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ORIGINAL ARTICLE

Simultaneous quantification of amino acids and Amadori products in foods through ion-pairing liquid chromatography–high-resolution mass spectrometry

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Abstract The formation of the Amadori products (APs) is the first key step of Maillard reaction. Only few papers have dealt with simultaneous quantitation of amino acids and corresponding APs (1-amino-1-deoxy-2-ketose). Chromatographic separation of APs is affected by several drawbacks mainly related to their poor retention in conventional reversed phase separation. In this paper, a method for the simultaneous quantification of amino acids and their respective APs was developed combining high-resolution mass spectrometry with ion-pairing liquid chromatography. The limit of detection was 0.1 ng/mL for tryptophan, valine and arginine, while the limit of quantification ranged from 2 to 5 ng/mL according to the specific sensitivity of each analyte. The relative standard deviation % was lower than 10 % and the coefficient of correlation was higher than 0.99 for each calibration curve. The method was applied to milk, milk-based products, raw and processed tomato. Among the analyzed products, the most abundant amino acid was glutamic acid (16,646.89 ± 1,385.40 μg/g) and the most abundant AP was fructosyl-arginine in tomato puree (774.82 ± 10.01 μg/g). The easiness of sample preparation coupled to the analytical performances of the proposed method introduced the possibility to use the pattern of free amino acids and corresponding APs in the evaluation of the quality of raw food as well as the extent of thermal treatments in different food products.

Keywords Maillard reaction · Amino acids · Amadori products · High-resolution mass spectrometry

Abbreviations

MR	Maillard reaction
APs	Amadori products
HRMS	High-resolution mass spectrometry
NFPA	Perfluoropentanoic acid

Introduction

The central hub of the Maillard Reaction (MR) is represented by the reaction between reducing sugars, such as D-glucose, and amino acids and proteins: this step is the basement from which the Maillard cascade starts off (Hodge 1953). Since the first articles by Nordin and Amadori highlighted the reaction between carbonyls and amines (Nordin and Kim 1958; Amadori 1929), many papers have dealt with this complex reaction to evaluate the key steps of the beginning of the MR. The initial phase is characterized by the formation of a cyclized Schiff base, a glycosylamine that via immonium ion can generate alternatively the α and β anomers. The acidic condition promotes the rearrangement of the N-glycosides where the isomerization reaction leads to the synthesis of the more stable 1-amino-1-deoxy-2-ketose; this process is known as Amadori

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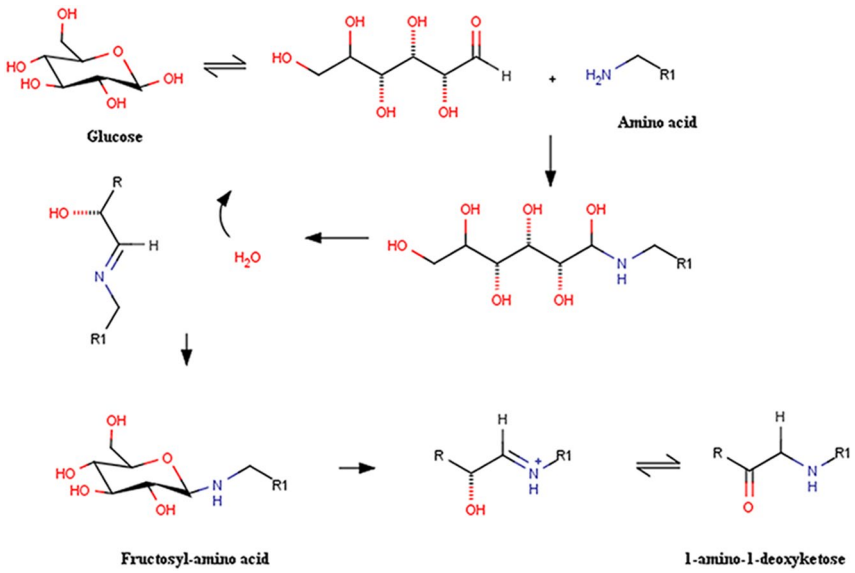


Fig. 1 Amadori product formation, adapted from Yaylayan and Huyghuesdespointes (1994)

rearrangement if the reducing sugar is an aldose or Heyns rearrangement if the reducing sugar is a ketose. The final result of this reaction is the formation of the Amadori rearrangement products (APs) or Heyns rearrangement products (HPs, 1-amino-2-deoxy-1-aldose) as it is shown in Fig. 1 (Yaylayan and Huyghuesdespointes 1994; Mossine and Mawhinney 2010). The extent of APs' and HPs' concentration depends on the chemical reaction leading to their formation which includes 1,2- and 2,3-enolisation, β-elimination, the migration of the carbonyl group and retroaldol reactions (Molero-Vilchez 1997). The formation of APs was mainly investigated under the mild conditions typical of long-term stored food, the high temperature/short time of the UHT treatment and also under the physiological condition of living organisms (Singh et al. 2001; Ahmed 2005; van Boekel 2006; Wnorowski and Yaylayan 2000; Baisier and Labuza 1992; Rao et al. 2012).

A complex deal for food chemistry is the simultaneous detection of free amino acids and their respective APs since it faces many difficulties such as the high polarity, the chemical and chromatographic behavior, the derivatization procedures required for their detection (Yaylayan and Huyghuesdespointes 1994; Srinivas and Harohally 2012; Wrodnigg and Eder 2001; Horvat and Jakas 2004). A number of papers reported the detection and quantification of free amino acids in foods and biological fluids (Kirschner and Green 2009; Kaspar et al. 2009). Focusing only on HPLC-based procedures several fully validated methods involve many different techniques: post-column and pre-column derivatization with UV or fluorescence detector (Bosch et al. 2006; GonzalezCastro et al. 1997; Arnold et al. 1994; Petritis et al. 2002), mass spectrometry with single or triple quadrupole (Yang et al. 2006; Langrock et al. 2006; Ozcan and Senyuva 2006; Petritis et al. 2000), high-resolution mass spectrometry with time of flight (TOF), Fourier-transform ion cyclotron resonance (FTICR) or high-resolution mass spectrometry (HRMS) analyzer (Gokmen et al. 2012; Han et al. 2008; Dunn et al. 2005). This last technique offers some advantages overcoming the most common problems related to the constraints mentioned above. The full spectra acquisition mode and tandem mass spectrometry allow a simplified extraction and a short chromatographic approach while the high resolution enables unambiguous identification of amino acids matching the theoretical *m/z* with experimental *m/z* up to fifth decimal digits (Dunn et al. 2005).

HRMS-based methods can also be applied to the quantification of APs which is still one of the most desired goals of those investigating the MR. The chemical features along with the lack of commercial source of APs' standards represent the first disadvantages for the building of a fully validated method. Moreover, the chemical or enzymatic hydrolysis necessary to quantify the APs bound to the proteins introduce high uncertainty and variability. Some of the strategies used for quantification of APs, mainly of Nε-(1-deoxy-D-fructos-1-yl)-L-lysine or glycated proteins, are summarized in Table 1.

