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Characterization of the major whey proteins from milk of Mediterranean water buffalo (Bubalus bubalis)

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1 **Abstract**

2 In this work, the whey protein fractions from 120 Mediterranean water buffalo individual milks

3 were analysed by microchip electrophoresis (MCE), reverse-phase high-performance liquid

4 chromatography (RP-HPLC) and mass spectrometry (ESI-MS). Validation procedures were carried

5 out for both MCE and HPLC. The chromatographic analysis allowed the complete separation of the

6 whey protein fractions, resulting in a well-defined peak structure; the adopted RP-HPLC and ESI-

7 MS protocols provided identification of β-lactoglobulin (18,266 Da), α-lactoalbumin (14,236 Da)

8 and serum albumin (66,397 Da). The calculated mean concentrations were 4.04 g/l, 2.45 g/l and

9 0.35 g/l, respectively.

10

14

1. Introduction

Whey is a biological fluid derived from milk. It is rich in proteins and peptides which play a crucial role in the innate immunity of the progeny (Zimecki & Kruzel, 2007). The protein components of whey include serum albumin (SA), α-lactalbumin (α-LA), β-lactoglobulin (β-LG) and immunoglobulins (Fox, 1989). Besides, the minor proteins include lactoferrin, lactoperoxidase and lysozyme, which have important antimicrobial and carrier functions (Marshall, 2004; Parodi, 2007). The primary structures are known for β-lactoglobulin and α-lactalbumin; their genetic properties and factors responsible for quantitative variability in their expression, are reported in the literature (Farrell et al., 2004). The physicochemical characterization of whey proteins can be achieved by a combination of electrophoretic, chromatographic and spectroscopic methods (Andreotti, Trivellone, & Motta, 2006; Bonfatti, Grigoletto, Cecchinato, Gallo, & Carnier, 2008; Bonizzi, Buffoni, & Feligini, 2009; Bordin, Cordeiro Raposo, de la Calle, & Rodriguez, 2001; Bramanti, Sortino, Onor, Beni, & Raspi, 2003; D'Ambrosio et al., 2008; Elgar et al., 2000; El-Zahar et al., 2004; Vallejo-Cordoba, González-Córdova, & Olguin-Arredondo, 2008). On the other hand, recent advances in chip-based separation of proteins (microchip electrophoresis, MCE) have provided an easier system than conventional gel electrophoresis with higher throughput (Anema, 2009). Despite cow milk proteins having been extensively studied, there still is a substantial lack of characterization, and particularly of quantitative information, about whey proteins from water buffalo. Several studies on whey proteins have determined the primary structures of genetic variants of b-LG (Vohra, Kumar Bhattacharya, Dayal, Kumar, & Sharma, 2006) and a-LA (Chianese et al., 2004; Fan, Li, & Wu, 2000; Ramesha, Khosravinia, Gowda, & Rao, 2008). Even after these reports, quantitative data about whey proteins from Mediterranean water buffalo (the milk of which is mostly used to produce the highly-valued PDO Mozzarella cheese) are evidently unavailable. Milk protein content can be influenced by physiological and nutritional factors, but several studies have indicated that genetic context plays the most important role in the control of milk composition (Martin, Szymanowska, Zwierchowski, & Leroux, 2002). The main goal of this work was to obtain new data on the quali-

1 quantitative composition of the major whey proteins in Mediterranean water buffalo milk, useful for

2 animal selection purposes. An additional aim of this work was to test the performance of the MCE

3 technique for the routine analysis of water buffalo whey proteins.

4

5 **2. Materials and methods**

6

7 *2.1. Materials and reagents*

8 Commercial purified whey proteins, β-LG (purity 80%), α-LA (purity 85%), SA (purity 98%) from

9 bovine milk, and trifluoroacetic acid (TFA) were supplied by Sigma–Aldrich (St. Louis, MO, USA).

10 HPLC-grade water and acetonitrile were purchased from VWR International (West Chester, PA,

11 USA). MCE of whey protein was performed using the Bioanalyzer Protein 80 kit (Agilent

12 Technologies, Waldbronn, Germany) that contains chips and proprietary reagents (sieving gel

13 matrix, protein dye concentrate solution, marker protein buffer solution and protein molecular mass

14 ladder solution). All the other chemicals were purchased from Bio-Rad (Hercules, CA, USA).

