulated) and in control samples the values linearly increased. The highest values were obtained after 8 min: 12.3 ± 0.5, 19.1 ± 0.7 and 19.4 ± 1.0 mg/100 g of protein for EAA milk, CTL milk and FAA milk. In milk lipidic coating (COA), CML reached its maximum after 4 min then it slowly decreased down to 15.195 ± 0.6 mg/100 g of protein. The concentration of CML in EAA milk was always lower than other recipes, in particular the highest reduction was toward COA milk after 4 min (55%). Specifically, the reduction of CML in EAA milk ranged from 10 to 41% towards FAA milk. In a closed system, as the lab scale UHT milk here proposed, the behavior of CEL was very close to the one reported for CML. Specifically, only COA milk exhibited a slight decrease at the end of the thermal treatment 5.58 ± 0.44 and 5.64 ± 0.51 after 8 and 6 min respectively. The concentration in other samples increased throughout the thermal treatment, the highest values were obtained after 8 min: 5.96 ± 0.40, 7.58 ± 0.15 and 4.17 ± 0.37 mg/100 g of protein for CTL, FAA and EAA milk, respectively. A significant reduction of CEL was observed in EAA milk: specifically in relation to FAA milk they were 53%, 33%, 51% and 45%, after 2, 4, 6 and 8 min, respectively. The

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concentration of total lysine decreased in line with the thermal loading in all the samples. The values ranged from 8.66 ± 0.36 g/100 g of protein before the thermal treatment to 4.19 ± 0.11 g/100 g of protein in FAA milk.

The effectiveness of encapsulation as a tool for the control of a chemical reaction has been extensively studied by our group.[27] Troise and Fogliano introduced the possibility to encapsulate AA not only for nutritional purposes, but also to prevent to the formation of MRPs in infant formula and fortified milk in presence of AA.[5] The relationship between volatiles, AA and encapsulation was investigated also by Ödzemir and Gökmen: acacia gum and maltodextrin coatings of AA significantly reduced furan formation up to 57% at 120 °C in model systems.[19] Beside the formation of volatiles, off-flavor, amide-AGEs and reactive dicarbonyls, AA plays an active role in the formation of other markers of the MR in milk, such as furosine, CML and CEL.[9, 14] As highlighted in **Figure 3**, the concentration of lysine significantly decreased at the end of the thermal treatment as a consequence of the attack of the ε-amino moiety of lysine that favored an amine-induced β-cleavage, α-fragmentation or decarboxylation with the final formation of MRPs (Tukey test, $\alpha = 0.05$, **table 3**).[8, 14, 28] The presence of encapsulated AA showed a protective effect on the lysine amino group, thus leading to a significant increase over the UHT treatment ($\alpha < 0.05$), up to 24 and 29% higher than FAA milk after 2 and 4 min, respectively, while after 6 and 8 min, upon the release of AA from capsules the differences were not significant.

Furosine is formed from the Amadori compounds, fructose-lysine and lactose-lysine, it is the most studied marker of the MR in milk products.[29] In our milk model systems, the presence of encapsulated AA promoted a significant reduction of furosine after 2 and 4 min of heat treatment. At this stage no conclusion can be drawn about the relationship between AA and formation of fructose-lysine. It was hypothesized that the alteration of the equilibrium in AA/DHAA in FAA milk and in the late stage of the thermal treatment in EAA milk may promote oxidizing conditions that could lead to an increase of the glycation of amino acids, resulting in a reduction of furosine concentration.[8] At the end of the thermal treatment the differences among furosine concentrations in the four formulations were not significant.