Traditionally, the APs were indirectly quantified through furosine analysis (Finot et al. 1968; Henle et al. 1995), even if the acidic hydrolysis, the MR extent and the similarity between lactosylamine and Nε-(1-Deoxy-D-fructos-1-yl)-L-lysine negatively influence the conversion of furosine into APs (Pischetsrieder and Henle 2012). Several strategies have been evaluated for the detection of APs in food including a large use of mass spectrometry detection (Staempfli et al. 1994; Davidek et al. 2003, 2005; Meltretter et al. 2009;

Table 1 Overview of Amadori products' detection and some reference methods previously proposed

Marker	Technique	Detection	Reference
β-Lactoglobulin	Neutral loss scan	LC/ESI-MS/MS	Morgan et al. (1998)
Glycated tetrapeptide, Fru-Lys	Enzymatic hydrolysis and LC	LC/MS	Vinale et al. (1999)
Fru-Gly	GC and Pyrolysis/GC	GC/MS	Wnorowski and Yaylayan (2000)
Fru-Glu, Fru-Pro	High-performance ion exchange chromatography	ESI-MS/MS and ESI-MS	Davidek et al. (2005)
Fructosamino-, ribulosamino, and glucosamino- modified peptides	Positive ion mode	nanoESI-QqTOF- and MALDI-TOF/TOF-MS	Frolov et al. (2006)
Fru-His and Fru-Arg	Gas/Liquid Chromatography; Trimethylsilyl Derivatives	GLC-MS/MS	Mossine and Mawhinney (2007)
Glycated HSA	Neutral loss scan	RP-LC/ESI/MS/MS Q-ToF	Gadgil et al. (2007)
Fru-Lys	SPE/ion-pairing liquid chromatography	LC/ESI-MS/MS	Hegele et al. (2008)
Lactulosyl-L-lysine	Positive ion mode	MALDI-TOF-MS	Meltretter et al. (2009)
Fru-Gly; Fru-Asn	LC-ESI-MS/MS	MS/MS; MRM mode	Liu et al. (2013)
Fru-Phe, Fru-Met, Fru-His, Fru-Ile, Fru-Leu, Fru-Val, Fru-Tyr	Stable isotope dilution assay; positive ion mode	LC/MS/MS	Meitinger et al. (2014)

Liu et al. 2013) and HPLC diode array detection via formation of furoylmethyl derivatives (del Castillo et al. 1999; Sanz et al. 2001). The analysis of the typical fragmentation pathway and the neutral loss scan of APs by tandem mass spectrometry along with gas or liquid chromatography are the key procedures which have been extensively applied (Mossine and Mawhinney 2010; Silván et al. 2006; Meitinger et al. 2014; Gadgil et al. 2007).

In this paper, we used HRMS combined with ion-pairing liquid chromatography to develop a method for the quantification of free amino acids and corresponding APs using a fast sample preparation and without any derivatization. The simplified approach with a linear chromatographic gradient and the full spectra scanning and acquisition mode allowed a rapid and sensitive analysis which was applied on different foods.

Materials and methods

Chemicals

Acetonitrile and water for LC/HRMS analysis along with methanol and acetic acid for fructosyl-L-amino acids synthesis were obtained from Merck (Darmstadt, Germany). The ion-pairing agent perfluoropentanoic acid (NFPA), all the standard amino acids, D-glucose, sodium pyrosulfite, dimethylformamide, morpholine, sodium hydroxide and Nα-Fmoc-protected lysine were purchased from Sigma-Aldrich (Saint-Louis, MO). The calibration solution (see "liquid chromatography/high resolution mass spectrometry" section) was obtained from Thermo Fisher Scientific (Bremen, Germany).

Fructosyl-L-amino acids (Amadori Products, APs) synthesis

Fructosyl-L-amino acids (Nε-(1-Deoxy-D-fructos-1-yl)-L-amino acids) were prepared refluxing in methanol a mixture of amino acid and an excess of anhydrous D-glucose following the course of the reaction by TLC accordingly with the synthetic protocols previously described in the literature for the various derivatives. Specifically, fructosyl-L-histidine and fructosyl-L-asparagine were synthesized according to the procedure of Mossine and Mawhinney (Mossine and Mawhinney 2007), using a 3.3:1 glucose/amino acid molar ratio in the presence of sodium pyrosulfite and acetic acid, whereas fructosyl-L-phenylalanine was obtained as described by Glinsky et al. using a 4:1 glucose/phenylalanine ratio (Glinsky et al. 1996). The preparation of fructosyl-L-aspartic acid was performed in analogy to the synthetic route presented by Abrams et al. by refluxing the mixture of L-aspartic acid and D-glucose in the presence of one mole of sodium hydroxide per mole of amino acid (Abrams et al. 1955). In case of fructosyl-L-lysine, the synthesis of which was accomplished by the two-step procedure described by Vinale et al. (1999) starting from a Nα-Fmoc-protected derivative, a further step for the removal of the Fmoc moiety was performed by treating the Nα-Fmoc-protected fructosyl-L-lysine with morpholine (8 equivalents) in a 9:1 dimethylformamide/methanol mixture (Vinale et al. 1999). The results of the structural analysis are in good agreement with previously reported criteria of purity and structural confirmation for all the fructosyl-amino acids. Each amino acid was named using the three letter code while the respective Nε-(1-Deoxy-D-fructos-1-yl)-L-amino acids (APs) were

identified using the three letter code along with the notation "Fru-".

Food samples

Amino acids and APs were monitored in different food products: UHT milk, low lactose milk, milk-based caramel powder and raw tomatoes (three varieties: "Locale Chieti", "Locale Lazio" and "Stella Pisa", named T1, T2, T3, respectively). Furthermore, the method was validated in three different canned tomato samples: Cherry tomatoes (CT), yellow Cherry tomatoes (variety: "Datterini", YDT), and in a tomato paste (TP). All the samples were purchased in a local market and a simplified sample preparation procedure was followed. Tomato samples were ground in a knife mill Grindomix 200 (Retsch, Haan, Germany) and 100 mg was mixed with 0.3 mL of deionized water; 0.1 mL of UHT milk and low lactose milk were diluted three times in water, while 100 mg of milk-based caramel powder was mixed with 0.9 mL of water. Samples were centrifuged (14,800 rpm, 20 min, 4 °C), and then the supernatants were filtered using regenerated cellulose filters (RC 0.45 µm, Phenomenex, Torrance, CA) and analyzed.

Liquid chromatography–high-resolution mass spectrometry (LC/HRMS)

For the chromatographic separation of amino acids and their respective APs, the mobile phases consisted of 5 mM perfluoropentanoic acid (NFPA) in water (solvent A) and 5 mM NFPA in acetonitrile (solvent B). The following linear gradient of solvent B (min/%B): (0/2), (2/2), (5/50), (7/50), (9/2), (12/2), (15/2) was used. The flow rate was set to 200 µL/min and the injection volume was 5 µL. Chromatographic separation of amino acids and APs was achieved through a thermostated (30 °C) Kinetex 2.6 µm (100 × 2.1 mm) core-shell C-18 column (Phenomenex, Torrance, CA). The Accela 1250 UPLC system (Thermo Fisher Scientific, Bremen, Germany) was directly interfaced to an Exactive Orbitrap high-resolution mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) and analytes were detected through a heated electrospray interface (HESI) operating in the positive mode and scanning the ions in the *m/z* range of 60–500. The resolving power was set to 50,000 full width at half maximum (FWHM, *m/z* 200) resulting in a scan time of 1 s. The automatic gain control was used in balanced mode (1×10^6 ions); maximum injection time was 50 ms. The interface parameters were as follows: spray voltage 3.8 kV, capillary voltage 10 V, skimmer voltage 15 V, capillary temperature 275 °C, heater temperature 200 °C, sheath gas flow 30 and auxiliary gas flow 3 arbitrary units. Before intraday analysis the instrument was externally calibrated by infusion of a solution that consisted of caffeine, Met-Arg-Phe-Ala (MRFA), Ultramark 1,621, and acetic acid in a mixture of acetonitrile/methanol/water (2:1:1, v/v/v). The exact mass of diisooctyl phthalate ($[M + H]^+$: 391.28429) was used as lock mass for the recalibration of the instrument during the analysis.

