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Quantitative determination of dityrosine in milk powders by liquid chromatography coupled to tandem mass spectrometry using isotope dilution

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Abstract

An analytical method to quantify dityrosine (DiTyr) in milk powder samples is presented. The assay is based on isotope dilution liquid chromatography coupled to electrospray ionisation tandem mass spectrometry (LC–ESIMS/MS). The sample preparation entails acid hydrolysis of milk proteins followed by a solid phase-extraction (SPE) step. Neither artifactual formation nor degradation of DiTyr were observed during the proteolysis step. Mass spectral detection was performed in the positive ion mode by recording five transition reactions for DiTyr, in order to unambiguously confirm the presence of DiTyr by correct ion ratios. Under the analytical conditions used, the limit of detection (LOD) and limit of quantification (LOQ) for DiTyr were estimated at ca. 2 and 6 μmol DiTyr per mol of Tyr (using ca. 500 μg of milk proteins), with a mean recovery of ca. 90%. Quantification was conduced in eight different commercial milk powder samples, and the level of DiTyr ranged from below the LOQ up to 393.0 ± 9.1 μmol DiTyr per mol of Tyr.
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Keywords: Protein oxidation; Dityrosine; Tandem mass spectrometry; Isotope dilution

1. Introduction

From a general point of view, thermal treatment improves food safety, organoleptic qualities, and storage time of dairy products. However, heat treatment, light exposure and oxygen may adversely lead to a decrease of product quality, safety, and nutritional value, by means of oxidative damage to food lipids and proteins. For instance, lipid oxidation is a major cause of quality deterioration during the processing and storage of lipid-rich foods [1]. Therefore, oxidation in milk has traditionally focused on assessment of lipid oxidation, mainly by monitoring secondary lipid oxidation products such as malondialdehyde [2] or volatile aldehydes or ketones (off-flavours) [3–5]. Another important target of oxidative damage in milk products is the protein entity. In particular, the well-known Maillard reaction, occurring during food manufacture and storage, is responsible for decreasing protein digestibility and nutritional supply [6]. In this case, carbonyl-intermediates, resulting from oxidized glycated amino acid residues, could in turn modify proteins, yielding "glycoxidation" products [7]. Oxidation could also occur more "directly" on proteins. For instance, protein oxidation could yield to formation of dityrosine (DiTyr) by metal-catalysed oxidation [8]. DiTyr, which is basically a cross-link between two tyrosine residues (either intra- or inter-macromolecules), could be formed from either hydroxyl or tyrosyl radicals [9], as well as from peroxyl radicals [10]. For these reasons, DiTyr has often been used as a stable biomarker of radical-mediated protein oxidation for in vivo studies [11,12].

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Moreover, it has also been shown that lactoperoxidase, an enzyme present in milk, could lead to the formation of DiTyr in the presence of hydrogen peroxide [13]. Regarding food proteins, the apparition of such cross-link could have further incidence on protein digestibility or protein structure as previously noted by Tilley et al. relatively to the gluten network [14].

Due to the natural fluorescence of DiTyr, several analytical methods, using HPLC with fluorescence detection, were developed and used to quantify DiTyr in biological systems [8,15,16] as well as in some food systems such as gluten [14] or mozzarella cheese [17]. Although sensitive and specific, such analytical method could be confounded by coeluting but structurally distinct molecules. In contrast, mass spectrometry-based methods such as GC–MS provide specific structural information, thereby reducing the potential for confusion with extraneous compounds that coelute with the target analyte during chromatography. In addition, using a mass spectrometer as detector enables the use of a stable, isotopically labelled internal standard, which, apart from its heavy isotope, is structurally identical to the analyte of interest and therefore behaves identically during sample preparation and chromatographic analysis. Thus, including such standard corrects for analyte loss during the analytical work-up and therefore increases the precision and accuracy of quantitative measurements. One of the weakest point of GC–MS techniques is the derivatisation step (time consuming, reaction yield). Indeed, such chemical reaction often requires a heating step that could potentially either affect the stability of oxidized amino acids or catalyse an artifactual formation of oxidized amino acids. For these reasons, in addition to GC–MS methods [18,19], LC–MS/MS methods were also developed to quantify DiTyr in biological samples [20,21].

