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Milk authenticity by ion-trap proteomics following multi-enzyme digestion

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ABSTRACT

The practice of adding adulterating substances in milk in order to raise profits is unfortunately worldwide. In addition, higher priced milk, coming from minor dairy species, is often illegally integrated with the lower priced cow milk. The presence of species-specific proteins, different from those declared in label, may be a serious problem for people with allergies. The development of proper analytical methods is therefore essential to protect consumer benefits and product authenticity.

In this study, a proteomic approach for the detection of adulteration processes of specific milks in mixtures is proposed. Few microliters of milk samples have been digested with trypsin and chymotrypsin and analyzed by nanoLC-ESI-IT-MS/MS. A post-database processing was performed to obtain confident peptide sequence assignments, allowing the detection of milk adulteration at a level lower than 1%. Species-specific peptides from bovine β-lactoglobulin and αS1 casein were identified as suitable peptide markers of milk authenticity.

1. Introduction

Food fraud is recognized as a worldwide risk. The addition of low cost ingredients or additives and the removal or replacement of authentic substances create not only economical lost, but also health hazards. One of the most widely known food fraud is adulteration of dairy products (Moore, Spink, & Lipp, 2012) that mainly consists in the addition of: i) chemicals containing high percentage of fat, protein or carbohydrate such as melamine, sucrose, etc.; ii) substances extracted from milk itself, such as cheese whey and milk powder; iii) water, which can dilute the normal milk resulting in the decrease of its nutritional quality (Liu, Ren, Liu, & Guo, 2015). Another common form of milk adulteration is based on the addition of lower priced cow milk to a more valuable milk from goat, buffalo, yak or camel. In addition to an increased economical profit to the detriment of consumers, this fraudulent substitution can cause severe adverse health effects in people suffering from milk allergies.

Several analytical methods, including capillary electrophoresis (Herrero-Martínez, Simó-Alfonso, Ramis-Ramos, Gelfi, & Righetti, 2000), polymerase chain reaction (Guerreiro, Fernandes, & Bardsley, 2012), enzyme-linked immunosorbent assays (Hurley, Elyse Ireland, Coleman, & Williams, 2004) and two-dimensional gel electrophoresis (2-DE) (Hinz, O'Connor, Huppertz, Ross, & Kelly, 2012; Roncada et al., 2012), have been used to evaluate the content of mixtures of milk of different species. Among these techniques, multiplex PCR could be used to simultaneous detection of cow, sheep, goat, and buffalo milk in different types of dairy products, allowing to differentiate cheeses made from different animal sources at a level of 0.1%. (De et al., 2011; Gonçalves, Pereira, Amorim, & van Asch, 2012). Although DNA based methods are characterized by rapidity, sensitivity, and moderate simplicity in amplifying DNA, some drawbacks could occur. Indeed, a preliminary step for DNA extraction is always required and it is important to know the exact sequences, which flank both ends of the target DNA region (Luykx & van Ruth, 2008). Additionally, crossed reactions may take place when DNA fragments of similar species within a sample were used, and lower amplification is obtained from the more fragmented DNA (Kamal & Karoui, 2015).

During the past decades, proteomics has been extensively used in the field of research on milk proteins in relation to the technological treatments and species–species differences (Abd El-Salam, 2014; Bernardi et al., 2015; Calvano, De Ceglie, Monopoli, & Zambonin, 2012; Calvano, Monopoli, Loizzo, Faccia, & Zambonin, 2013; Chen, Chang, Chung, Lee, & Ling, 2004; Cunsolo, Muccilli, Saletti, & Foti, 2013; Di Girolamo et al., 2014; Hrbek, Vaclavik, Elich, & Hajslova, 2014; MacMahon, Begley, Diachenko, & Stromgren, 2012; Motta et al., 2014; Nicolaou, Xu, & Goodacre, 2011; Russo, Rega, & Chambery, 2016; Russo et al., 2012; Sforza et al., 2008; Yang et al., 2014). The presence of cow milk was detected in goat milk using β-lactoglobulin as the molecular marker (Chen et al., 2004) or by targeted proteomics methods based on MALDI-TOF (Calvano et al., 2012; Yang et al., 2014). Enhanced selectivity and sensitivity was obtained by multiple reaction monitoring (MRM) scan function in detecting buffalo mozzarella adulteration (Russo et al., 2012). Then, a targeted quantitative analysis of the phosphorylated β-casein f33-48 peptide, identified as a novel

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species-specific proteotypic marker, was proposed by LC coupled with a triple quadrupole (Russo et al., 2012).

The problem of an exact quantification of the adulteration level can be regarded as being of rather little concern, since a fraudulent addition of cow milk to higher priced milks under 5% adulteration level would not make any economic sense (Czerwenka, Műller, & Lindner, 2010). Nevertheless, it is really important to be able to detect allergens at low concentrations, since even a small, not-intentional allergen cross-contamination, accidently occurred during food processing and not indicated in the food label, could represent a serious burden for allergic patients. Moreover, for a quick assessment of food traceability and frauds control, it is essential to have cheap and easy-to-use screening tools. Therefore, in spite of the recent strategies proposed for the species identification in milk-based products, there is still a growing demand of new methodologies.