15

16 *2.2. Standard solutions*

17 Individual mother solutions of whey proteins were prepared by weighing 45 mg of b-LG, 17 mg of

18 a-LA or 23 mg of SA and dissolving the weighed amounts in 4 ml, 4 ml and 25 ml of water,

19 respectively. Equal volumes of the individual mother solutions (1.5 ml each) were combined to

20 obtain a mixed standard solution. Aliquots of 200 µl, 400 µl, 600 µl, 800 µl and 1 ml were diluted to

21 the volume of 1.2 ml with water in order to obtain a set of diluted standards; these were used to

22 construct six-level calibration curves along with the undiluted mixed standard solution itself. The

23 final concentrations of this set of standards, corrected for the purity degrees of each protein, are

24 shown in Table 1.

25

26 *2.3. Milk sampling*

1   Individual milk samples from 120 Mediterranean water buffaloes (genus Bubalus, species Bubalus

2   bubalis) were collected at four farm sites in the Campania region (southern Italy). The animals were

3   from different breeding stocks. All samples were collected from animals at 120 d post partum. After

4   collection, samples were immediately frozen and kept at -20 °C until analysis. Prior to

5   determinations, milk was thawed overnight at 4 °C and the whey was obtained by centrifugation of

6   raw milk (8 ml) at 57,438 g for 90 min at 4 °C.

7

8   *2.4 Microchip electrophoretic analysis (MCE)*

9   A subset of 40 randomly chosen individual whey samples was analyzed by microchip

10   electrophoresis (MCE). The analyses were performed using an Agilent 2100 Expert Bioanalyzer,

11   operated by the 2100 Expert software, in conjunction with Agilent Protein 80 series II kits. The kit

12   reagents were prepared according to the manufacturer's instructions. In brief, the required gel–dye

13   mix was prepared by mixing 600 µl of spin-filtered sieving matrix with 25 µl of dye, whereas the

14   destaining solution was spin-filtered sieving matrix without any addition. To obtain reducing

15   conditions, a 3.5% (by volume) solution of β-mercaptoethanol was added to the kit sample buffer.

16   Two hundred microlitres of whey samples were diluted with 400 µl of water and filtered through a

17   0.45 µm-pore cellulose membrane (Phenomenex, Torrance, CA, USA), then prepared as follows.

18   Four microlitres of diluted samples were mixed with 2 µl of Protein 80 denaturing solution in 0.5 ml

19   tubes and heated at 95 °C for 5 min. After cooling at room temperature for 5 min, and shortly

20   spinning tubes at 11,000g for 30 s at 4 °C, the resulting solutions were further diluted with 84 µl of

21   water, vortexed and loaded onto the chips. All chips were loaded with two samples, in two repeats

22   each, and the full set of calibration standards. The instrumental operational parameters for the MCE

23   analysis, i.e. electrode programme, chip temperature and fluorescence detection, were set by the

24   Bioanalyzer software (which also allowed electrophoretogram acquisition and processing) upon

25   user's specification of the utilized kit.

26

1 *2.5. Reversed phase-high-performance liquid chromatography*

2 Whey samples were prepared by diluting 200 µl of ultracentrifuged whey with 400 µl of HPLC-

3 grade water. The diluted samples were filtered through a 0.45 µm-pore cellulose membrane and

4 directly analyzed in two repeats per sample. The chromatographic equipment consisted of a Waters

5 system (Waters, Milford, MA, USA), including two pumps (model 515, Waters), a manual injector

6 (Rheodyne, Cotati, CA, USA) equipped with a 20 ll loop, and a UV detector (model 2487, Waters).

7 The chromatographic separation was performed in reversed-phase mode, using a Jupiter C4 column

8 (250 x 4.6 mm, 300 Å-sized pores, 5 µm-sized particles; Phenomenex) maintained at 30 °C.