Method performances

A stock solution of each amino acid listed in Table 2 was prepared dissolving 10 mg of each standard in 1 mL of a mixture of water/acetonitrile/acetic acid (90.00/9.99/0.01). Each solution was diluted and stored at −20 °C until usage. Before starting the analysis two different mixtures one with amino acids and another with APs were prepared and HRMS method was tuned by infusion of these two mixtures first directly in the ion source, then in the chromatographic stream. Two sets of calibration curves for the 20 amino acids and for the 5 APs were built in the range 5–5,000 ng/mL according to the limit of detection (LOD) and the limit of quantitation (LOQ) (Armbruster and Pry 2008). Three replicates of 0.5 ng/mL solutions were injected into the LC/HRMS system to verify the lowest concentration for which the signal-to-noise ratio was higher than three. The r^2 value was calculated plotting the area counts against the injected concentration and it was always higher than 0.99 in the abovementioned range. Each point of the calibration curves was injected three times in the same day (intraday assay for the repeatability) and three times in three different days (interday assay for the reproducibility): the accuracy was reported as the discrepancies between six calibration curves each of them performed intraday and interday. The slope (or the sensitivity of the method) among the six curves was performed and compared to each point of each calibration curve. The results were expressed as relative standard deviation (RSD) (%). The concentration of non-synthesized APs was calculated according to the chemical class of each amino acid as listed in Table 3: the calibration curve of Fru-Lys was used for Fru-Arg and Fru-Pro; Fru-Phe was used for all hydrophobic and aromatic APs, Fru-Gln was calculated by Fru-Asn calibration curve and Fru-Glu by Fru-Asp; Fru-Cys, Fru-Met, Fru-Thr and Fru-Ser and Fru-Gly were calculated using the calibration curve of Fru-Asn. The recovery test was performed spiking a mixture of tomato juice with a known amount of amino acids and APs (final concentration 1 µg/mL) and taking into account the overestimation due to amino acids and APs already present in the sample. The recovery was calculated plotting the area of the current associated with each *m/z* of each amino acid and Amadori products in the tomato matrix (with and without the standard)

Table 2 High-resolution mass spectrometry (HRMS) performances of amino acids and Amadori products

Type	Amino acids	Rt (min)	Exact mass $[M + H]^+$	Experimental mass $[M + H]^+$	Error (ppm)	Amadori products	Rt (min)	Exact mass $[M + H]^+$	Experimental mass $[M + H]^+$	Error (ppm)
Aliphatic	Ala	2.41	90.05496	90.05529	−3.66	Fru-Ala	1.65	252.10778	252.1077	0.33
	Val	5.31	118.08626	118.08645	−1.61	Fru-Val	2.79	280.13908	280.1389	0.65
	Leu	6.47	132.10191	132.1019	0.08	Fru-Leu	5.35	294.15473	294.15469	0.15
	Ile	6.35	132.10191	132.1019	0.08	Fru-Ile	5.35	294.15473	294.15469	0.15
Aromatic	Phe	6.65	166.08626	166.08641	−0.90	Fru-Phe	6.12	328.13908	328.13876	0.99
	Trp	6.90	205.09715	205.09738	−1.12	Fru-Trp	6.79	367.14997	367.14924	2.00
	Tyr	5.80	182.08117	182.08125	−0.44	Fru-Tyr	2.92	344.13399	344.1338	0.56
Polar	Cys	2.00	122.02703	122.02751	−3.93	Fru-Cys	1.48	284.07985	284.07955	1.07
Neutral	Met	5.54	150.05833	150.0584	−0.47	Fru-Met	2.96	312.11115	312.11219	−3.32
	Ser	1.89	106.04987	106.05009	−2.07	Fru-Ser	1.45	268.10269	268.10344	−2.79
	Thr	2.06	120.06552	120.06564	−1.00	Fru-Thr	1.46	282.11834	282.11838	−0.13
	Asn	1.83	133.06077	133.06091	−1.05	Fru-Asn	1.48	295.11359	295.11350	0.32
	Gln	1.92	147.07642	147.07651	−0.61	Fru-Gln	2.78	309.12924	309.12969	−1.45
Acidic	Asp	1.77	134.04478	134.04491	−0.97	Fru-Asp	1.37	296.09760	296.09761	−0.02
	Glu	2.15	148.06043	148.06051	−0.54	Fru-Glu	1.69	310.11325	310.11377	−1.67
Basic	Arg	6.59	175.11895	175.11879	0.91	Fru-Arg	5.43	337.17177	337.17172	0.16
	His	6.08	156.07675	156.07686	−0.70	Fru-His	4.80	318.12957	318.12973	−0.49
	Lys	6.41	147.11280	147.11266	0.95	Fru-Lys	5.35	309.16562	309.16562	0.01
Unique	Pro	2.11	116.07061	116.07086	−2.15	Fru-Pro	1.54	278.12343	278.12341	0.08
	Gly	2.09	76.03930	76.03964	−4.47	Fru-Gly	1.57	238.09212	238.09186	1.11

Rt (retention time); error (ppm) was calculated as the ratio between the difference of the theoretical mass minus the experimental mass and the theoretical mass. This ratio was multiplied per one million to obtain the ppm. All the analytes were detected in positive ionization mode ($[M + H]^+$)

toward the concentration of the standard according to the following formula:

$$R = \left(\frac{C_a}{C_s}\right) \times 100 = \frac{(C_o - C_b)}{C_s} \times 100,$$

where R is the recovery, C_a is the concentration of the spiked analyte in the samples, C_s is the concentration of the spiked solution, C_o is the observed concentration of the spiked matrix and C_b is the basal concentration of each compound without the spiked solution.

The tolerance range for mass accuracy of the amino acids and APs (i.e., the experimental mass of each analyte had to fall within the maximum permitted tolerance) was fixed at 5 ppm (Michalski et al. 2012). Each sample was injected four times and the results were reported as µg/g FW (fresh weight) for tomatoes and µg/mL of samples for milk liquid products according to the matrix. Data were recorded and analyzed using Xcalibur software version 2.1 (Thermo Fisher Scientific, Bremen Germany), while the FancyTyle schemas were built using XLStat 3D-Pro statistical software (Addinsoft, New York, NY).