In this work, an ion-trap based proteomic method for the detection of milk adulteration by a low-cost cow milk is proposed. On purpose additions of 1–50% levels of cow milk in goat milk were carried out to simulate fraudulent additions or unintentional contaminations. Then, few microliters of cow-goat milks were submitted to a simultaneous multi-enzyme in-solution digestion by trypsin-chymotrypsin. A direct analysis of the peptide mixtures was performed by nanoLC-ESI-IT-MS/MS, coupled with a post-processing internal validation of MS/MS dataset, based on peptide ion and normalized delta scores. Then, milk animal source was quickly determined by the direct analysis of the tryptic/chymotryptic digests, avoiding the laborious protein separation by two-dimensional electrophoresis. The final objective of our non-targeted approach was the identification of bovine species peptide markers. Hence, the proposed proteomic method represents the first step for the development of rapid screening methods to be used to evaluate milk authenticity.

2. Materials and methods

2.1. Chemicals

Formic acid (FA) (≥98%), ammonium bicarbonate, 1,4-dithiothreitol and iodoacetamide were purchased from Sigma-Aldrich. Water and acetonitrile (ACN) (LC-MS CHROMASOLV®, ≥99.9%) were from Fluka. Modified trypsin (porcine) and chymotrypsin (bovine) were purchased from Princeton Separations (Adelphia, NJ, USA).

2.2. Samples

Goat and cow UHT milk samples of a single commercial brand were selected for the experiments from local supermarket and analyzed in quadruplicate (n = 4). Binary mixtures of them were prepared in duplicate by adding cow milk in goat milk at a percentage of 1%, 2%, 5%, 20% and 50%.

2.3. Enzymatic digestion of proteins from milk

The procedure for the in-solution enzymatic digestion of milk samples was that described by Azarnia et al. with slight modifications (Azarnia, Boye, Mongeo, & Sabik, 2013) by applying a multi-enzyme approach (Nardiello, Palermo, Natale, Quinto, & Centonze, 2015). Briefly, 50 μL of milk (goat and cow pure commercial milk and their binary mixtures) were mixed with 100 μL of 50 mM ammonium bicarbonate. Then, to an aliquot of 10 μL of the obtained solution, 50 μL of 50 mM ammonium bicarbonate were added. After addition of 2 μL of 0.1 M dithiothreitol, the solution was incubated for 30 min at 50 °C and then cooled down at room temperature. A volume of 4 μL of 0.1 M iodoacetamide was added and the solution was incubated in the dark at room temperature for 40 min. Afterwards, the mix trypsin/chymotryspin (1:1, w:w) prepared in 25 mM ammonium bicarbonate was added to the sample solution at a 1:50 (w:w) ratio. After an overnight incubation at 37 °C, the digestion was stopped by adding 5 μL of a 5% FA water solution.

2.4. Liquid chromatography and mass spectrometry analysis

Chromatographic analyses were performed by a nanoLC apparatus, Ultimate 3000 (Dionex LC-Packings, Amsterdam, The Netherlands), which consisted of an autosampler, a low pressure gradient micro-pump series equipped with flow managers, a column thermostat and a UV detector set at 214 nm. The UV flow cell was connected to an ESI-Ion Trap HCTultra ETD II Basic System (Bruker Daltonics Srl, Bremen, Germany). The nanoLC-ESI-IT-MS/MS system was controlled by softwares Chromeleon CHM-1 (Dionex) and Hystar 2.3 (Bruker Daltonics). A PepMap C18 nano trap column (300 μm i.d. × 5 mm, 5-μm particle size, LC Packings) was used for concentrating and desalting the injected sample. Chromatographic separations were carried out by a PepMap C18 analytical column (15 cm length × 75 μm i.d., 3-μm particle size, 100-Å pore diameter; LC Packings). The mobile phase consisted of 0.1% formic acid (FA) in water (eluent A) and ACN/0.1% FA in water (80:20 v/v) (eluent B). Sample elution through the analytical column was obtained at a flow rate of 0.300 μL min^{-1}. The optimized elution gradient was the following: 6 min isocratic step at 96% A and 4% B; 120 min linear gradient to 10% A and 90% B; 10 min isocratic step at 10% A and 90% B; 1 min with the initial mobile phase composition (96% A and 4% B). The system was then re-equilibrated for 43 min, with a total run-time of 180 min. The microliter pick-up injection mode was selected, and 4 μL of sample were mixed to the eluent A to overfill the 10 μL sample loop. A 10-port switching valve was used to combine sampling and switching functions. Then, the gradient elution was coupled with a single injection mode, since the opening isocratic step, with trap and analytical column mutually disconnected, allowed an efficient sample pre-concentration and desalting. Therefore, no loading process onto the trap column and no multiple injections for each protein digest were required prior to separation: this aspect is especially valuable for the analysis of small sample amounts. After 6 min from the sample injection, the 10-port valve was switched in order to connect online the trap column with the analytical column.

Mass spectrometry analyses were performed by using a high capacity ion trap, coupled to a nano-ESI source, operating in the positive ion mode. The following parameters were set: spray voltage 4.5 kV; sheath gas (nitrogen) flow rate 10 L min^{-1}; capillary voltage 1.5 V; heated capillary temperature 160 °C. A full scan MS acquisition in the 300–1500 m/z range was performed with the acquisition of Base Peak Chromatogram (BPC) and Total Ion Current (TIC) profile. MS/MS analysis by Collision-Induced Dissociation (CID) was performed using unattended data-dependent acquisition mode and the auto-MS/MS event (scan range 100–3000 m/z) was carried out with a number of precursor ions of 3. The minimal signal required for precursor ion selection was set to an absolute threshold of 10000. The Ion Charge Control (ICC) was on and set at a target of 200,000 and a maximum accumulation time of 100 ms. The duty cycle time was 180 ms and 220 ms for the MS and MS/MS mode, respectively. The fragmentation was performed by activating the options MS/MS fragmentation amplitude (MS/MS FragAmpl™ 1.00 V) and smart fragmentation (SmartFrag™ 30–200%).