9 Gradient elution and peak detection were performed according to Enne et al. (2005).

10 Chromatograms were acquired and processed by the Empower 2 software (Waters).

11

12 *2.6. Mass spectrometry and chromatographic analysis of whey proteins*

13 The determination of the molecular mass of the chromatographic peaks was carried out on an

14 Agilent 1100 series HPLC chromatographer (Agilent, Santa Clara, CA, USA) combined with a

15 Micromass Q-TOF mass spectrometer (Waters), which was equipped with a nanospray source and

16 operated by the Mass-links version 4.1 (Waters) software. The chromatographic separation

17 conditions were as described above. Experiments were run using positive ion detection, applying a

18 capillary voltage of 2–5 kV and a cone voltage of 30–35 V; the capillary temperature was set at

19 220 °C. Determinations were carried out on two buffalo samples and one standard solution and

20 were repeated twice each.

21

22 *2.7. Quantification of whey proteins*

23 Whey proteins in samples were quantified by HPLC through the construction of calibration curves

24 by the external standard method. The calibration was performed by analyzing each mixed standard

25 in 10 repeats and calculating a linear regression of mean peak areas over concentration, in order to

26 obtain a six-level, 60-points calibration curve for each whey protein. Validation was performed as

follows: to estimate repeatability, a standard solution corresponding to the level 3 of the calibration curves was analyzed, in 10 repeats, on the same analytical day; repeatability was expressed as the relative standard deviation of peak times and areas over this dataset. Ruggedness (intermediate precision) was estimated by repeating the same set of analyses for 3 days and calculating the overall relative standard deviation for retention times and peak areas. Accuracy was evaluated by recovery assays. Briefly, a bovine whey sample was subdivided into four 10 ml aliquots; one was taken as blank and known amounts of commercial whey proteins were added to the other three, as follows. The first aliquot was fortified with 4 mg of SA, 8 mg of α-LA and 20 mg of β-LG; the second one was with 5 mg of SA, 10 mg of α-LA and 25 mg of β -LG, and the third one was with 6 mg of SA, 12 mg of α-LA and 30 mg of β -LG. The four aliquots were diluted, as above, and analyzed in triplicate; the recovery rate was determined for each protein as the ratio between the peak area due to the addition (i.e. the differences between the fortified aliquots and the blank) and the peak area predicted by the calibration curves for the same addition, taking into account the purity degree of the commercial whey proteins. Student's t-tests were applied to check whether the recovery rates were significantly different from 100%. In order to test the performance of MCE, quantification of whey protein in a 40-sample subset was also done, using the same set of standards as adopted for HPLC. The electrophoretic quantification was calibrated by loading the set of standards onto each chip, along with samples (two samples in two repeats per chip); the chip-specific calibration curves obtained on each run were then used to quantitate the samples loaded onto the same chip only. The MCE protocol was submitted to validation according to the same procedure as adopted for HPLC. Statistical analysis was performed by means of the instrumentation software and of the R language for statistical computing, version 2.8.1 (R Development Core Team, 2008).

3. Results and discussion

3.1. Electrophoretic characterization of whey fractions

1   Electrophoretic separation of water buffalo whey samples was performed by the MCE technique;

2   the aim was to obtain protein separation and quantification. This technique allowed very fast

3   separation of the major whey proteins as the run time was about 3 min per chip well. The resolution

4   achieved by this method enabled the complete separation of the three major whey proteins (Fig. 1).

5   The electrophoretograms are characterized by three major peaks and a number of lesser peaks,

6   possibly corresponding to minor whey proteins or degradation products. Overall, the separation

7   performance of MCE appeared very similar to traditional SDS–PAGE, which also indicated a slight

8   difference in SA migration between standards and samples. This small variation, however, did not

9   interfere with protein identification through sizing. Such features make MCE a convenient

10   alternative to SDS–PAGE (Goetz et al., 2004) with considerably higher throughput. Protein

11   quantification over a 40-sample subset was performed on a per-chip basis by loading the standards

12   onto each chip and using the software ''calibrated quantification'' feature to quantitate the samples

13   loaded onto the same chip, so that chip-specific calibration curves (Table 1) were used. The