Results

LC/HRMS analysis

Pure APs used in the present study were obtained following well-established synthetic protocols previously described in the literature for the various compounds. All the abovementioned parameters were checked to ensure the highest signal and the lowest mass error. Two different solutions of standard amino acids and APs were infused first directly into the ion source then into the chromatographic flow to tune the optimal detection conditions and investigate the interferences due to the solvent and the ion-pairing agent. The mass spectrometry conditions were optimized to avoid any in source fragmentation. Taking into account the specificity of each analyte, it was decided to use a low potential, of 15 and 10 V for capillary voltage and skimmer voltage, respectively, and slow flow of auxiliary gas. These conditions avoided the fragmentation of the molecules filling the C-trap (number of ions injected for each scan: 1×10^6) with the parental ions without the formation of daughter ions ensuring the highest abundance of each signal (Davidek et al. 2005). The presence of a recalibrating agent such as diisooctyl phthalate favored

Table 3 Amino acids and Amadori products' analytical performances

Amino acids	LOD (ng/mL)	LOQ (ng/mL)	r^2	RSD (%)	Recovery	APs	LOD (ng/mL)	LOQ (ng/mL)	r^2	RSD (%)	Recovery (%)
Ala	0.5	2	0.990	7.01	105	Fru-Phe	1	5	0.998	7.49	90
Val	0.1	2	0.986	5.51	89	Fru-Asn	0.5	5	0.989	8.30	88
Leu	0.5	2	0.987	4.98	90	Fru-Asp	1	5	0.985	8.31	95
Ile	0.5	2	0.987	4.98	90	Fru-His	2.5	5	0.991	4.32	92
Phe	0.5	2	0.992	7.25	101	Fru-Lys	1	5	0.990	7.31	95
Trp	0.1	2	0.994	5.35	95	[1]Fru-Ala	1	5	0.998	7.49	90
Tyr	0.5	2	0.990	4.21	91	[1]Fru-Val	1	5	0.998	7.49	90
Cys	1.0	5	0.988	7.89	85	[1]Fru-Leu	1	5	0.998	7.49	90
Met	1.0	5	0.991	7.55	101	[1]Fru-Ile	1	5	0.998	7.49	90
Ser	0.5	2	0.989	6.88	98	[1]Fru-Trp	1	5	0.998	7.49	90
Thr	0.5	2	0.993	4.01	92	[1]Fru-Tyr	1	5	0.998	7.49	90
Asn	0.5	2	0.988	5.53	91	[2]Fru-Cys	0.5	5	0.989	8.30	88
Gln	0.5	2	0.986	3.30	92	[2]Fru-Met	0.5	5	0.989	8.30	88
Asp	0.5	2	0.991	8.01	95	[2]Fru-Ser	0.5	5	0.989	8.30	88
Glu	0.5	2	0.997	6.05	95	[2]Fru-Thr	0.5	5	0.989	8.30	88
Arg	0.1	2	0.987	6.09	88	[2]Fru-Gln	0.5	5	0.989	8.30	88
His	0.5	2	0.993	5.55	89	[3]Fru-Glu	1	5	0.985	8.31	95
Lys	0.5	2	0.991	7.21	105	[4]Fru-Arg	1	5	0.990	7.31	95
Pro	1.0	2	0.993	7.01	100	[4]Fru-Pro	1	5	0.990	7.31	95
Gly	0.1	2	0.991	6.55	101	[2]Fru-Gly	0.5	5	0.989	8.30	88

For the non-synthesized APs, the reference APs used for the calibration curve were inserted. 1: quantified using Fru-Phe as reference curve; 2: quantified using Fru-Asn as reference curve; 3: quantified using Fru-Asp as reference curve; 4: quantified using Fru-Lys as reference curve

LOD limit of detection; *LOQ* limit of quantification; r^2 mean of the coefficient of correlation of the six calibration curves performed; *RSD* (%) relative standard deviation among the calibration curves

the reduction of the discrepancies between the theoretical mass and the experimental mass. The mass error was always in the range ±5 ppm; the highest was that of glycine, with a mass error of −4.47 ppm likely due to its low *m/z*. The specificity and the chemical features of each analyte were one of the crucial points of this study: the above described conditions proved to be optimal for the simultaneous detection of amino acids and APs. In Figs. 2 and 3, the typical chromatographic profiles of amino acids and APs were reported. The pair ions with an opposite charge improved the retention of the target molecules and the separation of free amino acids from the APs. The retention pattern followed the typical reversed phase behavior: polar amino acids eluted first, while the hydrophobic and the side chain-charged amino acids eluted later. Interestingly, all APs eluted before the respective amino acids, according to the increase of the polar solubility due to the ketose or aldose residues (Yaylayan and Huyghuesdespointes 1994).

Method development and performances

Method performance was tested against the following quality parameters: mass accuracy, retention time, carryover, linearity of the calibration, coefficient of correlation (r^2), limit of detection (LOD), limit of quantification (LOQ) repeatability, reproducibility and recovery. The results of the analytical performances are reported in Table 3. The first point was to check the absence of any contaminant with the same exact mass of the target compounds by injecting several times a solution of water: acetonitrile 70:30. LOD is the lowest analyte concentration to be reliably distinguished from the noise and at which detection is feasible, while LOQ is the lowest concentration at which the analyte can be reliably detected and defined goals for bias and imprecision are acceptable. In other words, in amino acids detection LOQ should be at least five times LOD (Armbruster and Pry 2008). After ten replicates, the solution 0.1 ng/mL for all the amino acids and the synthesized APs resulted in no signal, while the linearity was achieved in the range 5–5,000 ng/mL and 5–2,000 ng/mL for amino acids and APs, respectively. The reproducibility and repeatability of the method in the optimized conditions were tested by intraday and interday tests; both showed a relative standard deviation lower than 10 % for each analyte. Carryover effects were tested after each calibration curve, monitoring the presence of each analyte in a solution