CML and CEL followed a similar pattern during the UHT process with exception to CML in COA milk. In the presence of lipidic coating, CML reached its maximum after 4 min then it rapidly decreased revealing a similar trend to the one reported by Nguyen and coworkers. In certain conditions, CML is not a thermally stable compound [30] and the most important source of CML formation is the oxidative cleavage of the Amadori compounds with the consequent C-2 and C-4 fragmentation (Hodge pathway), the cleavage of the Schiff base (Namiki pathway), the oxidative

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glycosylation (Wolff pathway).[2] Moreover, we have demonstrated that the ascorbylation of the ε-amino group of lysine occupied a central role in the milk model system, as reported previously.[28, 31] In this respect, the formation of L-threose from AA and DHAA promoted the formation of the corresponding Schiff base upon the reaction with lysine residues or free lysine. The Schiff base underwent both the Amadori rearrangement leading to the formation of L-tetrulose-lysine then CML. Alternatively, after an oxidation step, CML can be formed from glycoaldehyde/alkylimine/glyoxal pathway,[15] even if the contribution of glyoxal was estimated as negligible via multiresponse reaction network in sodium caseinate/lactose model system.[30]

The protective effect of lipidic capsules on CML formation was significative throughout the thermal treatment with the only one exception represented by the first step ($\alpha < 0.05$). By sequestrating the AA inside the lipidic coating the reaction mechanisms leading to the formation of CML were delayed or completely blocked with a reduction ranging from 10% to 51%. It is worthy to highlight that the reduction here obtained were of the same order of magnitude towards previous work by our group for sodium chloride in lipidic coating and HMF in biscuits and for furan and AA in maltodextrin and acacia gum.[18, 19]

The reduction of CEL was particularly influenced by the presence of encapsulated AA, as revealed in **Figure 3**. It was hypothesized that the formation of methylglyoxal (MGO) was increased in the presence of AA and the α-dicarbonyl was particularly relevant for the formation of CEL.[14] CEL is mainly formed via the MR with MGO formation alongside the release of lysine, deriving from the degradation of APs.[30] Other pathways can be included: the Cannizzaro rearrangement and sequential hydration and dehydration reactions (formation of CEL from MGO); decomposition of the sugar to form MGO, which then reacts with protein to form CEL (formation of CEL via retro-aldol fragmentation of 1-deoxyglucosone and 3-deoxyglucosone); direct reaction of amino groups with the triose phosphate, followed by elimination of the phosphate group, or spontaneous or amine-catalyzed decomposition of triose phosphates to MGO (formation of CEL from triose phosphates).[32] In presence of AA, MGO is one of the products of the degradation of 3,5-diketogulonic acid (issued from the isomerization of 2,3-diketogulonic acid), tartronic acid and glyceric aldehyde.[14] In particular, as demonstrated by Larisch and co-workers and by Schultz and co-workers two pathways may occur. Under anaerobic conditions, decarboxylation of AA led to 3-desoxy-L-xylosone which gave after retro-aldol-cleavage MGO. In the presence of oxygen dehydroascorbic acid is formed as primary degradation product. After hydrolysis of the lactone and decarboxylation xylosone underwent retro-aldol-cleavage to give glyceraldehyde which eliminated water and formed MGO.[33, 34] Also in this case, the capsules exerted a protective effect with

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significative reductions during the thermal treatment. The formation of CEL from AA should be further investigated since up to now only the mechanism via MGO has been shown.[32] Specifically, several α-dicarbonyl structures arising from the degradation of AA can modify lysine side chains, thus leading to the formation of CEL and other MRPs.[28, 35]

In order to confirm the results on bound MRPs, the formation of free amide-AGEs was investigated upon the reaction between AA and lysine along with protein bound CML, CEL and furosine, an aqueous model system, consisting of lysine and AA. AA and lysine were incubated at 140° C for 2, 4, 6 and 8 min in order to simulate the same thermal loading used for milk and verify the formation of derived amide-AGEs via HRMS targeted screening of the chemical structures reported in **Figure 1** by using a database developed in Exact Finder environment. The extracted ion chromatograms shown in **Figure 4** and the trends shown in **Figure 5** reveled that five amide-AGEs can be formed in the presence of free lysine and AA: glycerinyl-lysine; oxalyl-lysine; xylonyl-lysine, threonyl-lysine e lyxonyl-lysine. Once these compounds were detected in aqueous solution, they were investigated also in EAA milk and FAA milk by using the database previously developed.