14   corresponding results are shown in Table 2. The instrumental response showed nearly linear

15   behaviour, leading to average r^2 coefficients of 0.95 for α-LA, 0.94 for β-LG and 0.93 for SA over

16   all the chip runs. The mean values of buffalo α -LA, β -LG and SA concentrations indicated a

17   higher mean concentration of α -LA and β -LG than the literature data obtained for bovine whey

18   proteins by both HPLC (Farrell et al., 2004) and MCE (Anema, 2009); however, a high variability

19   (up to 39% for SA) was observed in the MCE results over the 40 analyzed samples.

20

21   *3.2. Identification of whey protein by chromatographic analysis and mass spectrometry*

22   The described RP-HPLC protocol allowed effective resolution of the individual whey proteins in

23   about 24 min with good precision of the retention times, thus allowing a reliable recognition of the

24   chromatographic peaks associated with SA, α-LA and β -LG proteins in the buffalo whey (Fig. 2).

25   In order to achieve an unambiguous identification of whey proteins from water buffalo samples, the

26   molecular masses of the chromatographic peaks, separated by the HPLC protocol, were determined

by ESI-MS. The results were: 66,397 Da for peak 1, 14,236 Da for peak 2 and 18,266 Da for peak 3, corresponding to SA, α-LA and β-LG, respectively. As expected, the main difference between standards and samples was represented by the β -LG which exhibits genetic polymorphism in cow whereas it is genetically invariant in Mediterranean water buffalo. Besides, genetic polymorphisms, at the β -LG locus, have been observed in Asian riverine buffalo (Vohra et al., 2006). Furthermore, water buffalo α -LA exhibited a secondary small peak eluting before the major one, as shown in Fig. 2 (referred to as peak 2s). The presence of a minor additional peak may arise because of glycosylation: in fact, several differently glycosylated forms of bovine α -LA have been described by Slangen and Visser (1999). In addition, calcium ion loss at low pH, leading to a conformational change with different folding and hydrophobicity, has been observed in human α-LA (Håkansson et al., 2000) and subsequently confirmed in cow (Expósito & Recio, 2006). The molecular mass measured for peak 2s (16,663 Da) is in accordance with the results reported by Slangen et al. (1999), thus supporting the glycosylation scenario. The glycosylated alpha-lactalbumin was detected in all whey samples. A further peak occurring in buffalo samples only and eluting after β-LG, as first reported in Resmini, Pellegrino, Andreini, and Prati (1989), was also detected in all samples and considered as the buffalo-specific Bx compound (referred to as peak 5 in Fig. 2); this peak was not quantified but only characterized by its migration time (24.64 min with standard deviation of 0.22 min) and molecular mass (18,447 Da).

3.3. Quantification of water buffalo whey proteins

Protein quantification of 120 individual whey samples was performed by HPLC through the external standard calibration method. The standards were analyzed at six concentration levels, in ten repeats each, and the mean total peak areas generated by each protein were calibrated over concentration by linear regression, yielding the equations and the corresponding statistical parameters summarized in Table 3. The set of mixed standard solutions was designed to reflect the commonly known proportions between the whey proteins in milk while ensuring suitable