2.5. Data analysis and database search

Extraction of mass spectra peak-lists from chromatograms, mass annotation and deconvolution were performed by using Data Analysis 4.0 (Bruker Daltonics). The acquired MS and MS/MS datasets were submitted to database searches by using Biotools 3.2 (Bruker Daltonics) and MASCOT search engine (Matrix Science, London, UK). The data analysis files were used to search entries under the Other Mammalia category of Swiss-Prot database, assuming that peptides were monoisotopic and carbamidomethylated at cysteine residues. A maximum

number of 2 missed cleavages were allowed and, for both precursor peptide ion and MS/MS tolerance, a peptide tolerance of 0.3 Da was set in the error window to match the peptide mass values. The option "automatic error tolerant" search was checked to discover unsuspected chemical and post-translational modifications, sequence variants and non-specific cleavage products. MS/MS spectra of matched peptides were carefully evaluated in terms of peptide ion score (PIS), rank and normalized delta score (nΔs, i.e. the difference between the best and the second best ion score, divided by the best score) as described in our previous work (Nardiello, Natale, Palermo, Quinto, & Centonze, 2016).

3. Results and discussion

3.1. Database post-processing validation of MS/MS data sets of milk samples

Samples of pure commercial UHT milk (100% goat and 100% cow) were analyzed as a starting point to get a reference list of peptides, before analyzing goat-cow milk mixtures. A direct analysis of milk tryptic/chymotryptic digests was carried out by nanoLC-ESI-IT-MS/MS. Then, an in-depth evaluation of peptide scoring parameters was performed to assess the goodness of fit between experimental and theoretical data. Indeed, as previously reported, in order to partially overcome the intrinsic limits of ion-trap and obtain a confident sequence assignment by a low resolution MS/MS dataset, the description of user-specified rules is essential (Nardiello et al., 2016). Certainly, the definition of a scoring criterion is a fundamental issue in proteomics when a flexible database searching based on the error tolerant mode is adopted in combination with a low-specificity enzyme (combined and simultaneous use of trypsin and chymotrypsin) to maximize sequence coverage and capture additional information. Since specific software packages, such as Percolator, cannot be used in ion-trap proteomics/error tolerant searching mode, when focused on the specific protein characterization in small real sample data sets, we proposed a cut-off level based on a linear combination between peptide ion score (PIS) and normalized delta score (nΔs), as an acceptance threshold for confident sequence assignments. Therefore, for each Peptide Spectrum Matches (PSM) of the complete peptide list obtained for the milk samples by MASCOT database searching, the linear combination nΔs + %PIS was calculated. Then, these values were listed in a spreadsheet and filtered on the base of the threshold value of 0.73, established under the adopted experimental set-up (enzymatic digestion by trypsin/chymotrypsin, error tolerant searching against Swiss Prot/Other Mammalia database). Finally, the validated PSMs were reviewed by rejecting unacceptable error tolerant modifications because meaningless or associated to forced single-point mutations and alterations leading to the formation of isobaric or near-isobaric residues in the ion trap. (Further details and a schematic workflow diagram for reviewing the MASCOT peptide lists are available in our previous work, Nardiello et al., 2016). A mini database displaying the primary structure of the six most abundant milk proteins (β-lactoglobulin, αS1-casein, β-casein, α-lactalbumin, αS2-casein, k-casein) from cow, buffalo, goat and sheep was inserted in a worksheet and used to check the correspondence of each validated PSM to one (species-specific peptides) or more (degenerate peptides) animal species.

The whole post-searching validation process of the MS/MS data set was applied to the analysis of UHT goat milk. The MASCOT protein view report showed the identification of the six milk proteins with high scores up to 4000. For each identified protein, the MASCOT protein suggested the presence of four variants coming from cow, buffalo, goat or sheep, associated with comparable score values for the animal couples goat/sheep and cow/buffalo. Although the highest score was always associated to proteins from goat, the effective milk animal source could not be deduced on the simple basis of the MASCOT score values and the analyzed sample could be ascribed, indifferently, to cow, buffalo, goat or sheep milk or to mixes of them. Nevertheless, after post-processing validation of the MS/MS dataset, with the elimination of the not-validated peptide sequences, only for the hits corresponding to the caprine proteins, the presence of species-specific peptides was observed. Then, through the validated PSM list displaying 10 goat species-specific peptides (3 from β-casein in the region f144-163 of the mature protein sequence, 3 from β-lactoglobulin -f123-138, 2 from αS2-casein -f91-101, 1 from αS1-casein -f4-22 and α-lactalbumin -f80-93, none from k-casein), it was possible to deduce the milk origin. On the contrary, the validated PSMs associated to the milk proteins from buffalo, goat and sheep actually corresponded to degenerate peptides that are common to all the four animal species and were sequenced by the same MS/MS spectrum queries already processed for the caprine protein hit.