To see whether DiTyr could constitutes a good marker of milk proteins oxidation, we have investigated the validity of using LC–MS/MS technique as a tool to accurately quantify this chemical in milk powder samples. Milk proteins were first hydrolysed by hydrochloric acid, and DiTyr further recovered from the amino acid hydrolysates by solid phase-extraction (SPE). Quantification was performed by liquid chromatography coupled to electrospray tandem mass spectrometry (LC–ESIMS/MS) using isotope dilution. The usefulness of choosing labelled analytes to obtain reliable quantitative results will be underlined. Moreover, it will be demonstrated that, even when a highly selective technique such as LC–MS/MS is used, much attention should be paid to unambiguously confirm the identity of the target analyte.

2. Experimental

2.1. Chemicals

Hydrochloric acid 37% (corresponds to 12N) and formic acid were purchased from Merck (Darmstadt, Germany). All other solvents were of analytical or HPLC grade. Trifluoroacetic acid (TFA), L-tyrosine and horseradish peroxidase were obtained from Fluka (Buchs, Switzerland). α,α'-d_2-L-tyrosine (d_2-Tyr, 99.9% D) and α,β,γ,1,2,3,4,5,6-$^{13}C_9$-L-tyrosine ($^{13}C_9$-Tyr, 97–98% ^{13}C) were from Cambridge Isotope Laboratories (Andover, MA). Dityrosine and d_4-dityrosine (d_4-DiTyr) were enzymatically synthesized from Tyr and d_2-Tyr following an adaptation of the method of Malencik et al. [22], as previously described [20].

2.2. Milk powder samples

Eight different milk powder samples (A–H) were purchased from local supermarkets and were used for method development.

2.3. Preparation of milk powder extracts

Amounts of milk powders equivalent to ca. 20 mg of total proteins (based on compositional data given by the provider) were hydrolysed in 50 mL of 6N HCl for 24 h at 110 °C. The resulting hydrolysates were then quantitatively transferred in 100 mL volumetric flasks, and the volume completed to the mark with water. A 2.5 mL aliquot (equivalent to ca. 500 μg proteins) of the resulting solutions were spiked with d_4-DiTyr (15 ng) before being evaporated to dryness (SpeedVac concentrator from Savant, Farmingdale, NY). The addition of d_4-DiTyr after acid hydrolysis, as single internal standard, was rendered possible by the fact that neither artifactual formation of DiTyr nor DiTyr breakdown occurs during the acid hydrolysis step (see Section 3). Dried residues were then reconstituted in 2.5 mL of 0.1% TFA before being applied to Supelclean LC-18 SPE cartridge (Supelco, Buchs, Switzerland), previously conditioned by 5 mL of methanol and 10 mL of 0.1% TFA. The cartridges were then washed with 2 mL of 0.1% TFA, and DiTyr and d_4-DiTyr species further eluted with 2 mL of 25% methanol. The eluates were then concentrated to dryness in a SpeedVac, and resuspended in 100 μL of water containing 0.1% formic acid prior to LC–ESIMS/MS analysis.

2.4. Quantification of DiTyr by LC–ESIMS/MS

Hydrophilic interaction liquid chromatography (HILIC) [23] was used for DiTyr analysis and was performed on a TSK-Gel Amide 80 column (1.5 mm i.d. × 25.0 cm, 5 μm, 80 Å) preceded by a TSK-Gel Amide 80 guard column (2.0 mm i.d. × 1.0 cm), both from Tosoh BioSep (Tokyo, Japan). Because one of our future goal is to achieve the simultaneous analysis of DiTyr and different polar modified amino acid residues (mainly derived from Maillard reaction) that are not retained on reverse-phase (RP) columns if not previously derivatized, HILIC was used in place of RP-HPLC that is commonly reported for DiTyr analysis [20,21,24]. The solvents used were water pH 2.7 (adjusted with formic acid,

Table 1
Transition reactions and respective collision energies used for DiTyr analysis