concentration ranges for their simultaneous quantification. The instrument response was linear over the chosen concentration range for all the proteins with r^2 coefficients of at least 0.998 and a satisfactory prediction uncertainty (RSD less than 2.4% for all the proteins). The quantitative results achieved for the three major whey proteins are shown in Table 4. Compared to the literature data on bovine whey proteins, Mediterranean water buffalo showed, on average, higher concentrations of α-LA, whereas β-LG and SA were very close to the values reported for cow milk. The quantitative variability over the sampled buffaloes, as determined by HPLC, was in the range 17–27%, for all three whey proteins. No data on the absolute quantification of water buffalo whey proteins by HPLC or other techniques were found in the literature to draw a comparison. Farm-wise mean protein concentrations were calculated and one-way ANOVA was used to test the presence of significant differences between farms (α = 0.05). The results indicated that the farm-wise groupings were significant for all the proteins. One farm, referred to as ''farm1'' (means ± SD: α-LA = 2.82 ± 0.69 g/l; β-LG = 4.92 ± 1.01 g/l; SA = 0.43 ± 0.13 g/l) featured several significant differences with respect to the others. In particular, ''farm1'' showed significantly higher α-LA concentration than did ''farm2'' (2.33 ± 0.41 g/l) and ''farm4'' (2.43 ± 0.44 g/l), significantly higher SA concentration than ''farm3'' (0.32 ± 0.09 g/l) and ''farm4'' (0.34 ± 0.08 g/l) and, most interestingly, significantly higher β-LG concentration than all the other farms (''farm2'' = 4.12 ± 0.58 g/l; ''farm3'' = 3.81 ± 0.60 g/l; ''farm4'' = 3.96 ± 0.67 g/l). No significant differences not involving ''farm1'' were observed, suggesting that the milk production from this farm is influenced by site-specific factors (for example, genealogy of animals).

3.4. Validation of HPLC and MCE protocols

The performances of the HPLC protocol were tested in terms of precision and accuracy. The results of the validation procedure are reported in Table 5. The method showed repeatability and ruggedness of retention times below 1.5%, thus enabling a straightforward recognition of protein peaks. The chromatographic results featured satisfactory and nearly uniform repeatability and

1   ruggedness of chromatographic areas (the former at levels below 2.4% and the latter not exceeding

2   3.6%) for all the peaks, indicating that the method offers acceptable quantification precision.

3   Accuracy was evaluated by means of recovery assays. The protocol achieved recovery rates close to

4   100% for all the whey proteins with acceptable standard deviations (8.8% or less), so that none

5   significantly differed from the theoretical expectation (for a = 0.05). The same validation scheme

6   was applied to MCE, leading to the results shown in Table 5. The repeatability and ruggedness of

7   migration times were satisfactory, whereas the same parameters calculated for peak areas were in

8   the range of roughly 7–11%, depending on the protein. The observed repeatability and ruggedness

9   of the MCE separation are in accordance with those reported by Anema (2009) for α-LA and β-LG

10   and by Goetz et al. (2004) for SA, respectively. On the other hand, the recovery assays performed

11   by MCE led to recovery rates quite distant from 100% (up to 183% in the case of SA); in spite of

12   this, the Student's t-test did not indicate significant differences from the theoretical expectation (for

13    $\alpha = 0.05$) because of the large associated standard deviations. It is interesting to note that the former

14   are calculated on a single chip (repeatability) or using 30 repeats over 3 chips (ruggedness),

15   respectively. On the other hand, as no more than 10 analyses can be run on a single chip, the

16   recovery assays could only be performed using several chips because the calibration standards had

17   to be loaded on every run. Therefore, the exceedingly large standard deviations and distances from

18   100% observed in the MCE recovery assays can only be due to between-chip variation. This, of

19   course, implies that chips are scarcely comparable to each other, thus limiting the use of MCE as a

20   quantitative technique. The validation procedures highlighted substantial dissimilarities between

21   HPLC and MCE; the two methods are not strictly comparable, either by separation principle or by

22   separation and quantification performances. In particular, MCE does not feature the same

23   quantification reliability as does HPLC because of its larger standard deviations, thus allowing the

24   occurrence of discrepancies in the quantification of samples, such as those observed in the case of

25   β-LG (Tables 2 and 4).

26

4. Conclusions

In this study, the Mediterranean water buffalo whey proteins were analyzed to achieve reliable identification and quantification of the α-LA, β-LG and SA by using a RP-HPLC method; well-established techniques, such as gradient reverse-phase HPLC and electrospray ionization mass spectrometry, were exploited. The analytical findings indicate that α-LA is, on average, more abundant in water buffalo than in cow, whereas β-LG and SA show similar mean values. The proteins did not show heterogeneity in chromatograms although a peak corresponding to glycosylated α-LA was observed in buffalo samples. The results we report address the lack of data concerning the Mediterranean water buffalo whey protein profile, as no previous data are available in the literature.