Analogues considerations are also valid for the analysis of 100% cow milk, leading to the identification of the six most abundant proteins, each as coming from cow (first hit) or buffalo, goat and sheep milks (secondary hits). Therefore, the simple MASCOT search results protein view did not allow to fully discriminate between the animal species. Anyway, once again, only for the proteins from cow milk, amino acidic sequences specifically belonging to the bovine species were identified. Indeed, all the amino acidic sequences associated to the other animal species referred to the degenerate peptides coming from the same MS/MS spectra already valid and sequenced for the bovine origin proteins. Then, the characterization of the cow species-specific peptides allowed the exclusion of the milk proteins from the other animal species, leading to the final identification of the analyzed sample as of bovine origin, as reported in the milk label. In Fig.1 the primary structure of the six identified proteins in pure goat and cow milk are shown with highlighting the validated species-specific peptides. In the aligned milk proteins, other mismatches in amino acid sequence between goat and cow milk can be observed among the validated amino acidic strings. Anyway, these peptides cannot be included in the exclusive lists of goat and cow species-specific peptides; indeed, after their localization in the specific protein regions using our home-made milk protein database, it was noted that the same sequences are shared in the proteins of the animal couples goat/sheep and cow/buffalo.

3.2. Protein analysis in binary goat-cow milk mixtures

The evaluation of milk origin was performed on binary mixtures of goat/cow UHT milk samples, with an adulteration level ranging from 1 to 50%. As an illustrative example, in Fig. 2 the chromatographic profiles of goat milk adulterated by 20% cow milk are shown. Despite of the matrix complexity, good results in terms of peak widths and resolution were observed, also corroborated by the excellent MASCOT scores for the protein hits (ranging from 193 to 1520) and the high number of identified peptides (n = 102), corresponding to protein sequence coverages in the range 33–82%. Such an information amount can be quickly obtained by a single LC-MS/MS run, without resorting to two-dimensional electrophoresis that is usually performed for protein separation before ion-trap proteomic analysis. Additionally, the post-processing validation times, based on the evaluation of scoring parameters easily deduced from the MASCOT protein view, are quite short. Indeed, data analysis of the eluted peptides can start before the end of the chromatographic run, during the time needed for column washing and re-equilibration to the initial mobile phase composition. Then, although the gradient program is 180 minute-long, both database searching and post-search validation can be performed as soon as MS/MS spectra of milk peptides are acquired during the analysis, usually within 60 min from the injection.

In each goat-cow milk binary sample, the identification of the six most abundant milk proteins (β-casein, αS2-casein, αS1-casein, k-casein, α-lactalbumin, and β-lactoglobulin) was obtained. For all the mixtures, a conclusive correspondence of the milk proteins to the effective two animal sources was established only by post-processing validation of the MS/MS dataset. Indeed, among the validated PSMs the

β-casein

CASB_CAPHI (code: P33048)

```
1   MKVLILACLV ALALAREQEE LNVVGETVES LSSSEESITH INKKIEKFQS
51  EEQQQTEDEL QDKIHPFAQA QSLVYPFTGP IPNSLPQNIL PLTQTPVVVP
101 PFLQPEIMGV PKVKETMVPK HKEMPFPKYP VEPFTESQSL TLTDVEKLHL
151 PLPLVQSWMH QPPQPLSPTV MFPPQSVLSL SQPKVLPVPQ KAVPQRDMPI
201 QAFLLYQEPV LGPVRGPFPI LV
```

CASB_BOVIN (code: P02666)

```
1   MKVLILACLV ALALARELEE LNVPGEIVES LSSSEESITR INKKIEKFQS
51  EEQQQTEDEL QDKIHPFAQT QSLVYPFPGP IPNSLPQNIP PLTQTPVVVP
101 PFLQPEVMGV SKVKEAMAPK HKEMPFPKYP VEPFTESQSL TLTDVENLHL
151 PLPLLQSWMH QPHQPLPPTV MFPPQSVLSL SQSKVLPVPQ KAVPYPQRDM
201 PIQAFLLYQE PVLGPVRGPF PIIV
```

α-S1-casein

CASA1_CAPHI (code: P18626)

```
1   MKLLILTCLV AVALARPKHP INHRGLSPEV PNENLLRFVV APFPEVFRKE
51  NINELSKDIG SESTEDQAME DAKQMKAGSS SSSEEIVPNS AEQKYIQKED
101 VPSERYLGYL EQLLRLKKYN VPQLEIVPKS AEEQLHSMKE GNPAHQKQPM
151 IAVNQELAYF YPQLFRQFYQ LDAYPSGAWY YLPLGTQYTD APSFSDIPNP
201 IGSENSGKTT MPLW
```

CASA1_BOVIN (code: P02662)

```
1   MKLLILTCLV AVALARPKHP IKHQGLPQEV LNENLLRFFV APFPEVFGKE
51  KVNELSKDIG SESTEDQAME DIKQMEAESI SSSEEIVPNS VEQKHIQKED
101 VPSERYLGYL EQLLRLKKYK VPQLEIVPNS AEERLHSMKE GIHAQQKEPM
151 IGVNQELAYF YPELFRQFYQ LDAYPSGAWY YVPLGTQYTD APSFSDIPNP
201 IGSENSEKTT MPLW
```