The area counts of oxalyl-lysine was higher in FAA milk than EAA milk; reaching a maximum at 6 min of UHT treatment and then rapidly decreased. In EAA milk, there was a slight increase in oxalyl-lysine after 2 min of treatment then it remained constant until 8 min. Interestingly, there were similar values for glycerinyl-lysine in FAA milk and EAA milk at time 0. The signals of glycerinyl-lysine increased in FAA at 4 min of UHT treatment and subsequently decreased rapidly thereafter, indeed at the end of the thermal treatment the signal for glycerinyl-lysine was higher in FAA milk than EAA milk. Threonyl-lysine in FAA milk and EAA milk until 6 min for both samples. This continued to increase only in FAA milk at 8 min of UHT treatment while levels decreased in EAA milk, possibly suggesting degradation in the presence of lipid capsules. The two isomers xylonyl-lysine and lyxonyl-lysine were separated by the chromatographic method. Xylonyl-lysine was the only amide-AGE found to be higher in EAA milk than FAA milk at time 0; in both model systems and the signal slightly decreased around 4 and 6 min then it increased over the thermal treatment in particular in FAA milk. Lyxonyl-lysine showed the lowest signal and exhibited a similar profile in EAA and FAA. The presence of this amide-AGE was negligible prior to thermal treatment and increased in both EAA and in FAA milk.

Several routes may lead to the reduction of available lysine in the presence of AA in the milk model system. On one hand, the mechanisms described previously suggest that AA and its degradation products may react with the ε-amino group of lysine leading to the formation of CML and CEL and may promote the formation of Amadori compounds. On the other hand, even if free amino acids

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and free lysine are present at lower concentration than total lysine or proteins, it is possible to hypothesize a reaction network between AA and lysine leading to the formation of a plethora of products, such as amide-AGEs, carbonyl compounds, α-dicarbonyls, carboxylic acids and volatiles. Smuda and Glomb suggested the formation of the hydrate form of 2,3-diketogulonic acid as the central hub for the formation of amide-AGEs. The first steps include the nucleophilic attack of a hydroxyl anion followed by a decarboxylation. A second nucleophilic attack by the ε-amino group of lysine promotes the formation of hemiaminal whose decarboxylation lead to xylonyl-lysine and lyxonyl-lysine formation. Conversely, the amine induced α cleavage favors the formation of threonyl-lysine from 2,3-diketogulonic acid, while glyceryl-amide and oxalyl-amide can be formed from 2,3-xylodiulose and 2,4-diketogulonic acid/2,3-diketogulonic acid, respectively.[14] For the first time, amide-AGEs were tentatively identified in milk and also the formation of carboxylic amides, such as formyl-lysine, lactoyl-lysine and acetyl-lysine can be hypothesized even if these last compounds mainly arise from sugars. However, AA could be indirectly linked to the formation of carboxylic acid amide: the alteration of the redox potential during the conversion into DHAA can promote the formation of 1-deoxyhexo-2,3-diulose and its isomers the key intermediates for the formation of formyl-lysine, lactoyl-lysine and acetyl-lysine upon β-cleavage.[36] In this respect it can be assumed that the presence of cations promoted the degradation of free AA and Amadori compounds and the consequent amine induce β-cleavage, α fragmentation and decarboxylation.[37]

Conclusions

The use of encapsulated AA successfully reduced the formation of MRPs in fortified milk and promoted the increase in total lysine. By optimizing the ingredient formulation and processing methods, it was possible to finely disperse the capsules in milk and then to test their stability during the thermal treatment. The capsules worked as sequestrating agents by removing AA from the reaction mixture, and preventing oxidation of AA, thus leading to the control of Amadori products, CML and CEL formation. As revealed by HRMS it is possible also to block the formation of amide-AGEs, even if their chemical behavior in milk systems at high temperatures warrants further investigation. Moreover, the interplay between the formation of Amadori compounds and the presence of AA/DHAA in complex environments, such as milk, should be carefully compared to the formation of other reactive intermediates and end products in order to improve the effectiveness of encapsulation as a potential mitigation strategy of MRPs.