	Transitions (*m/z*)	Collision energy (eV)
DiTyr	361 → 315	21
	361 → 283	23
	361 → 269	28
	361 → 254	28
	361 → 237	30
d_4-DiTyr	365 → 319	21
	365 → 287	23
	365 → 258	28

solvent A) and acetonitrile pH 2.7 (solvent B) and delivered at 200 μl/min by a HP series 1100 (Hewlett-Packard, Palo-Alto, CA, USA) HPLC system. The gradient was as follows: 20% A for 3 min, 70% A at 10 min, isocratic at 70% A for 10 min and return to 20% A in 3 min followed by a re-equilibration period of 17 min. The detection was performed by positive electrospray ionisation tandem mass spectrometry on a TSQ 7000 triple quadrupole mass spectrometer equipped with the API 2 interface (Thermo, San Jose, CA, USA). The spray voltage was set at 3.8 kV and the capillary temperature at 360 °C, whereas the sheath gas pressure was 90 psi (1 psi = 6894.8 Pa). Acquisition was performed in the multiple reaction monitoring (MRM) acquisition mode, using argon as collision gas (pressure 2.8 mTorr, with 1 Torr = 133.3 Pa). The MRM transitions recorded for DiTyr and d_4-DiTyr, and their optimised corresponding collision energies are summarized in Table 1. DiTyr quantification was achieved using a 8-points external calibration curve (area ratio versus concentration ratio) using the MRM transitions *m/z* 361 → 254 and *m/z* 365 → 257 for DiTyr and d_4-DiTyr, respectively (see Section 3 for more detailed information). The calibration curve ranged from 0 to 5 ng of DiTyr injected on-column (1.5 ng of d_4-DiTyr). The DiTyr amount was then normalized to the content of the precursor amino acid Tyr. Tyr was quantified in the hydrolysates using an amino acids analyzer (High Performance Analyzer System, Beckman Coulter, Fullerton, CA, USA). Each milk powder extract was prepared in duplicate and a volume of 10 μL injected twice.

2.5. *Fragmentation pathway of protonated DiTyr by multistep MS^n experiments*

The fragmentation pathway of DiTyr was confirmed by performing multistep collision induced dissociation (CID) experiments on a LCQ Deca ion trap mass spectrometer (Thermo, San Jose, CA, USA). The spray voltage and the capillary temperature were set at 4.0 kV and 200 °C, respectively. A sheath gas was also applied at a flow rate of 35 arbitrary units. Sequential MS^n spectra were recorded using a 3 Th wide window to select the precursor ions that were dissociated by applying relative excitation energies from 20 to 30%.

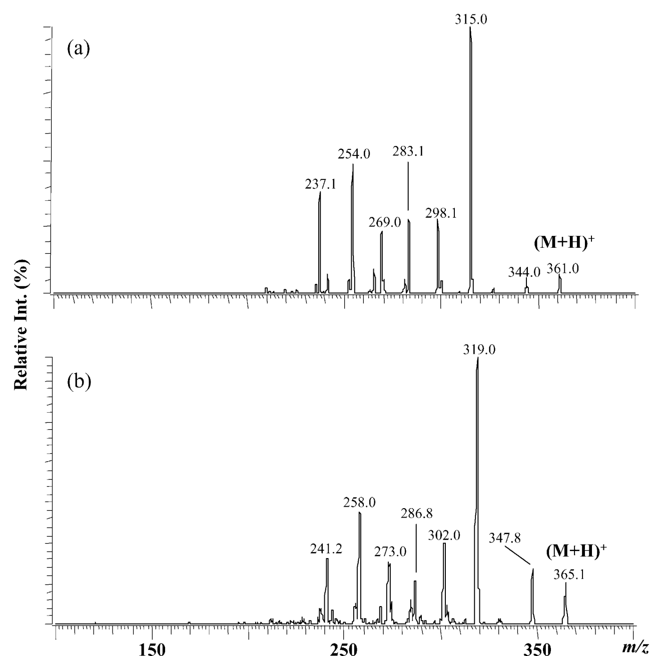

Fig. 1. CID mass spectra of (a) DiTyr and (b) d_4-DiTyr (both acquired on a triple quadrupole instrument with a collision energy of 25 eV).