α-S2-casein

CASA2_CAPHI (code: P33049)

```
1   MKFFIFTCLL AVALAKHKME HVSSSEEPIN IFQEIYKQEK NMAIHPRKEK
51  LCTTSCEEVV RNANEEEYSI RSSSEESAEV APEEIKITVD DKHYQKALNE
101 INQFYQKFPQ YLQYPYQGPI VLNPWDQVKR NAGPFTPTVN REQLSTSEEN
151 SKKTIDMEST EVFTKKTKLT EEEKNRLNFL KKISQYYQKF AWPQYLKTVD
201 QHQKAMKPWT QPKTNAIPYV RYL
```

CASA2_BOVIN (code: P02663)

```
1   MKFFIFTCLL AVALAKNTME HVSSSEESII SQETYKQEKN MAINPSKENL
51  CSTFCKEVVR NANEEEYSIG SSSEESAEVA TEEVKITVDD KHYQKALNEI
101 NQFYQKFPQY LQYLYQGPIV LNPWDQVKRN AVPITPTLNR EQLSTSEENS
151 KKTVDMESTE VFTKKTKLTE EEKNRLNFLK KISQRYQKFA LPQYLKTVYQ
201 HQKAMKPWIQ PKTKVIPYVR YL
```

k-casein

CASK_CAPHI (code: P02670)

```
1   MMKSFFLVVT ILALTLPFLG AQEQNQEQPI CCEKDERFFD DKIAKYIPIQ
51  YVLSRYPSYG LNYYQQRPVA LINNQFLPYP YYAKPVAVRS PAQTLQWQVL
101 PNTVPAKSCQ DQPTTLARHP HPHLSFMAIP PKKDQDKTEV PAINTIASAE
151 PTVHSTPTTE AIVNTVDNPE ASSESIASAS ETNTAQVTST EV
```

CASK_BOVIN (code: P02668)

```
1   MMKSFFLVVT ILALTLPFLG AQEQNQEQPI RCEKDERFFS DKIAKYIPIQ
51  YVLSRYPSYG LNYYQQKPVA LINNQFLPYP YYAKPAAVRS PAQILQWQVL
101 SNTVPAKSCQ AQPTTMARHP HPHLSFMAIP PKKNQDKTEI PTINTIASGE
151 PTSTPTTEAV ESTVATLEDS PEVIESPPEI NTVQVTSTAV
```

α-lactalbumin

LALBA_CAPHI (code: P00712)

```
1   MMSFVSLLLV GILFHATQAE QLTKCEVFQK LKDLKDYGGV SLPEWVCTAF
51  HTSGYDTQAI VQNNDSTEYG LFQINNKIWC KDDQNPHSRN ICNISCDKFL
101 DDDLTDDIVC AKKILDKVGI NYWLAHKALC SEKLDQWLCE KL
```

LALBA_BOVIN (code: P00711)

```
1   MMSFVSLLLV GILFHATQAE QLTKCEVFRE LKDLKGYGGV SLPEWVCTTF
51  HTSGYDTQAI VQNNDSTEYG LFQINNKIWC KDDQNPHSSN ICNISCDKFL
101 DDDLTDDIMC VKKILDKVGI NYWLAHKALC SEKLDQWLCE KL
```

β-lactoglobulin

LACB_CAPHI (code: P02756)

```
1   MKCLLLALGL ALACGIQAII VTQTMKGLDI QKVAGTWYSL AMAASDISLL
51  DAQSAPLRVY VEELKPTPEG NLEILLQKWE NGECAQKKII AEKTKIPAVF
101 KIDALNENKV LVLDTDYKKY LLFCMENSAE PEQSLACQCL VRTPEVDKEA
151 LEKFDKALKA LPMHIRLAFN PTQLEGQCHV
```

LACB_BOVIN (code: P02754)

```
1   MKCLLLALAL TCGAQALIVT QTMKGLDIQK VAGTWYSLAM AASDISLLDA
51  QSAPLRVYVE ELKPTPEGDL EILLQKWENG ECAQKKIIAE KTKIPAVFKI
101 DALNENKVLV LDTDYKKYLL FCMENSAEPE QSLACQCLVR TPEVDDEALE
151 KFDKALKALP MHIRLSFNPT QLEEQCHI
```

Fig. 1. Primary structure of proteins from goat and cow milk; the gray strings are the signal peptides. Protein code according to Swiss Prot database. Bold sequences correspond to validated peptides identified by LC-ESI-IT-MS/MS analyses of pure goat and cow milk samples. Goat and cow species-specific peptides are highlighted.

presence of both goat and cow species-specific peptides was observed, whereas for the hits associated to sheep and buffalo milk, only degenerate peptides were obtained. In Fig. 3 the bar chart showing the total number of validated peptides against the percentage of cow milk is reported (including the extreme values, 0%, pure goat milk, and 100%, pure cow milk). As expected, the number of the identified cow species-specific peptides increased progressively up to a maximum value of 13 at 100% adulteration level. The complete list of the cow species-specific peptides is shown in Table 1. Peptides were accepted and included in the list only if associated to validated PSMs in each replicate of the multiple injection (at least twice) of the same sample. Only two peptides were observed since starting from the lowest adulteration level of 1%: the sequences LSFNPTQLEEQCHI from β-lactoglobulin (f149–162 of the mature form protein) and HQGLPQEVLNENLLR from αS1-casein (f8-22). As an example, Fig. 4 shows the MS/MS spectra of the two peptides together with the corresponding fragmentation pattern and delta scores, demonstrating a reliable sequence assignment. It is worth noting that these peptides are included in the IgE binding epitopes (LNENLLRFFVAPFPEVFGKE, NENLLRFFVAPFPEVFGKEK, VFGKEKVNELSKDIGSESTE from αs1-casein, and ALPMHIRLSFNPTQ-LEEQCHI from β-lactoglobulin) of the two major allergens (exactly bovine αs1-casein and β-lactoglobulin) in transient and persistent