The authors declare no conflict of interest.

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DOI: 10.1039/C6FO00151C

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DOI: 10.1039/C6FO00151C

Table 1: Ingredients used in the milk formula. In bold the quantity of AA, encapsulated AA and coating in the four recipes. Milk with encapsulated AA (EAA), milk with free AA (FAA), milk with empty capsules, palmitic acid blend (COA), control milk (CTL).

Ingredient 100 g of milk	FAA	EAA	COA	CTL
Milk skimmed powder	1.2	1.2	1.2	1.2
Whey powder	2.5	2.5	2.5	2.5
Lactose monohydrate	5	5	5	5
Oil blend	3.3	3.3	3.3	3.3
Sucrose esters	0.1	0.1	0.1	0.1
Ascorbic acid	**0.04**	/	/	/
Coating	/	/	**0.04**	/
Encapsulated ascorbic acid	/	**0.08**	/	/
Mineral blend	0.04	0.04	0.04	0.04
Calcium/Potassium citrate	0.15	0.15	0.15	0.15
Potassium phosphate	0.02	0.02	0.02	0.02
Citric acid	0.02	0.02	0.02	0.02
Magnesium/Potassium chloride	0.02	0.02	0.02	0.02
Water	87.6	87.6	87.6	87.6

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491 **Table 2:** Analytical performances of the LC-HRMS method for the identification of AA and
492 DHAA. The mass accuracy was calculated dividing the mass error (i.e.: the difference
493 between the theoretical mass and the experimental mass) by the theoretical mass. The results
494 were reported in ppm by multiplying by 10^6. LOQ (limit of quantitation), LOD (limit of
495 detection), r^2 (coefficient of determination).

Compound	AA	**DHAA**
Molecular ion	$[M-H]^-$	$[M-H]^-$
Exact mass *(m/z)*	175.02481	173.00916
Mass accuracy (ppm)	1.3	2.0
LOD (ppb)	1	1
LOQ (ppb)	10	10
r^2	>0.991	>0.992
RSD %	9	6
Recovery	93%	95%

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Table 3: Concentration of lysine (g/100 g of protein), furosine, CML and CEL (mg/100 g of protein) in the different recipes during the UHT thermal treatment. Separate tests were performed for the four markers and different letters correspond to significative differences (Tukey test, $\alpha = 0.05$). The test was performed within each marker throughout the thermal treatment.