3. Results and discussion

3.1. *Fragmentation pathway of protonated DiTyr by multistep MS^n experiments*

The fragmentation pattern of protonated DiTyr under low-energy conditions is illustrated by the CID spectrum of Fig. 1a. This spectrum is characterized by a rather important number of fragment ions, the most intense being the ion at *m/z* 315. The latter ion is obtained by the loss of formic acid HCOOH (46 u), as also observed for other modified Tyr residues such as nitrotyrosine [25]. Beside this prominent fragment ion, an ion at *m/z* 344, resulting from the loss of an ammonia moiety (17 u), is also observed. From these two primary fragment ions, neutral losses of CO_2 (44 u), HCOOH or NH_3 could be seen, the latter two being competitive. The postulated fragmentation pathway of DiTyr is presented in Fig. 2. The filiation of the different fragment ions was confirmed by sequential MS^n experiments performed on an ion trap mass spectrometer.

On the basis of a fragmentation optimisation, DiTyr quantification was performed by monitoring the transition reactions listed in Table 1.

3.2. *Performance of the method*

3.2.1. *Specificity and confirmation criteria achieved by tandem mass spectrometry*

The detection and/or quantification of components present at trace level in food or biological matrices always represent challenging tasks. Measurements reliability is achieved when both sensitivity and specificity are obtained. Regarding the