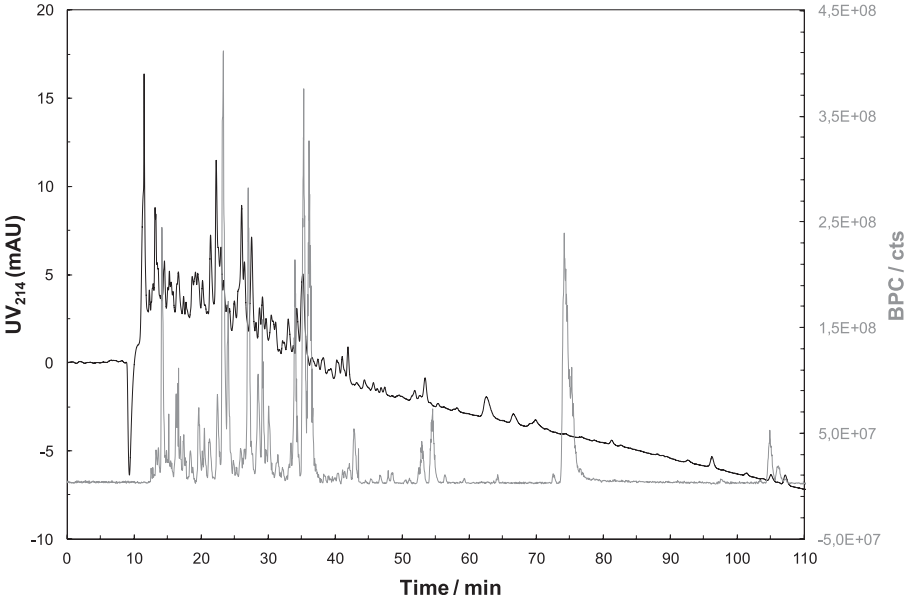

Fig. 2. Direct shot-gun nanoLC-ESI-IT-MS/MS analysis, UV profile at 214 nm (black line) and Base Peak Chromatogram, BPC (gray line) of a goat milk sample adulterated by adding cow milk at a percentage of 20%.

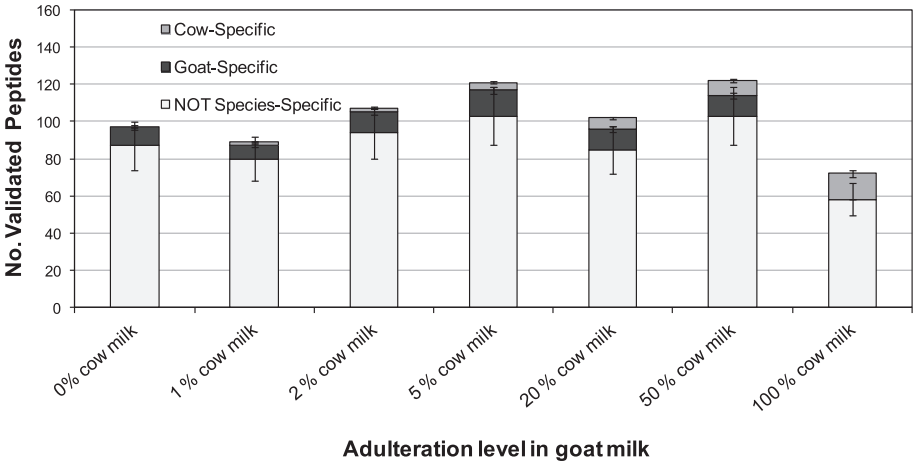

Fig. 3. Total number of identified peptides (not species-specific, cow species-specific and goat species-specific) by validated PSMs in binary goat-cow milk mixtures. The error bars are the associated standard deviations (n = 4).

Table 1
Cow specific peptides identified in binary mixtures of cow-goat milk.

Sequence	m/z	Position*	Protein	Adulteration Level**
R.LSFNPTQLEEQCHI.-	858.3; 572.5	149–162	β-lactoglobulin	1%; 2%; 5%; 20%; 50%; 100%
K.GYGGVSLPEWVCTTF.H	836.8	17–31	α-lactalbumin	5%; 50%; 100%
Y.GGVSLPEWVCTTF.H	726.8	19–31	α-lactalbumin	100%
K.HQGLPQEVLNENLLR.F	880.4; 587.2	8–22	αS1-casein	1%; 2%; 5%; 20%; 50%, 100%
K.YKVPQLEIVPNpSAEER.L	651.2; 976.5	104–119	αS1-casein	20%; 50%; 100%
Y.KVPQLEIVPNpSAEER.L	894.8; 596.9	105–119	αS1-casein	100%
K.VPQLEIVPNSAEER.L	790.8	106–119	αS1-casein	100%
L.GTQYTDAPSFSDIPNPIGSENSEK.T	852.0	170–193	αS1-casein	100%
Y.TDAPSFSDIPNPIGSENSEK.T	1052.9	174–193	αS1-casein	20%; 50%; 100%
K.NMAINPSKENLCSTF.C	863.3	25–39	αS2-casein	20%; 50%; 100%
K.FALPQYLK.T	490.3	174–181	αS2-casein	5%; 20%; 50%; 100%
W.MHQPHQPLPPTVMFPPQSVL.S	761.1	144–163	β-casein	50%; 100%
W.QVLSNTVPAK.S	528.7	77–86	k-casein	100%

* Referred to the mature protein sequence.
** Percentage of cow milk in the binary mixtures of cow-goat milk.

allergic patients (Monaci, Tregoat, van Hengel, & Anklam, 2006).