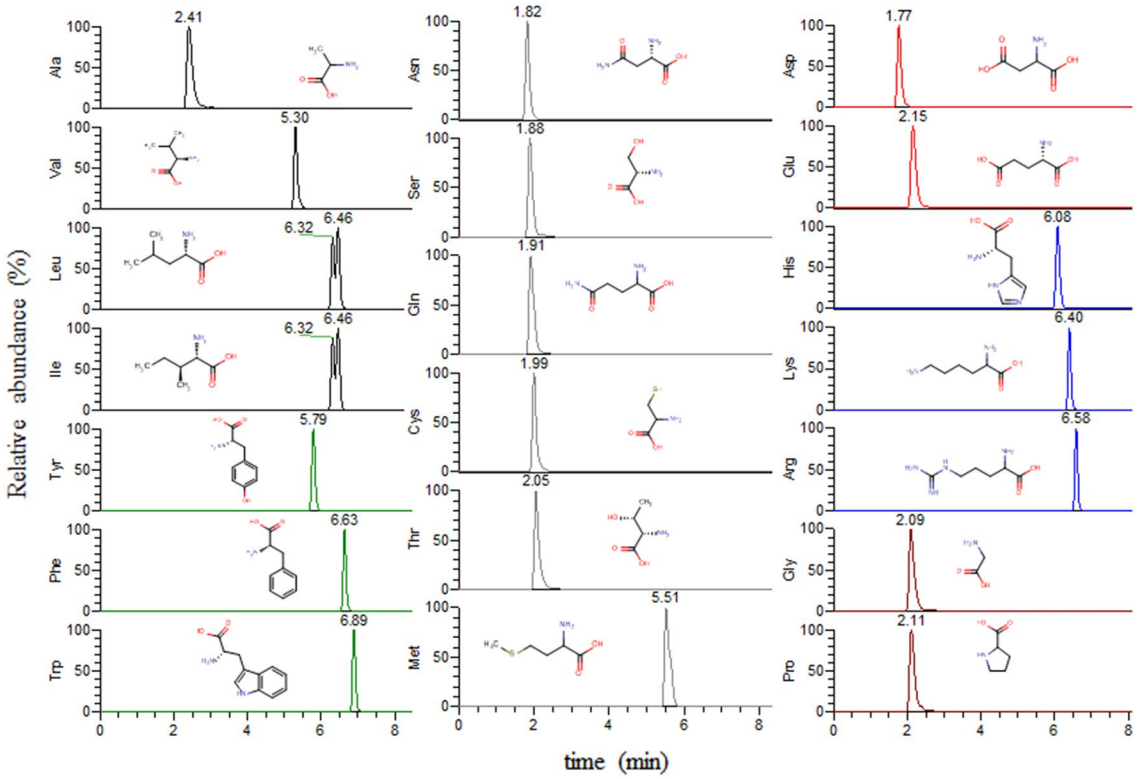


Fig. 2 Chromatographic profile of amino acids, *black* (hydrophobic), *green* (aromatic), *gray* (polar neutral), *red* (acidic), *blue* (basic), *brown* (unique) (color figure online)

of water/acetonitrile 70:30. The recovery test was performed spiking three different raw tomato samples with a known amount of a mixture of APs and amino acids (final concentration 1 µg/mL). The area of each peak of spiked sample was compared to the area count of a not spiked sample without any amino acid addition. Results confirmed the performance of HRMS: the detection of the defined *m/z* ratio turned out to be extremely accurate even if a complex mixture was injected. As a consequence the simplified extraction procedure surpassed the drawbacks due to matrix effect. The lowest recovery value of 85 % was measured for cysteine, where some losses can be ascribed to the high reactivity of thiol group. A recovery of 105 % was found for alanine and lysine, while for the five APs the recovery was 88, 90 and 92 % for fructosyl-asparagine, fructosyl-phenylalanine, fructosyl-histidine, respectively, while for fructosyl-aspartic acid and fructosyl-lysine the recovery was 95 %.

Amino acids and APs' content in food samples

To verify the reliability of the method, content of amino acids and APs was monitored in two liquid matrices (UHT milk and low lactose milk), three semisolid matrices (raw tomato and thermal-treated tomato) and a powdered sample (milk-based caramel product). Furthermore, the method was used to analyze a canned tomato sampling which consisted in "Cherry" tomatoes (CT), "Datterini" (YDT) and tomato paste (TP) and the effect of the thermal impact on tomato samples was illustrated by the FancyTyle approach recently developed (Troise et al. 2014).

Results on amino acids and APs are listed in Tables S1 and S2 of supplementary data while the ratio between APs and the sum of APs and free amino acids is reported in Table 4 and it will be described and discussed in the following subparagraphs.

Milk products

The concentration of free amino acids in low lactose milk and UHT milk was roughly similar, as revealed by the ratio between the sum of free APs and the total amount of APs and amino acids. Some slight differences can be ascribed to the origin and the production processes. In UHT milk the ratio ranged from 0.17 % for proline to 54.55 % for tryptophan and the total ratio was 3.93 %, while for low lactose milk it ranged from 1.04 % for proline up to 84.62 % for cysteine and the total ratio was 16.84 %. The concentration

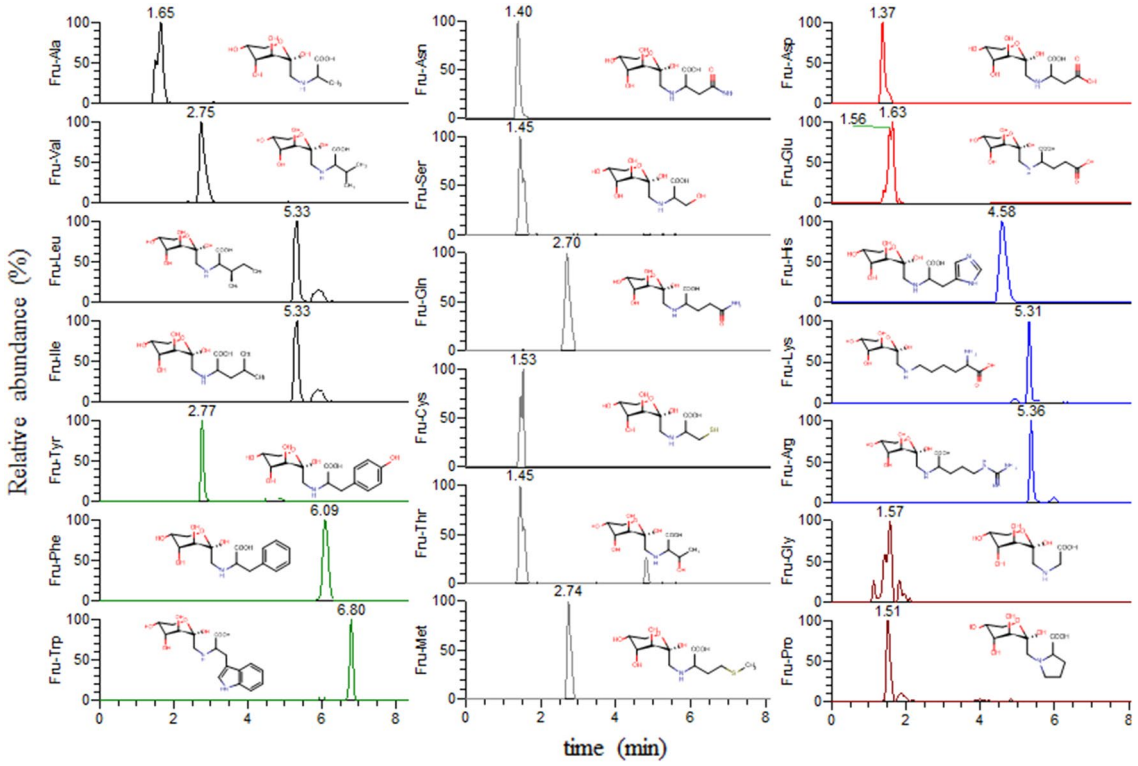


Fig. 3 Chromatographic profile of Amadori products in tomato juice: *black* (hydrophobic APs), *green* (aromatic APs), *gray* (polar neutral APs), *red* (acidic APs), *blue* (basic APs), *brown* (unique APs) (color figure online)