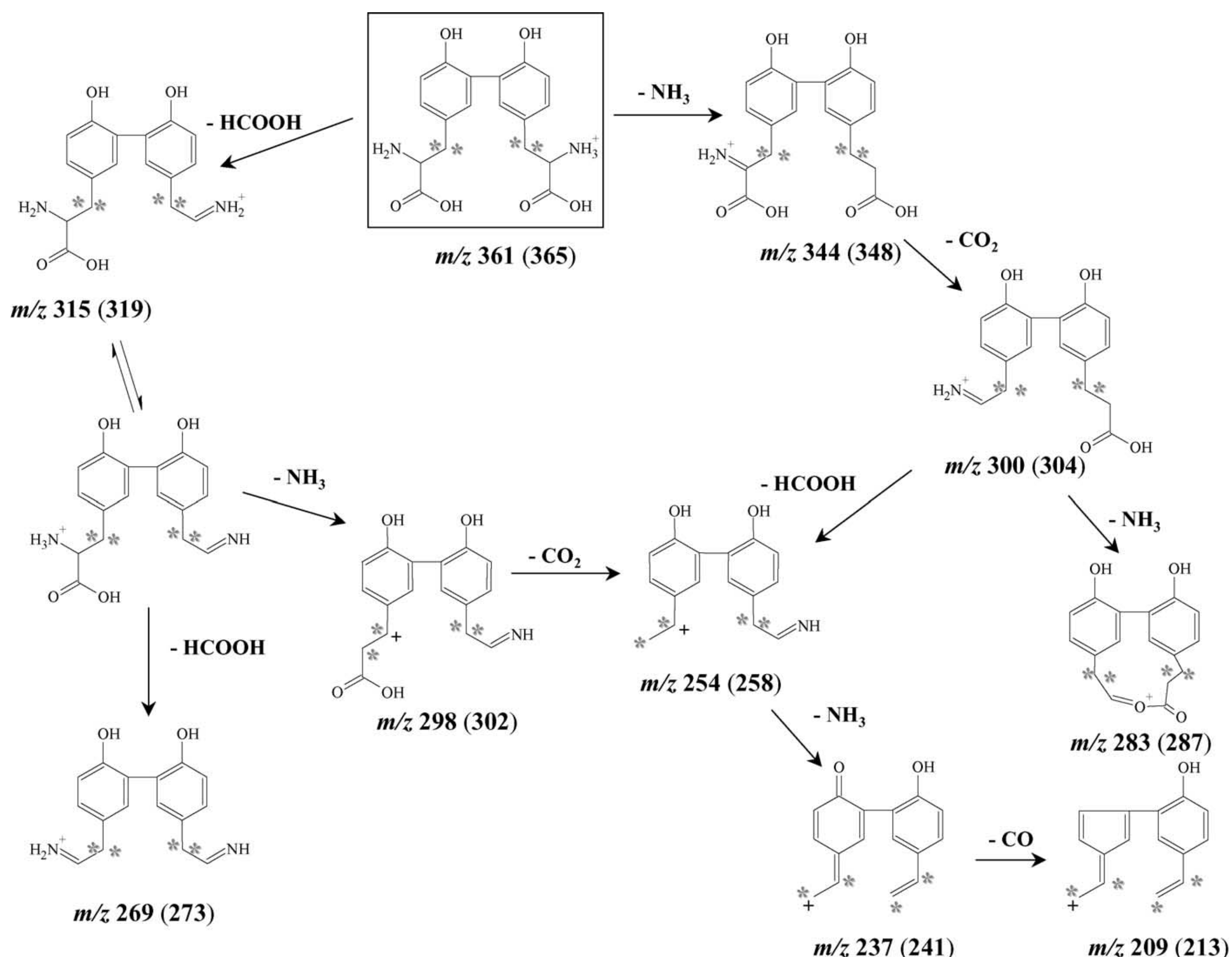

Fig. 2. Postulated fragmentation pathway of protonated DiTyr. The grey stars account for the deuterium atoms.

sample preparation, many endogenous compounds could be co-extracted with the analyte of interest. Therefore, lowering the chemical background will improve the sensitivity of the method. In other words, the highest sensitivity would be obtained with the highest specific extraction. From the mass spectrometric point of view, an excellent specificity is obtained by monitoring MRM transitions characteristic of the target analyte. What is commonly done is to monitor the most intense transition for quantification to get higher sensitivity. For instance, in the case of protonated DiTyr, the single monitoring of the transition reaction *m/z* 361 → 315 is often performed for quantification purposes [20,21,24]. In these cases, confirmation of the analyte identity is also achieved by comparison of its retention time with the one of the labelled internal standard (both chemical compounds coelute). Nevertheless, monitoring several other transition reactions for the target analyte, in addition to the quantification one, could help in achieving a higher level of specificity. Indeed, for a considered analyte, the area ratio of two transition reactions must be the same when calculated from a milk extract or a standard solution, thus calculating such ratio for a given extract will validate or invalidate the purity of a LC–MS/MS peak.

When the "confirmation transition"/"quantification transition" ratios are calculated for a given milk powder sample (using for quantification the transition reactions *m/z* 361 → 315 and *m/z* 365 → 319 for protonated DiTyr and d_4-DiTyr species, respectively) and further compared to those obtained from standard DiTyr solutions (average values from several standard solutions of different concentrations), the results obtained are significantly different (Table 2). The maximum tolerated differences for these area ratios were defined according to European decisions regarding the performance of analytical methods and interpretation of results developed mainly for the analysis of veterinary drugs [26]. For example, a deviation of 25% is tolerated when the confirmation transition represents between 20 and 50% (based on fragment ions intensities) of the quantification transition. So, under these conditions, the transition ratios can be considered as different between the milk extract and standard solutions (Table 2),

Table 2
Calculated transition reaction ratios for a milk powder extract vs. those obtained from standard solutions of DiTyr

Transition (*m/z*)	Area ratio considering *m/z* 361 → 315 as quantification transition		Area ratio considering *m/z* 361 → 254 as quantification transition	
	Sample (n = 2)	Standards (n = 14)	Sample (n = 2)	Standards (n = 14)
361 → 315	–	–	–	–
361 → 254	0.26	0.45	–	–
361 → 283	0.14	0.21	0.52	0.47
361 → 269	0.16	0.23	0.60	0.53
361 → 237	0.16	0.34	0.60	0.65

when the transition *m/z* 361 → 315 is used for quantification. However, when the transition *m/z* 361 → 254 (second most intense transition) is considered for quantification, the ratios calculated from the milk extract match perfectly those of standard solutions (Table 2). This demonstrates that, for this particular milk sample, the most intense transition must not be used for quantification, since a coeluting compound pollutes the chromatographic peak.

To further illustrate the importance of area ratios, Fig. 3 represents the different MRM transitions monitored for DiTyr from a certain milk powder extract. If we use only the most intense transition *m/z* 361 → 315 for DiTyr quantification (with the transition *m/z* 365 → 319 for d_4-DiTyr), a value of 19.7 ng/mg protein is calculated. When considering *m/z* 361 → 254 for quantification, the amount of DiTyr is now calculated at 13.5 ng/mg of proteins, which constitutes a value ca. 30% lower than the former one (19.7 ng/mg proteins). As such contamination of the transition *m/z* 361 → 315 was often observed with milk powders and to further avoid misleading results, we have then used the transition *m/z* 361 → 254 as quantification transition and three other transitions (*m/z* 361 → 283, 361 → 269, 361 → 237) as confirmation ones. The corresponding transition reactions were monitored for d_4-DiTyr for both quantification and confirmation purposes. Obviously, under such conditions, the method sensitivity is lowered (two- to three-fold) since the most intense transition is not used but DiTyr is unambiguously quantified. These results tend to demonstrate the usefulness of monitoring several other transitions in addition to the quantification one, when analysis of complex matrices such as foods is performed.