Our results confirm the role of the peptides LSFNPTQLEEQCHI and HQGLPQEVLNENLLR as candidate species-specific markers in detecting the presence of bovine milk in food products, even at a lower adulteration percentage than that reported in the literature (Calvano et al., 2012; Russo et al., 2012). Indeed, we observed the presence of both these specific peptides at an adulteration level of 1%, against the identification of the αS1-casein and β-lactoglobulin peptides at 5% and 10%, respectively. In addition, our proposed signature peptides have the advantage of belonging exclusively to the cow species-specific strings, not shared in milk proteins from different animal sources. Therefore, they are effective molecular markers of milk adulteration/contamination by low-cost bovine milk. Their analytical determination could be successfully applied to detect similar adulterations in different kinds of milk-based products, expanding the application range compared to the proteotypic marker (the phosphorylated β-casein peptide FQSEEQQQTEDELQDK), recently used for the detection of adulteration in buffalo mozzarella (Russo et al., 2012). Indeed, this f33-48 peptide from β-casein is a degenerate sequence (common to the species cow, goat and sheep) and different by a single residue from the corresponding peptide from buffalo milk (FQSEEQQQMEDELQDK). Therefore, the β-casein peptide cannot be considered an universal marker for any kind of intentional or not contamination; it can be used only for the evaluation of the presence in cheese samples of undeclared milk of different origin from buffalo, then not allowing to distinguish the cow milk from that of goat or sheep.

A pseudo-quantitative evaluation was carried out by extracting the peptide characteristic ion current chromatogram (EIC) of the double charged ions at m/z 858.3 and 880.4 for the peptide sequence LSFNPTQLEEQCHI and HQGLPQEVLNENLLR, respectively. Then, the peak area integration from the EIC profile were performed for each milk binary mixture. The semi-quantitative determinations were performed on duplicate samples, each of them injected twice within 5 days, and the relative standard deviation (RSDr %) under repeatability conditions was calculated, obtaining values ranging from 9 to 28%, due to the variability of the MS measurements in ion trap. Fig. 5 reports the fits relevant to the two candidate peptide markers of cow milk in goat milk as a function of the percentage of cow milk; the correlation coefficients estimated for the linear relationship were higher than 0.9930.

In the complex overview of the strategies reported for the evaluation of milk authenticity, our proteomic approach is characterized by analysis speed and reduced instrumental costs. Indeed, the proposed method is quite rapid, with the only exception of the digestion step that, however, is usually performed overnight, and then does not represent a real extra-time of analysis. Moreover for the protein characterization few microliters of sample are required and directly analyzed by a single shot-gun analysis, without a previous separation. In addition to a direct evaluation of milk traceability at low level of adulteration, this method has confirmed the identification of two effective signature peptides related to bovine milk source, allowing the detection of hidden allergens at adulteration levels down to 1% This is an important aspect, since although adulterations lower than 5% are not economically convenient, sensitive analytical tools for the detection of minimal amounts of bovine milk are highly desirable to preserve consumers' health. Indeed, also an accidental contamination could lead to serious sanitary consequences, because consumers can be exposed to hidden allergens as, for example, cow milk proteins, especially αs1-casein and β-lactoglobulin. Proper analytical methods for the selective determination of