Table 4 Amadori products and amino acids ratio

Analytes ratio	UHT milk	LLM	Milk caramel	T1	T2	T3	CT	YDT	TP
Ala	0.89	62.35	23.68	1.93	2.49	3.03	13.94	25.91	77.51
Val	0.60	3.67	62.57	32.63	5.99	7.15	31.18	47.63	95.77
Ile/Leu	8.13	5.18	19.43	0.10	0.02	0.03	22.67	44.14	26.90
Phe	24.49	13.29	80.61	0.25	0.02	0.05	4.97	14.36	6.83
Trp	54.55	8.27	93.77	0.01	0.14	0.03	2.84	5.71	38.08
Tyr	18.52	n.d.	82.92	26.09	79.93	61.34	26.56	23.52	40.86
Cys	n.d.	84.62	n.d.	37.80	11.87	9.51	97.63	97.48	87.00
Met	n.d.	n.d.	98.45	1.97	1.96	1.90	18.52	36.29	97.76
Ser	20.11	5.51	19.34	79.00	85.33	89.78	78.49	86.19	89.13
Thr	23.14	19.40	51.35	11.75	15.42	20.49	14.32	28.74	34.64
Asn	1.85	n.d.	73.40	0.20	0.55	0.54	8.69	15.02	24.65
Gln	3.47	7.19	99.10	0.11	0.08	0.09	9.73	13.72	65.22
Asp	4.00	9.91	56.85	0.93	0.80	1.30	19.37	29.24	49.27
Glu	4.33	13.81	72.55	0.54	0.33	0.53	2.56	5.69	5.21
Arg	0.93	8.99	52.13	0.74	0.01	0.63	22.47	49.30	82.00
His	7.23	44.69	66.10	0.01	0.02	0.04	17.04	32.15	75.15
Lys	12.57	37.89	64.13	2.37	1.03	2.45	28.69	45.09	42.90
Pro	0.17	1.04	95.02	0.23	0.74	0.66	0.28	0.64	0.45
Gly	0.31	5.96	28.57	22.61	25.70	22.38	13.94	32.80	23.76
Total ratio	3.93	16.84	64.12	2.35	3.09	3.66	7.41	12.55	43.29

The results were reported as (%) of free APs toward the sum of free APs and amino acids. For the concentration of each analytes in the different products see supplementary tables S1 and S2. LLM (low lactose milk); T1, T2, T3 (raw tomatoes); CT (canned tomatoes), YDT (canned yellow "Datterini"), TP (tomato paste)

of glutamic acid was the highest, 159.89 ± 7.23 μg/mL in both milk products, while cysteine and methionine were almost absent. The concentration of aliphatic hydrophobic amino acids varied from 1.13 ± 0.02 μg/mL for isoleucine/leucine to 37.29 ± 0.20 μg/mL for valine in low lactose milk. Aromatic amino acids were particularly prone to the thermal oxidation and to the carbonyl attachment; their concentration was lower than 3 μg/mL. Polar neutral amino acids varied from 1.43 ± 0.06 μg/mL for serine in UHT milk to 9.30 ± 0.20 μg/mL for glutamine in low lactose milk. Basic amino acids, such as arginine and lysine together with proline and glycine represented a good percentage of the total amino acid pool. Specifically the concentration of lysine was 16.77 ± 0.14 μg/mL and 6.36 ± 0.03 μg/mL for UHT milk and low lactose milk.

In milk products the concentration of APs was influenced by the presence of free amino acids and reducing sugars, even though an indirect relationship for the two groups was present for few analytes. The APs ranged from 0.10 ± 0.002 μg/mL for fructosyl-leucine/isoleucine in UHT milk to 25.12 ± 0.15 μg/mL for fructosyl-glutamic acid in low lactose milk. On one hand, in UHT milk, along with acidic amino acids, the overall extent of glycation was evident for fructosyl-glutamic acid (7.23 ± 0.05 μg/mL) and for polar basic fructosyl-lysine (2.41 ± 0.03 μg/mL). The concentration of the other APs was in the range 0.01 ± 0.001, 0.56 ± 0.01 μg/mL for fructosyl-leucine/isoleucine and fructosyl-threonine, respectively. On the other hand, in low lactose milk, the concentration of APs varied between 0.11 ± 0.01 μg/mL for fructosyl-cysteine and fructosyl-tryptophan and 25.12 ± 0.15 μg/mL for fructosyl-glutamic acid revealing a direct effect of free glucose and galactose issued from hydrolysis of lactose.

In milk-based caramel product lysine showed the highest concentration: 47.15 ± 3.16 μg/g, while the hydrophobic amino acids ranged from 2.15 ± 0.04 μg/g to 5.35 ± 0.08 μg/g for phenylalanine and isoleucine/leucine, respectively. Interestingly, the highly reactive amino acids were influenced by the production process and their concentration varied from 0.001 ± 0.0001 μg/g for glutamine or 0.003 ± 0.00001 μg/g for methionine to 2.25 ± 0.03 μg/g for arginine. Cysteine was not detected likely due to the reactivity of thiol group.

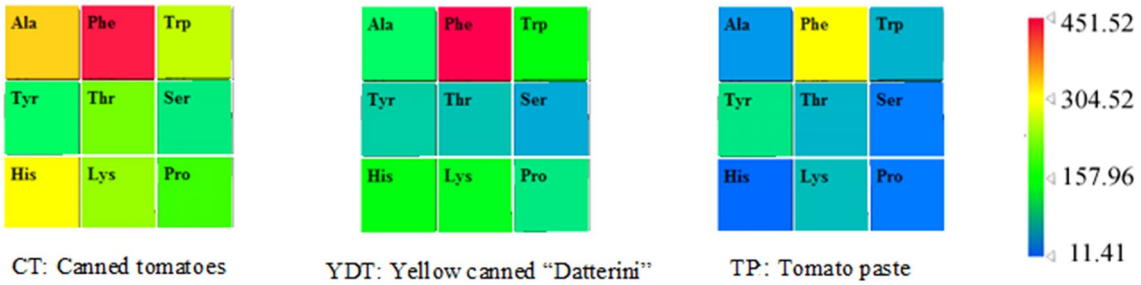
Fructosyl-lysine was 84.31 ± 1.93 μg/g, indicating that the severe thermal treatment determined an extensive lysine blockage. It was one of the most abundant APs in milk-based caramel, followed by fructosyl-proline and fructosyl-phenylalanine, whose concentrations were 8.01 ± 0.06 μg/g and 8.94 ± 0.07 μg/g, respectively. As highlighted in Table 4 the ratio of the two analytes classes showed the highest value among the analyzed products: 64.12 %. Despite a concentration of free amino acids lower than UHT and low lactose milk, the total amount of APs was two and nine times higher than UHT and low lactose milk, respectively. The ratio ranged from 19.34 % for serine and 99.10 % for glutamine indicating that the thermal treatment can be linked to the MR extent and other MRPs formation, mainly flavor compounds (Newton et al. 2012; van Boekel 2006).