In addition, as the obtained data are based upon the analysis of a suitable number of animals, this research represents novel information that can be applied to the definition of selection schemes for buffalo. In particular, the analysis of the protein concentrations, by farm, allowed us to identify one site producing milk with significantly higher levels of whey proteins than the others. Furthermore, we tested the ability of the microchip electrophoresis (MCE) to rapidly separate and characterize whey proteins. The key operational advantage of MCE is its speed of analysis, representing a significant and convenient improvement over traditional techniques, such as SDS–PAGE. The higher throughput and the ability to consistently estimate molecular mass make MCE a suitable technique for rapid screening of large sample sets and for routine protein sizing.

References

Andreotti, G., Trivellone, E., & Motta, A. (2006). Characterization of buffalo milk by 31P-nuclear magnetic resonance spectroscopy. Journal of Food Composition and Analysis, 19(8), 843–849.

Anema, S. G. (2009). The use of ''lab-on-a-chip'' microfluidic SDS electrophoresis technology for the separation and quantification of milk proteins. International Dairy Journal, 19(4), 198–204.

Bonfatti, V., Grigoletto, L., Cecchinato, A., Gallo, L., & Carnier, P. (2008). Validation of a new reversed-phase high-performance liquid chromatography method for separation and quantification of bovine milk protein genetic variants. Journal of Chromatography A, 1195(1–2), 101–106.

Bonizzi, I., Buffoni, J. N., & Feligini, M. (2009). Quantification of bovine casein fractions by direct chromatographic analysis of milk. Approaching the application to a real production context. Journal of Chromatography A, 1216(1), 165–168.

Bordin, G., Cordeiro Raposo, F., de la Calle, B., & Rodriguez, A. R. (2001). Identification and quantification of major bovine milk proteins by liquid chromatography. Journal of Chromatography A, 928(1), 63–76.

Bramanti, E., Sortino, C., Onor, M., Beni, F., & Raspi, G. (2003). Separation and determination of denatured alpha(s1)-, alpha(s2)-, beta- and kappa-caseins by hydrophobic interaction chromatography in cows', ewes' and goats' milk, milk mixtures and cheeses. Journal of Chromatography A, 994(1–2), 59–74.

Chianese, L., Caira, S., Lilla, S., Pizzolongo, F., Ferranti, P., Pugliano, G., et al. (2004). Primary structure of water buffalo a-lactalbumin variants A and B. Journal of Dairy Research, 71(1), 14–19.

D'Ambrosio, C., Arena, S., Salzano, A. M., Renzone, G., Ledda, L., & Scaloni, A. (2008). A proteomic characterization of water buffalo milk fractions describing PTM of major species and the

identification of minor components involved in nutrient delivery and defense against pathogens. Proteomic, 8(17), 3657–3666.

Elgar, D. F., Norris, C. S., Ayers, J. S., Pritchard, M., Otter, D. E., & Palmano, K. P. (2000). Simultaneous separation and quantification of the major bovine whey proteins including proteose peptone and caseinomacropeptide by reversed-phase highperformance liquid chromatography on polystyrene–divinylbenzene. Journal of Chromatography A, 878(2), 183–196.

El-Zahar, K., Sitohy, M., Dalgalarrondo, M., Choiset, Y., Métro, F., Haertlé, T., et al. (2004). Purification and physicochemical characterization of ovine betalactoglobulin and alpha-lactalbumin. Nahrung, 48(3), 177–183.

Enne, G., Elez, D., Fondrini, F., Bonizzi, I., Feligini, M., & Aleandri, R. (2005). High-performance liquid chromatography of governing liquid to detect illegal bovine milk's addition in water buffalo Mozzarella: Comparison with results from raw milk and cheese matrix. Journal of Chromatography A, 1094(1–2), 169–174.

Expósito, I. L., & Recio, I. (2006). Antibacterial activity of peptides and folding variants from milk proteins. International Dairy Journal, 16(11), 1294–1305.

Fan, B. L., Li, N., & Wu, C. X. (2000). Research on constructing phylogenetic trees of ruminants basing on the database of milk protein gene sequences. Yi Chuan Xue Bao, 27(6), 485–497.