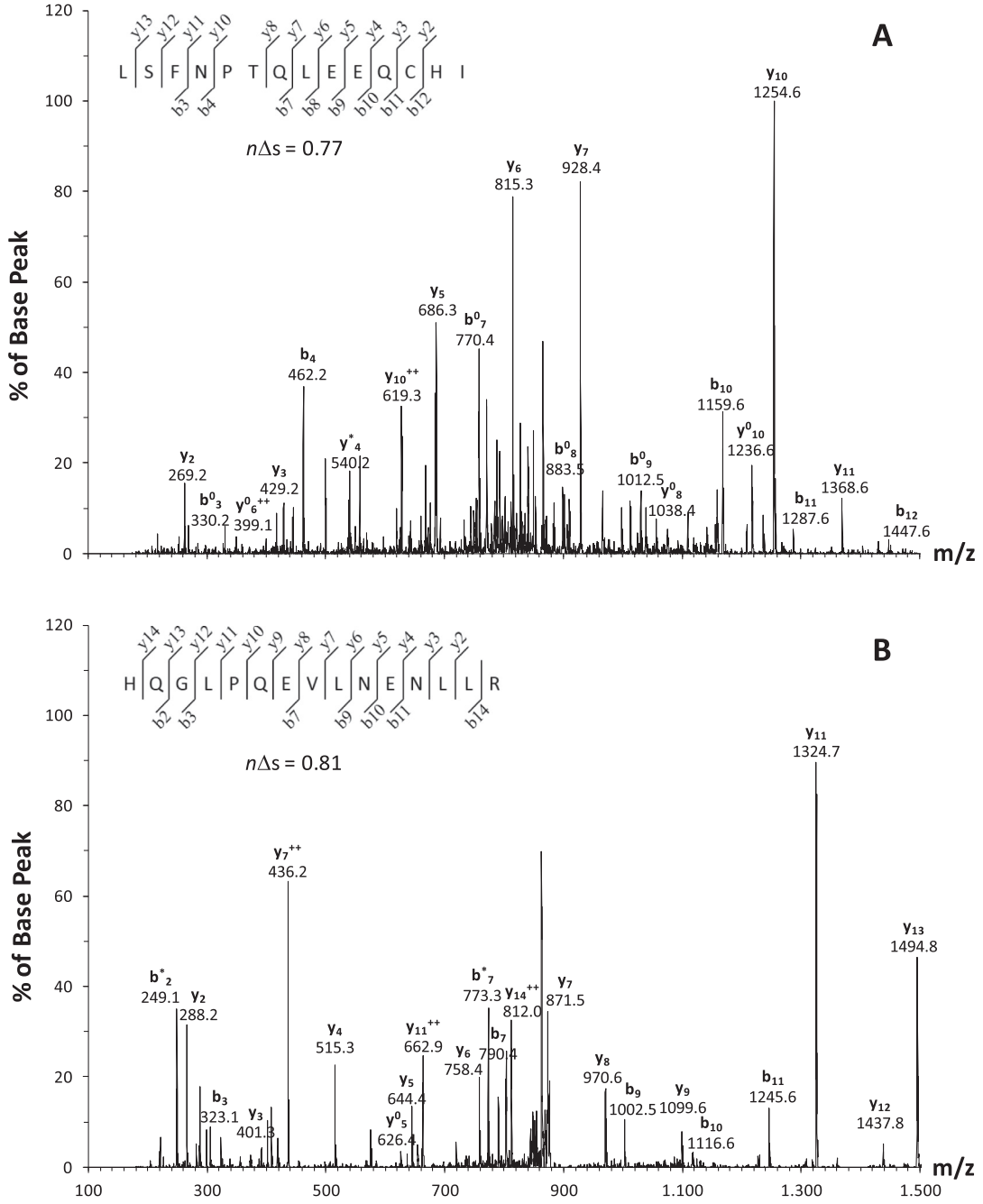

Fig. 4. MS/MS spectrum of the double charged peptide ion: (A) LSFNPTQLEEQCHI at m/z 858.3 from β-lactoglobulin and (B) HQGLPQEVLNENLLR at m/z 880.4 from αS1-casein in goat-milk adulterated by 1% cow milk.

the peptides LSFNPTQLEEQCHI and HQGLPQEVLNENLLR could be developed for online or large-scale analyses, then *ad-hoc* low-cost assays or on-line sensors could be quickly used for dairy process monitoring and product quality assessment. Therefore, the present study has had the double aim of proposing a single direct method for the detection of milk adulteration and establishing the basis for the development of targeted and easy-to-use screening analytical methods.

4. Conclusions

In this work, a proteomic approach is described for the species identification in milk samples by nanoLC – ESI-IT-MS/MS coupled with a database post-processing to validate peptide sequence assignments. Milk authenticity was quickly confirmed by a single direct analysis of the tryptic/chymotryptic digest, without resorting to two-dimensional electrophoresis, generally required for protein separation before MS analysis in ion-trap. The post-processing validation process is simply based on a evaluation of data, that can be easily obtained from the MASCOT report, with no need of specific statistical software. The identification of species-specific amino acidic sequences among the validated peptide spectrum matches has allowed to fully discriminate between the animal species. Bovine species-specific peptides coming from β-lactoglobulin and αS1 casein are proteotypic markers that allow to detect the presence of cow milk in goat milk at an adulteration level down to 1%.

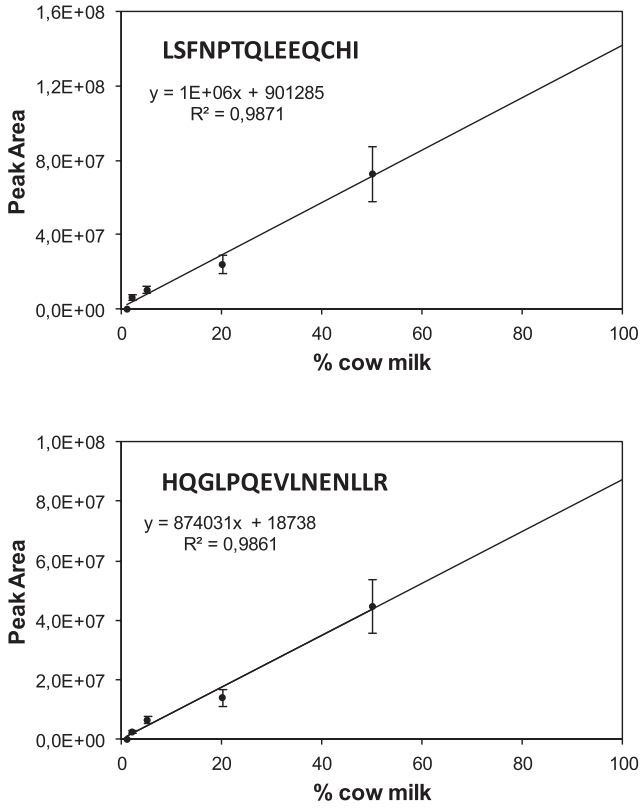

Fig. 5. Fits relevant to the specific markers of cow milk in goat milk against the percentage of cow milk. The error bars are the associated standard deviations (n = 4).

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