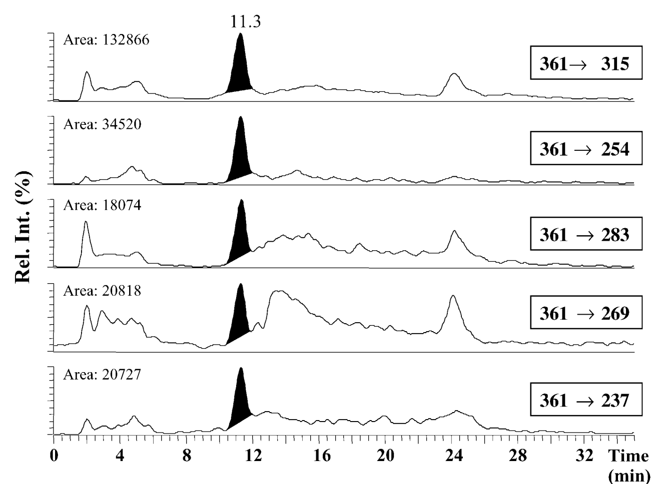

Fig. 3. LC–ESIMS/MS chromatograms recorded in the MRM mode for DiTyr from a milk powder extract. The MRM traces of d_4-DiTyr are not represented for clarity reasons.

3.2.2. Assessment of DiTyr stability and artifactual formation during the proteolysis step

Initially, we wanted to check the stability of DiTyr, notably during the acid hydrolysis step. Recoveries of 90–95% were obtained when d_4-DiTyr was added to a milk sample before acid hydrolysis (n = 3) or after acid hydrolysis and before SPE clean-up (n = 3). This is in good agreement with what has been reported by Huggins et al. [8]. These data demonstrate the stability of both DiTyr and d_4-DiTyr during the whole sample handling, and more especially during the drastic acid hydrolysis step. This enabled us to save considerable amounts of d_4-DiTyr, by spiking it after the acid hydrolysis.

In other preliminary experiments, the artifactual generation of DiTyr was also investigated. For such purposes, the milk powder sample that contains the highest quantity of DiTyr, was spiked with $^{13}C_9$-Tyr prior to acid hydrolysis and the formation of $^{13}C_{18}$-DiTyr and $^{13}C_9$, $^{12}C_9$-DiTyr assessed by monitoring the corresponding MRM transitions (Fig. 4). As observed from this figure, the amounts of DiTyr recovered from the milk powder, with and without $^{13}C_9$-Tyr spike, were similar. Thus, as also reported by others, DiTyr is not subject to artifactual formation during the acid hydrolysis step, in opposite to *o*-Tyr (formed from phenylalanine) for example [9].

Table 3
Amount of DiTyr found in the different milk powder samples studied

Sample	DiTyr amount (μmol/mol of Tyr)[b]
A	15.1 ± 0.6
B	<LOQ[a]
C	13.2 ± 3.4
D	12.1 ± 2.0
E	156.5 ± 1.1
F	15.2 ± 2.1
G	6.3 ± 0.0
H	393.0 ± 9.1

[a] DiTyr was detected but at a level inferior to the limit of quantification (i.e. 6 μmol/mol of Tyr).
[b] Values are mean ± standard deviation, two replicates are considered.