time (min)	EAA		CTL		FAA		COA	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Lysine	**g/100 g**							
t0	8.7	0.4^{A}	8.7	0.4^{A}	8.7	0.4^{A}	8.7	0.4^{A}
t2	7.3	0.1^{B}	6.8	$0.5^{B,C}$	5.9	$0.3^{C,D,E}$	6.4	$0.6^{B,C,D}$
t4	7.0	0.2^{B}	5.7	$0.2^{D,E,F}$	5.4	$0.3^{D,E,F,G}$	4.8	$0.4^{F,G,H}$
t6	5.8	$0.9^{C,D,E,F}$	5.3	$0.3^{E,F,G}$	4.5	$0.2^{G,H}$	5.1	$0.6^{E,F,G,H}$
t8	5.2	$0.3^{E,F,G,H}$	4.4	$0.4^{G,H}$	4.2	0.1^{H}	4.5	$0.4^{G,H}$
Furosine	**mg/100 g**							
t0	227.2	11.6^{H}	227.5	11.3^{H}	226.9	11.8^{H}	227.2	11.6^{H}
t2	237.5	11.7^{H}	313.3	28.8^{F}	297.2	$8.8^{F,G}$	301.1	$12.8^{F,G}$
t4	259.4	$12.3^{G,H}$	362.2	$24.4^{D,E}$	334.7	$38.7^{E,F}$	305.6	10.5^{F}
t6	429.7	$10.0^{B,C}$	465.8	22.0^{D}	446.9	10.2^{B}	520.6	5.0^{A}
t8	386.7	14.9^{D}	380.6	8.1^{D}	388.9	$5.3^{C,D}$	450.6	11.6^{B}
CML	**mg/100 g**							
t0	3.8	0.4^{G}	3.8	0.4^{G}	3.8	0.4^{G}	3.8	0.4^{G}
t2	6.2	0.8^{F}	7.2	$0.4^{E,F}$	6.8	0.6^{F}	8.7	$0.6^{D,E}$
t4	8.9	0.1^{D}	12.9	0.4^{C}	15.2	0.7^{B}	19.9	0.8^{A}
t6	10.3	0.4^{D}	15.9	1.0^{B}	16.3	1.1^{B}	19.2	0.9^{A}
t8	12.4	0.5^{C}	19.0	0.7^{A}	19.4	1.0^{A}	15.2	0.6^{C}
CEL	**mg/100 g**							
t0	1.2	0.2^{I}	1.2	0.2^{I}	1.2	0.2^{I}	1.2	0.2^{I}
t2	1.4	0.2^{I}	2.1	0.2^{H}	2.9	0.1^{G}	2.9	0.2^{G}
t4	1.9	$0.1^{H,I}$	4.5	$0.2^{D,E}$	2.9	0.2^{G}	3.7	0.2^{F}
t6	3.5	$0.2^{F,G}$	5.0	$0.4^{C,D}$	7.2	0.4^{A}	5.6	$0.5^{B,C}$
t8	4.2	$0.4^{E,F}$	6.0	0.4^{B}	7.6	0.2^{A}	5.6	$0.4^{B,C}$

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Figure captions

Fig.1: Reaction mechanisms between AA and lysine according to, Larisch and coworkers [33], Dunn and coworkers[15] and Smuda & Glomb [14].

Fig.2: Profile of AA (A) and DHAA (B) during the thermal treatment. EAA: milk with encapsulated AA, FAA: milk with free AA.

Fig.3: Evolution of furosine, CML, CEL and total lysine over the thermal treatment. The results are in mg/100 g of protein, while for lysine they are in g/100 g of protein. EAA: milk with encapsulated AA, FAA: milk with free AA, COA: milk with empty capsules, CTL: milk without free AA, encapsulated or empty capsules.

Fig.4: Extracted ion chromatogram of oxalyl-lysine (exact mass $[M+H]^+$: 219.09755), glycerinyl-lysine (exact mass $[M+H]^+$: 235.12994), threonyl-lysine (exact mass $[M+H]^+$: 265.13941), xylonyl-lysine (exact mass $[M+H]^+$: 295.14998) and lyxonyl-lysine (exact mass $[M+H]^+$: 295.14998) from the top to the bottom. Mass tolerance, 10 ppm, identification via isotopic pattern and retention time. The red line represents the center of the peak, while the gray hill is the Gaussian smoothed profile defined by the peak score (black solid line).

Fig.5: Evolution of oxalyl-lysine (exact mass $[M+H]^+$: 219.09755), glycerinyl-lysine (exact mass $[M+H]^+$: 235.12994), threonyl-lysine (exact mass $[M+H]^+$: 265.13941), xylonyl-lysine (exact mass $[M+H]^+$: 295.14998) and lyxonyl-lysine (exact mass $[M+H]^+$: 295.14998) in EAA milk (with encapsulated AA) and FAA milk (with free AA). The values are reported as area counts.