Tomato products

In raw tomato samples the ratio between APs and the sum of APs and free amino acids was almost similar among the three cultivars, with some exceptions for valine and arginine; it ranged from 0.01 for arginine in T2, histidine and tryptophan in T1 and 79.93 % for tyrosine in T2. The total ratio was 2.35, 3.09 and 3.66 % for T1, T2 and T3, respectively. On the opposite, in processed tomatoes, the total ratio was 7.41, 12.55 and 43.29 % for canned tomatoes, "Datterini" and tomato paste, respectively. In tomato samples, free glutamic acid showed the highest concentration in raw tomatoes ranging from 6,077.83 ± 121.00 to 8,002.18 ± 67.15 μg/g FW. The amount of other amino acids varied according to the different cultivars: specifically the concentration of acidic amino acids such as glutamic acid and aspartic acid was higher in T1 than in T2 and T3 and those were the highest values among the amino acid pattern, while lysine ranged from 256.69 ± 1.57 to 315 ± 0.91 and 382.20 ± 6.77 μg/g FW in T1, T2 and T3, respectively. The concentration of valine was the lowest among the hydrophobic amino acids (from 5.14 ± 0.17 to 23.08 ± 1.70 μg/g FW), while polar neutral amino acids were mainly characterized by the presence of asparagine and glutamine whose concentration was higher than 300 μg/g FW and 2,150 μg/g FW, respectively. Three different categories of thermal-treated products were analyzed to evaluate not only the concentration of amino acids, but also to explore the link between free amino acids, APs, MR and thermal treatments. The concentration of glutamic acid in these products was about three times higher than in raw tomatoes: it varied from 1,196.1 ± 53.09 to 16,646.89 ± 1,385.40 μg/g FW.

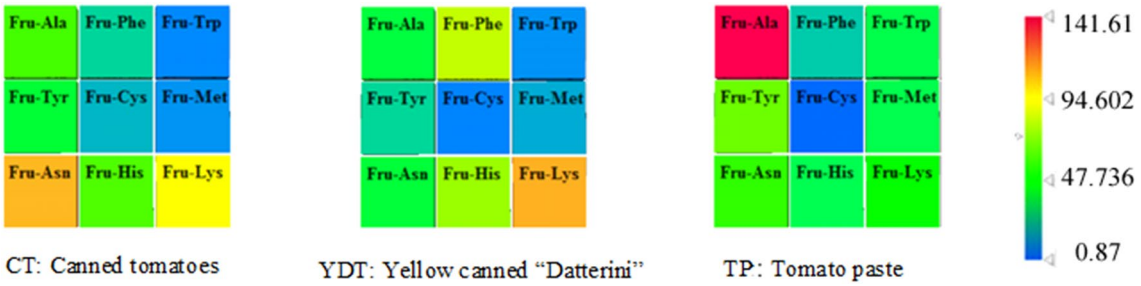
The concentration of APs in tomato samples was influenced by the concentration of free amino acids, reducing sugars, harvesting procedure, ripening and last but not the least by the thermal processes. In raw tomatoes, APs' concentration was quite constant among the three cultivars, even if some exceptions can be linked to the abovementioned variables. In general, concentration of APs ranged from 0.01 ± 0.0001 μg/g for fructosyl-tryptophan to 43.18 ± 0.13, 27.10 ± 1.70, 31.34 ± 0.47 and 15.18 ± 0.39 for fructosyl-glutamic acid, fructosyl-threonine, fructosyl-serine and fructosyl-tyrosine, respectively. As expected the values of APs in thermal-treated tomatoes were higher than in raw tomatoes. The concentration

Table 5 Amino acids FancyTyle in thermal-treated tomatoes



The quantitative colorimetric scale was reported in μg/g

Table 6 Amadori products FancyTyle in thermal-treated tomatoes



The quantitative colorimetric scale was reported in μg/g

of APs derived from hydrophobic amino acids in canned Cherry tomatoes was higher than in tomato paste, varying from 12.37 ± 0.02 μg/g for fructosyl-leucine/isoleucine in tomato paste to 37.25 ± 0.98 μg/g for fructosyl-alanine in yellow canned "Datterini". The concentration of fructosyl-glutamic acid, fructosyl-lysine and fructosyl-serine in tomato paste was 67.25 ± 0.01, 48.81 ± 0.02 and 20.14 ± 0.93 μg/g, respectively.

In Table 5, the differences among the samples were shown using a visual tool, named FancyTile (Troise et al. 2014). Up to now this representation was based on semi-quantitative data while here the scheme was constructed using the concentrations of nine amino acids. The FancyTile immediately highlights the impact of the thermal treatment on three products, namely canned Cherry tomatoes, canned yellow "Datterini" and tomato paste. Tomato paste showed the most severe thermal treatment: the free amino acid FancyTile showed all blue squares indicative of a low amount of free amino acids, while the free amino acid image of canned yellow "Datterini" and canned Cherry tomatoes had mostly green marked and yellow/orange tiles indicative of greater amount of them.

In Table 6, FancyTile representation was applied using the concentration of nine APs. On one hand, tomato paste showed a dominant green color with respect to the other two canned products confirming that the extent of glycation was more intense on this sample. On the other hand, not all the amino acids have the same trend: most of them and particularly fructosyl-alanine had the highest concentration in the tomato paste, fructosyl-lysine and fructosyl-asparagine had higher concentration in "Datterini" and in Cherry tomato, respectively, suggesting that in these samples the MR further progressed probably leading to the formation of flavor compounds and other products such as HMF, furan and dicarbonyls (Cosmai et al. 2013; Sanz et al. 2000).

Discussion

The present paper represents one of the first examples of simultaneous detection of amino acids and APs. The conventional reversed phase C18 and C8 columns were not sufficient to ensure the separation between free amino acids and their respective APs' counterparts and the results were unsatisfactory (Sanz et al. 2001). Previous papers reported the separation of APs with the use of ion exchange column or an ion exchange with electrochemical detection coupled to post-column addition of NaOH (Davidek et al. 2003, 2005; Reutter and Eichner 1989), with HILIC phase (Schlichtherle-Cerny et al. 2003), on a bare HILIC silica column at high temperature (Hao et al. 2007) and by ion pairing through heptafluorobutyric acid (Frolov and Hoffmann 2008).

In the present study, the use of an ion-pairing agent, such as NFPA, definitively separated the peaks of APs from

free amino acids, even if it is still not possible to separate APs from their corresponding Schiff bases; the two compounds have exactly the same *m/z* in positive ions and in most cases the same chemical and fragmentation behavior. It derives that the chromatographic profile is the same; thus, their accurate detection and quantitation are difficult.

The co-elution was avoided to prevent the simultaneous ionization of reducing sugars, APs and amino acids and the consequent overestimation or underestimation of the signal associated with the target compounds. The shapes of the peaks and the retention time among the other chromatographic performances were ensured by the core-shell materials of the chromatographic column adopted (Gritti et al. 2010). Moreover, the use of a core-shell column allowed to set the instrumental resolution to 50,000 (FWHM, *m/z* 200) and gain a good compromise between number of ions injected (balanced mode) and scan time. The exact mass of diisooctyl phthalate, one of the most common contaminants in mass spectrometry (Keller et al. 2008), was used as internal mass reference to allow the recalibration of the instrument. By this strategy the mass analyzer can correct each mass trace according to the detection of the *m/z* 391.28429. The result was the increase of the mass accuracy of each target compound. The same approach was followed in previous papers by our group (Troise et al. 2013, 2014).