Farrell, H. M., Jr., Jimenez-Flores, R., Bleck, G. T., Brown, E. M., Butler, J. E., Creamer, L. K., et al. (2004). Nomenclature of the Proteins of Cows' Milk–Sixth Revision. Journal of Dairy Science, 87(6), 1641–1674.

1 Fox, P. F. (1989). Developments in dairy chemistry, Vol. 4: Functional milk proteins. London, UK:

2 Elsevier. pp. 1.

3

4 Goetz, H., Kuschel, M., Wulff, T., Sauber, C., Miller, C., Fisher, S., et al. (2004). Comparison of

5 selected analytical techniques for protein sizing, quantification and molecular weight determination.

6 Journal of Biochemical and Biophysycal Methods, 60(3), 281–293.

7

8 Håkansson, A., Svensson, M., Mossberg, A.-K., Sabharwal, H., Linse, S., Lazou, I., et al. (2000). A

9 folding variant of alpha-lactalbumin with bactericidal activity against Streptococcus pneumoniae.

10 Molecular Microbiology, 35(3), 589–600.

11

12 Marshall, K. (2004). Therapeutic applications of whey protein. Alternative Medicine Review, 9(2),

13 136–156.

14

15 Martin, P., Szymanowska, M., Zwierchowski, L., & Leroux, C. (2002). The impact of genetic

16 polymorphisms on the protein composition of ruminant milks. Reproduction, Nutrition,

17 Development, 42(5), 433–459.

18

19 Parodi, P. W. (2007). A role for milk proteins and their peptides in cancer prevention. Current

20 Pharmaceutical Design, 1(8), 813–828.

21

22 R Development Core Team (2008). R: A language and environment for statistical computing. R

23 Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org.

24

1   Ramesha, K. P., Khosravinia, H., Gowda, S., & Rao, M. R. S. (2008). Alpha-lactalbumin gene

2   polymorphism: a preliminary study on two breeds of the river Buffalo (Bubalus bubalis). Asia

3   Pacific Journal of Molecular Biology and Biotechnology, 16(2), 47–52.

4

5   Resmini, P., Pellegrino, L., Andreini, R., & Prati, F. (1989). Evaluation of milk whey proteins by

6   reversed-phase HPLC. Scienza e Tecnica Lattiero-Casearia, 40, 7–23.

7

1   **Table 1**

2   Composition of the whey protein concentration standards

Standard level	Final protein concentration [g/l]		
	SA [purity 98%]	α-LA [purity 85%]	β-LG [purity 80%]
1	0.05	0.20	0.50
2	0.10	0.40	1.00
3	0.15	0.60	1.50
4	0.20	0.80	2.00
5	0.25	1.00	2.50
6	0.30	1.20	3.00

3

4

1 **Table 2**

2 Mean concentration of whey proteins in water buffalo samples, as determined by MCE.

Peak	Whey protein	Migration time mean ± SD, $n = 40$ [s]	Concentration mean ± SD, $n = 40$ [g/l]
1	α-LA	21.86 ± 0.05	2.20 ± 0.88
2	β-LG	24.28 ± 0.04	4.6 ± 1.5
3	SA	39.60 ± 0.04	0.40 ± 0.15

3

4

1 **Table 3**

2 HPLC Calibration curves.

Whey protein	Equation[a]	RSD[b] [%]	r^2	Standard errors	
				Slope	Intercept
SA	$y = 3.64 * 10^7 x - 1.14 * 10^5$	1.91	0.999	$5.73 * 10^5$	$1.11 * 10^5$
α-LA	$y = 3.65 * 10^7 x + 7.17 * 10^5$	2.06	0.999	$6.25 * 10^5$	$4.87 * 10^5$
β-LG	$y = 3.02 * 10^7 x + 3.06 * 10^5$	2.36	0.998	$6.03 * 10^5$	$1.17 * 10^6$

3

4 [a] y: peak area; x: analyte concentration expressed as g/l.