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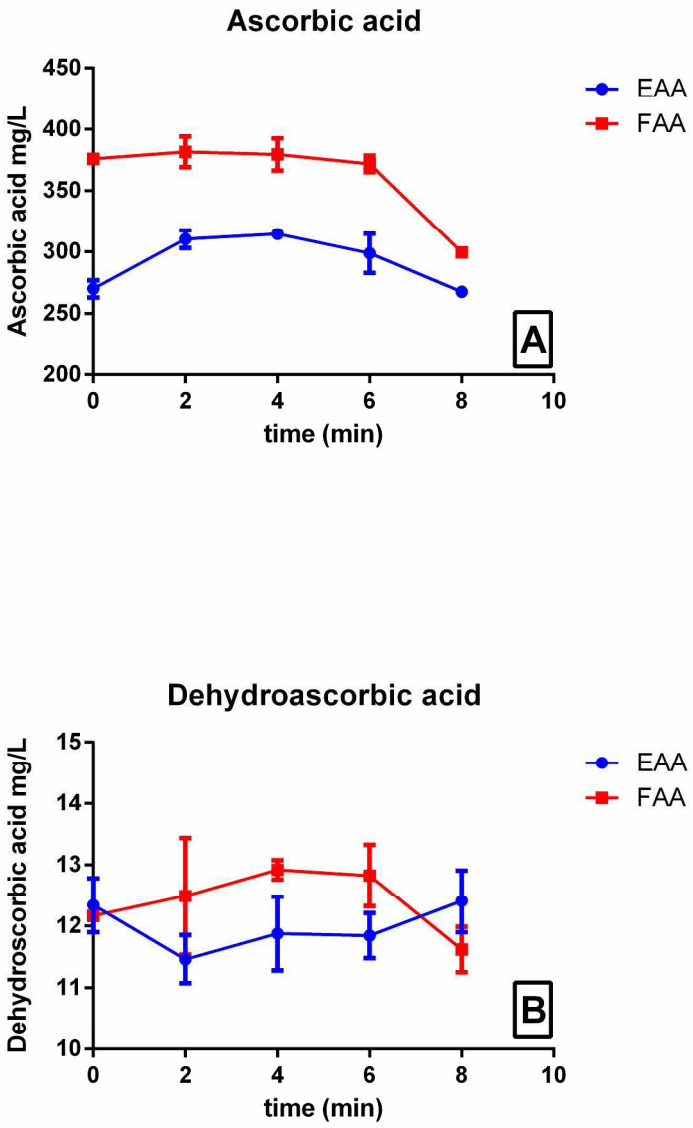

Fig.2: Profile of AA (A) and DHAA (B) during the thermal treatment. EAA: milk with encapsulated AA, FAA: milk with free AA.
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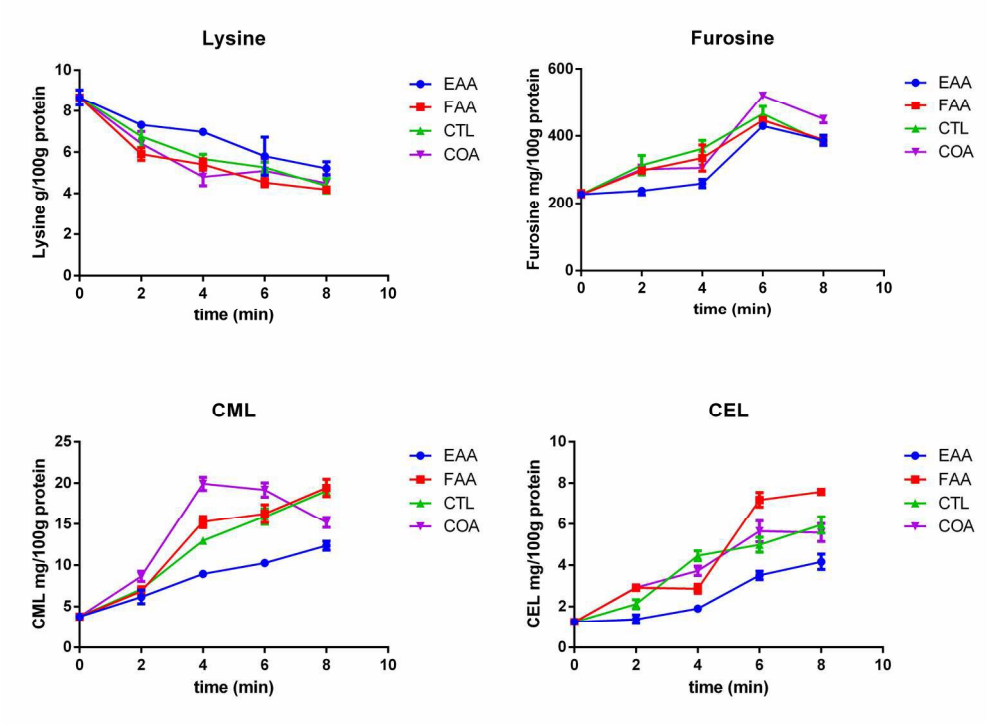