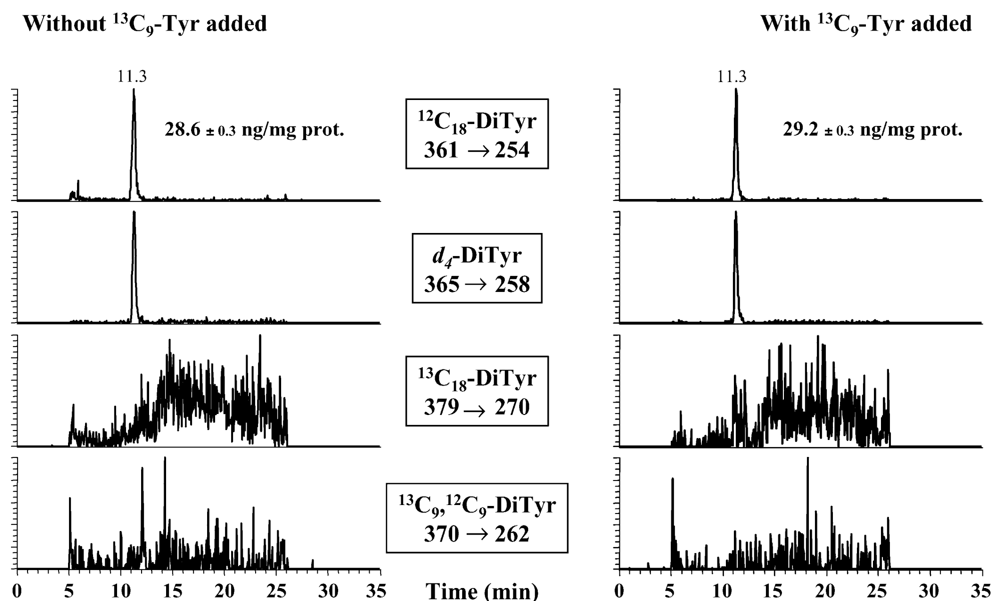

Fig. 4. LC–ESIMS/MS chromatograms recorded in the MRM mode for $^{12}C_{18}$-, $^{13}C_{18}$-, and $^{13}C_9$-, $^{12}C_9$-DiTyr species from a milk powder extract.

3.2.3. Method performance

Under the analytical conditions described above, the limit of detection (LOD) and limit of quantification (LOQ) for DiTyr were estimated at ca. 2 and 6 μmol DiTyr per mol of Tyr (using ca. 500 μg of milk proteins) by extrapolation from spiked milk powder samples. In addition, an injected amount of ca. 15 pg (i.e. ca. 42 fmol) of DiTyr could be detected on pure standard solutions. For instance, using GC–MS, Leeuwenburgh et al. were able to observe less than 5 μmol of DiTyr per mol of Tyr [19]. Our LOD and LOQ