5 [b] All the curves consist of 6 data points, each taken as the mean of 10 repeats.
6
7

1   **Table 4**

2   Concentration of whey proteins in water buffalo samples.

Peak	Whey protein	Rt mean ± SD [min]	Concentration mean ± SD, [g/l]	Cow (literature data[a]) [g/l]
1	SA	15.64 ± 0.047	0.35 ± 0.10	0.4
2	α-LA	18.26 ± 0.0067	2.45 ± 0.45	0.6–1.7
3	β-LG	22.73 ± 0.0056	4.04 ± 0.72	2–4

3

4   [a] Farrell et al., 2004.

5

1 **Table 5**

2 Validation of the HPLC and MCE protocols using bovine standards: precision and accuracy.

	Peak time RSD (%)		Peak area RSD (%)		Quantitation accuracy		
	Repeatability	Ruggedness	Repeatability	Ruggedness	Recovery rate (%)	RSD (%)	t-test ($t_{0.05,2}$ = 4.30)
HPLC							
SA	0.31	1.31	2.31	2.80	99.8	5.46	0.047
α-LA	0.037	1.20	2.35	3.18	109.6	3.90	4.252
β-LG	0.025[a]	0.82[a]	2.06[a]	3.04[a]	97.1	8.80	0.562
	0.034[b]	0.73[b]	2.07[b]	3.58[b]			
MCE							
SA	0.09	0.92	11.3	11.9	183	36	4.039
α-LA	0.23	0.25	7.16	6.85	130	46	1.111
β-LG	0.18	0.21	6.91	9.04	140	65	1.058

3

4 [a] First peak in elution order.
5 [b] Second peak in elution order.

6

1 **Fig. 1.** MCE electrophoretogram (emitted fluorescence vs. migration time) of whey from

2 Mediterranean water buffalo individual milk obtained by MCE using an Agilent 2100 Bioanalyzer

3 in combination with a Protein 80 kit. Mass markers and system peaks are also indicated.
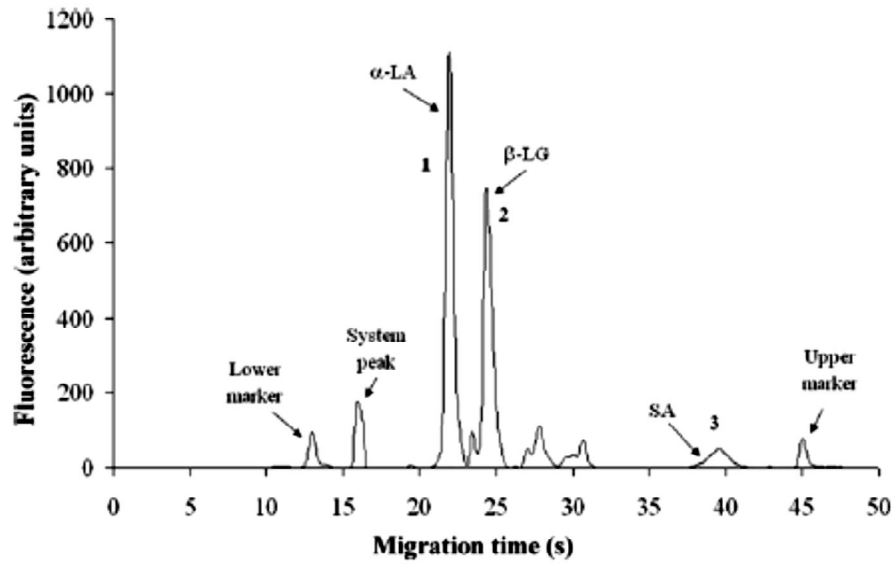
4

5

1 Fig. 2. Chromatogram of whey from Mediterranean water buffalo individual milk. Peak 2s is a

2 secondary α -lactalbumin peak observed in buffalo samples, which is considered a glycoform of α-

3 LA based on its molecular mass. Column: Phenomenex Jupiter C4 (250 mm x 4.6 mm, 300 Å pores,

4 5 μm particles); detection: UV, λ = 205 nm; mobile phase: water containing 0.1% trifluoroacetic

5 acid (TFA)/acetonitrile containing 0.1% TFA (refer to Section 2.5 for elution gradient details).

6