Fig.3: Evolution of furosine, CML, CEL and total lysine over the thermal treatment. The results are in mg/100 g of protein, while for lysine they are in g/100 g of protein. EAA: milk with encapsulated AA, FAA: milk with free AA, COA: milk with empty capsules, CTL: milk without free AA, encapsulated or empty capsules.
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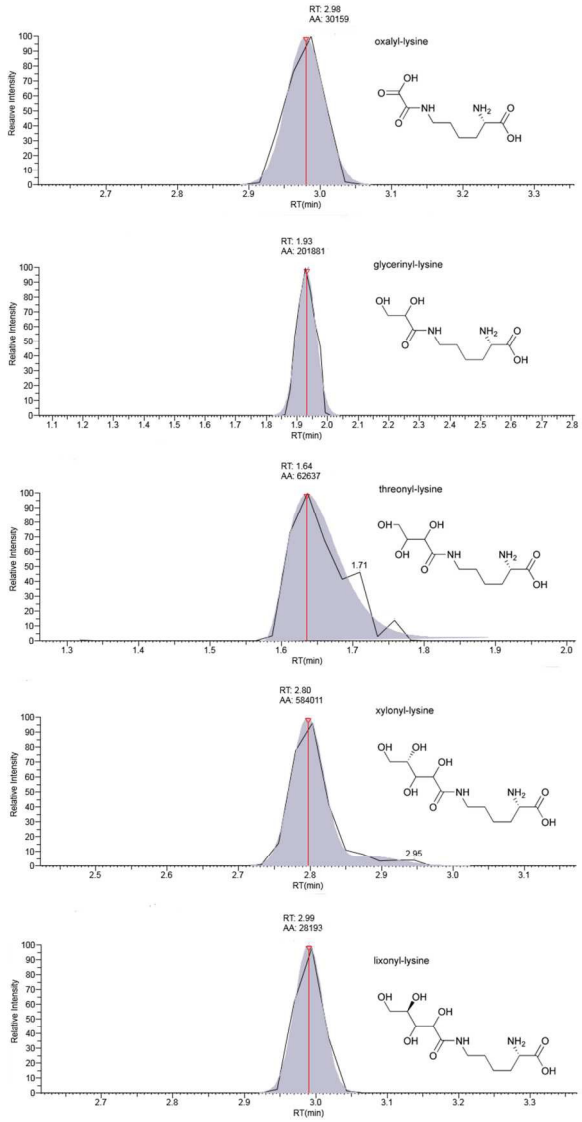