Looking at the different matrix it is possible to highlight the main advantages of the proposed method. Using the developed procedure, free amino acids and APs were measured in different foods to have preliminary information on the possibility of evaluating the importance of APs as a direct marker of the quality of the starting material and of the thermal impact. On one hand, the available literature data on the quantification of single amino acids in foods usually refer to the concentrations that can be measured after protein hydrolysis. As a consequence, working only with free amino acids allowed to rule out the main disadvantages due to the chemical transformation caused by the severe acidic treatment, such as: the conversion of asparagine and glutamine into aspartic acid and glutamic acid; the sulphoxidation of the thioether side chain of methionine; the modification of the phenolic group of tyrosine and phenylalanine; the degradation of the indole ring of tryptophan; the esterification reaction of tyrosine and serine with glutamic acid (Hunt 1985).

The assessment of thermal damage on free amino acid is based on the assumption that the formation of APs on free amino acids is well representative of the derivatization occurring at the side amino group of the amino acids present in the proteins. The results obtained in this study confirmed this hypothesis: in fact, the extent of amino acid modifications observed considering only the free amino acids moiety is well in line with those previously observed measuring amino acid derivatization after protein hydrolysis. This was verified, in particular, for the UHT milk sample where many data are available (Pischetsrieder and Henle 2012).

Investigating the concentration of lysine in milk samples was particularly useful to deepen the aspect linked to Nε-(carboxymethyl)-lysine (CML) and Nε-(carboxyethyl)-lysine (CEL) formation: data of this paper showed that lysine represented about 5 % of the total free amino acids which is similar to the percentage obtained after acidic hydrolysis on total protein (Delatour et al. 2009; Ferrer et al. 2003). The results were in good agreement with those obtained earlier in infant formula confirming the performances of the developed method in a complex matrix such as milk (Ventura et al. 2012; Agostoni et al. 2000; Ozcan and Senyuva 2006).

Because of the presence of glucose and galactose, low lactose milk showed a higher amount of APs than UHT milk for all compounds, with the only one exception represented by fructosyl-serine and fructosyl-threonine. Taking into account the different thermal treatments and storage condition, the differences between UHT milk and low lactose milk were particularly evident for aliphatic APs such as fructosyl-alanine and fructosyl-valine, and for acidic APs such as fructosyl-glutamic acid while for fructosyl-lysine the two concentrations were almost comparable. Specifically this last compound was directly implicated in the formation of CML and CEL (Nguyen et al. 2014). The reported values for fructosyl-lysine were higher than the one reported earlier (Hegele et al. 2008) and these differences can be related not only to the extraction procedure, but also to the acidic hydrolysis performed for the quantitation of protein-bound fructosyl-lysine. On the contrary, the concentration of fructosyl-lysine here reported was lower than the one calculated through the furosine method after acidic hydrolysis highlighting some discrepancies between the direct and indirect analytical methods (Fenaille et al. 2006). High-resolution mass spectrometry revealed a new scenario in the monitoring of the early stage of MR overcoming the drawbacks linked to acidic hydrolysis or to enzymatic digestion (Pischetsrieder and Henle 2012; Hunt 1985). However, for a complete picture of the milk thermal damage the measure of lactosylated derivatives would be necessary.

Some interesting points can be highlighted from the data on milk-based caramel and from the behavior of lysine in the three products. As expected, the overall amount of amino acids was around four times higher in UHT and low lactose milk than in caramel powder, while the derivatization ratio was very high (64.12 %) likely due to the severe thermal load and the abundance of reducing sugars and dicarbonyls. The derivatization rate of lysine jumped from 12.57 and 37.89 % in UHT and low lactose milk, respectively, to 64.13 % in caramel powder highlighting the direct effect of carbonyl groups on amino moiety.

Tomato products, mainly dried tomatoes, are one of the most studied foods as far as the formation of APs (Eichner and Wittmann 1990). Free amino acid concentrations in raw tomatoes are highly variable: the differences can be ascribed to several factors such as variety, cultivar, ripening and storage (Ozcan and Senyuva 2006; Odriozola-Serrano et al. 2013; Carli et al. 2009). This is confirmed by our results which are in some cases in line with those previously reported and in others very different. Our values are of the same order of magnitude toward previously reported papers ranging from 0.13 ± 0.01 μg/g for cysteine in tomato paste to 16,646.89 ± 1,385 for glutamic acid in canned "Cherry" tomatoes. Specifically, the concentration of amino acids in tomato paste, lower than canned tomatoes, can be related to the Maillard cascade, where the intermediate stage favored the dehydration, oxidation, fragmentation, and other reactions of the Schiff bases of basic and polar amino acids. The final stage of the Maillard reaction is characterized by the formation of stable volatile components, cross-linked products, mainly proteins, and aromatics and colored polymers, such as melanoidins. The concentration of glutamic acid was six and nine times lower than the ones revealed canned yellow "Datterini" and canned tomatoes, respectively. The concentration of the other amino acids in tomato paste was lower than in canned tomato, with the only exception being tyrosine (91.95 ± 1.97 μg/g FW) and glycine (6.77 ± 0.0 μg/g FW) toward canned "Datterini". According to the high reactivity of side chain, the thermal damage was particularly pronounced on lysine, glutamic acid, proline, methionine and alanine whose values were lower compared to those from canned tomatoes of almost three, eight, eight, fifty and seven times, respectively.

Fructosyl-glutamic acid in canned tomato was the most abundant APs and the concentration here reported was significantly different from that reported for dried tomatoes and fruits; this can be due to the differences in the raw material and the thermal process employed (Eichner and Wittmann R 1990; Davidek et al. 2005; Meitinger et al. 2014). It is followed by fructosyl-aspartic acid, fructosyl-asparagine and fructosyl-lysine.

The relevance of this paper can be evaluated also through the ratio between APs and the sum of APs and amino acids in tomato products that varied according to thermal treatment performed (Table 4): it ranged from around 3 % in raw tomatoes to 7.41, 12.55 and 43.29 % for "Cherry" tomatoes, canned "Datterini" and tomato paste. In particular, in the thermal-treated products the effect of thermal load was impressive for thermo labile amino acids such as tryptophan (2.84, 5.71 and 38.08 %), methionine (18.52, 36.29 and 97.76 %) and histidine (17.04, 32.15, and 75.15 %).

The results related to the free amino acids and corresponding APs in tomatoes are summarized in the two FancyTyles of Tables 5 and 6. Even if the starting materials and the thermal treatments were different, results suggested that histidine, alanine and lysine were particular sensitive parameters, while the aromatic phenylalanine underwent the highest reduction percentage. These results gave a preliminary overview on the potentiality related to the monitoring of free amino acids and APs, whose ratio can also be an interesting aspect for the evaluation of the starting raw material, of the general thermal impact and of the final quality not only in the hereby analyzed samples, but also in milk-based products, fruit juices, bread, biscuits, French fries and so on.

In conclusion, the present paper highlights the possibility to simultaneously quantify free amino acids and their respective APs following a simplified approach that avoids the extraction protocols or derivatization procedures. Moreover, the quantification of APs in several food matrices represents the key that may allow to skip complex, time-consuming and expensive procedures linked to the acidic hydrolysis of proteins and the derivatization procedures. By monitoring the ratio between free amino acids and APs there is the opportunity to create a deep fingerprinting of the quality of the starting material, of the thermal treatment and of the extent of the MR in the initial steps.

Conflict of interest The authors declare no conflict of interest.

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