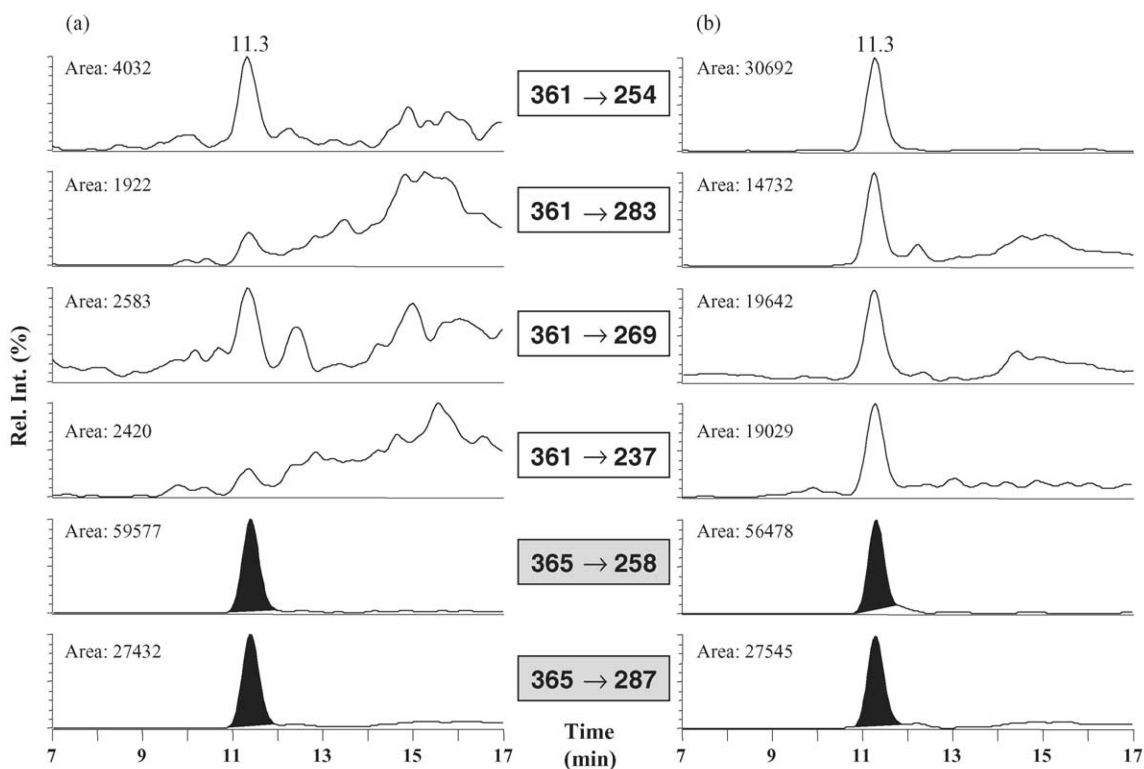

Fig. 5. LC–ESIMS/MS chromatograms recorded in the MRM mode for milk powder samples containing (a) 15.1 and (b) 156.5 μmol of DiTyr per mol of Tyr. The two transitions used for the d_4-DiTyr internal standard are highlighted by a grey background.

values may be improved with an additional step during the sample preparation to eliminate the chemical interference(s) observed on the *m/z* 361 → 315 transition reaction.

A 5-points calibration curve prepared by spiking a milk powder sample, showing a level of DiTyr inferior to the limit of quantification, was compared against a standard curve prepared in water, over the same concentration range. Both curves exhibited similar slope (i.e. 0.983 and 0.987 for the matrix and water curves, respectively; $r^2 > 0.99$ in both cases), thus demonstrating the absence of drastic matrix effects. Such a spiking procedure also brought the possibility of calculating DiTyr recoveries for different fortification levels (0, 575, 2875, 4600, and 9200 pg). Thus, the mean recovery was estimated at ca. 90%.

3.3. Quantification of DiTyr in commercial samples

Table 3 summarizes the results for the quantification of DiTyr in eight commercial milk powders (infant formulas), and Fig. 5 represents the LC–MS/MS chromatograms obtained for two samples considered as rather non-oxidized and oxidized. Five samples out of eight contain DiTyr amounts in the order of magnitude around 12 μmol DiTyr/mol Tyr in average, whereas one sample presents non-quantifiable amount of DiTyr. Two other milk powder samples exhibit DiTyr amounts ca. 10- and 30-fold higher. Leeuwenburgh et al. reported up to 300 μmol DiTyr/mol Tyr in low-density lipoproteins (LDL) isolated from human atherosclerotic lesions, which represent ca. a 100-fold increase compared to circulating LDL [19]. Based on the data gathered, it seems that DiTyr could constitute a useful marker of milk protein oxidation. To our knowledge, this study constitutes the first one that reports DiTyr contents of milk powder samples. Therefore, it is difficult to compare our data with others. Nevertheless, Balestrieri et al. reported DiTyr values for some milk-related foodstuffs, i.e. mozzarella cheese produced from either buffalo or bovine milk measured by HPLC-fluorescence [17], where its level was found ranging from 80 and 250 ng DiTyr/mg proteins without noticing any influence of the milk origin. These values represent quite high amounts of DiTyr compared to our data for milk powders. Indeed, the values of the most oxidized milk powder sample (sample H) could be estimated at ca. 29 ng DiTyr/mg proteins. This data comparison tends to suggest potential higher proteins oxidation in cheeses than in milk powders.

4. Conclusion

An unambiguous quantitative method has been developed for the determination of DiTyr in different milk powder samples (infant formulas). Tandem mass spectrometry using specific MRM transitions enable selective and confirmatory detection. Indeed, the usefulness of choosing several MRM transitions has been particularly emphasized for confirmatory purposes. DiTyr has been quantified accurately using d_4-DiTyr as internal standard. Moreover, it has been shown, using stable isotopes that DiTyr is stable during the proteolysis step and no artifactual generation occurs during this drastic procedure. Under these analytical conditions, DiTyr has been quantified in several milk powder samples, and the results tend to demonstrate that DiTyr could be potentially used as a good chemical marker of milk protein oxidation. Further experiments are on-going to adapt the developed methodology to other milk-based products, to further evaluate the influence of the processing/storage conditions on the DiTyr quantity.

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