Fig.4: Extracted ion chromatogram of oxalyl-lysine (exact mass [M+H]+: 219.09755), glycerinyl-lysine (exact mass [M+H]+: 235.12994), threonyl-lysine (exact mass [M+H]+: 265.13941), xylonyl-lysine (exact mass [M+H]+: 295.14998) and lyxonyl-lysine (exact mass [M+H]+: 295.14998) from the top to the bottom. Mass tolerance, 10 ppm, identification via isotopic pattern and retention time. The red line represents the center of the peak, while the gray hill is the Gaussian smoothed profile defined by the peak score (black solid line).
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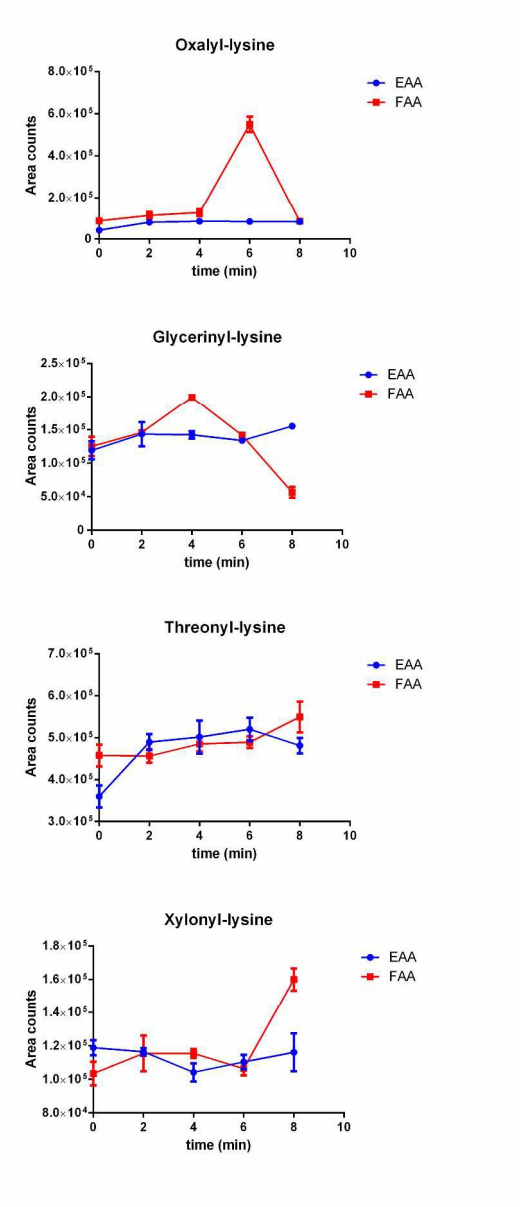

Fig.5: Evolution of oxalyl-lysine (exact mass [M+H]+: 219.09755), glycerinyl-lysine (exact mass [M+H]+: 235.12994), threonyl-lysine (exact mass [M+H]+: 265.13941), xylonyl-lysine (exact mass [M+H]+: 295.14998) and lyxonyl-lysine (exact mass [M+H]+: 295.14998) in EAA milk (with encapsulated AA) and FAA milk (with free AA). The values are reported as area counts.
211x490mm (300 x 300 DPI)

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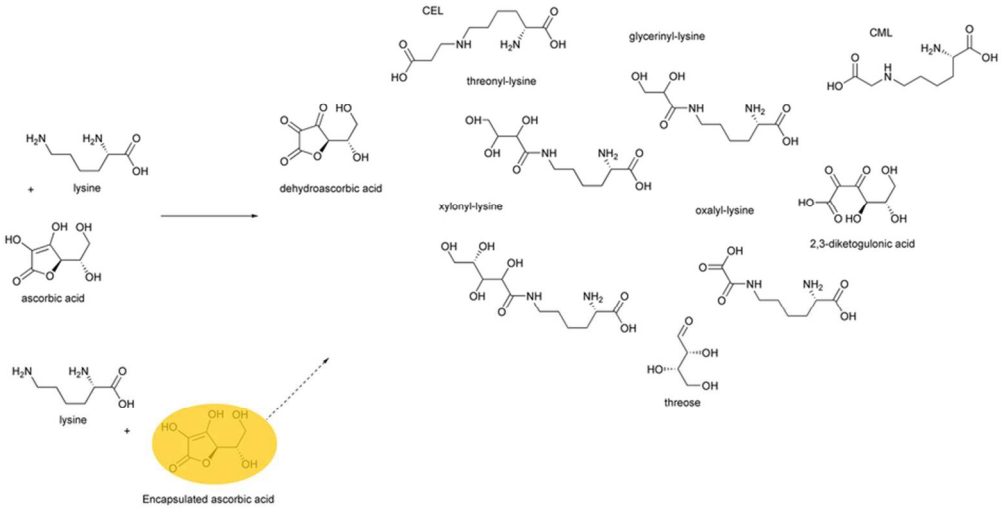

40x20mm (600 x 600 DPI)