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Micro-Electrophoretic Study of the Sarcoplasmic Fraction in the Dry-Cured Goat Raw Ham

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Abstract: The water-soluble protein fractions in the "Violino di capra" goat dry cured ham were studied by using the "Lab-on-a-chip" version of a size-based capillary electrophoresis, and the results were compared with those obtained from the analysis of the corresponding fraction of pork meat and raw ham. The system automatically determined the relative concentration of each protein present in the sarcoplasmic fraction samples, where the nearly all proteins ranged from 24 kDa to 62.5 kDa, and made possible a supposition of the proteolysis process taking place after ripening of the goat raw ham. We propose the use of such technique as high-quality and routine analysis method in the biochemistry of food proteins.

Keywords: Electrophoresis, meat, microfluidic, sarcoplasmic protein, ham.

INTRODUCTION

Traditionally, meat processing extends and improves the shelf life of the product, and affords the convenience of a prolonged product storage and/or transport. Processing reduces enzyme activities present in the raw product, retards meat oxidation, and prevents spoilage, thereby guaranteeing the safety of the final product. Methods for preserving meat include drying, salt curing, and smoking. Italy has a long history in the production of traditional fermented meat products [1], and almost every Italian region offers one or more of these much appreciated products, some of which awarded the Protected Designation of Origin and Protected Geographical Indication labels (http://europa.eu.int/comm/agriculture/qual/en/pgi_03en.htm). "Violino di capra" is a typical goat dry-cured ham, manufactured only in few regions of Italy and generally obtained from the breeds "Frisia" or "Fontalasca". The meat is prepared with spices (generally thyme, black and white pepper, bay leaf, cinnamon, cloves and coriander), massaged with brine salt for about 10 days, then ripened and aged for up to 6 months, depending on the weight.

This particular product offers a marketable alternative to fresh goat meat which, although of interesting nutritional value [2], is underutilized in Italy, due to general lack of appeal, and is commonly consumed only during particular religious events [3]. Generally, the palatability-related characteristics of dry-cured meat products, such as texture and flavour, are also due to extensive proteolytic phenomena that occur during ripening [4-6], as consequence of the activity of endogenous cytosolic enzymes [7-9] and, at least in part, of the microbial action [10], which act in synergistic way on the water and insoluble muscle fraction.

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In recent time, the study of the proximate composition, fatty acid profiles and volatile compounds was performed on fresh and cured and ripened goat thighs [3]. Here, we evaluated the water soluble protein fraction of the goat dry cured ham, comparing it with the pork raw ham one. The study was performed by using a new analytical approach, based on the "Lab-on-a-chip" version of size-based micro-capillary electrophoresis. The Lab-on-a-chip system integrates handling of sample, protein separation, staining/destaining, detection, and the analysis inside a single platform, to diminish the amount of sample, reagents, and hands-on time required to perform routine qualitative and quantitative protein analyses [11-13]. This microfluidics-based system offers significant convenience in terms of sample analysis and a throughput unmatched by some conventional methods of protein analysis, such as SDS-PAGE. The principle of the analysis is an electrophoretic process, where denatured proteins, negatively charged through the interaction of SDS molecules, are moved across micro-fabricated chips with distinct microfluidic channels. The channels are filled with a sieving polymer with the aim to separate the proteins according to their size, and an intercalating fluorescent dye, which stains the proteins. Before the laser induced fluorescence detection of the different proteins, a destaining step is integrated on the chip. By transferring to chip, an acceleration factor of 20 times is achieved in comparison to traditional SDS gel electrophoresis. In fact, through this system, 10 protein samples can be analyzed unattended within less than 30 minutes (maximum 3 min/sample), with a cost of only 20-25 € *per* chip. The software automatically evaluates the data and displays a detailed result table. To achieve accurate sizing of unknown protein samples, a sizing ladder is run on each chip, Proteins between 4.5 and 240 kDa can be analyzed with a resolution of 5% to 10% within a broad linear dynamic range. A software automatically evaluates the data and provides the relative concentration of the individual proteins. Such value is automatically determined based on a one-point calibration with the upper molecular weight marker, used as an internal

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quantitation standard in every sample [14]. The chip layout is shown in Fig. (**1**) [15]. Lab-on-a-chip methodology (*via* protein analysis) has rapidly established as a viable alternative tool to conventional methods for protein identification, sizing and quantification [16] It has been already used to identify grain varieties and wheat types [17], to monitor the protein changes taking place during ripening of Naples-type salami, a southern Italian fermented sausage [18], and to determine the protein profile of different *Lactobacillus* spp. in table olives [19].

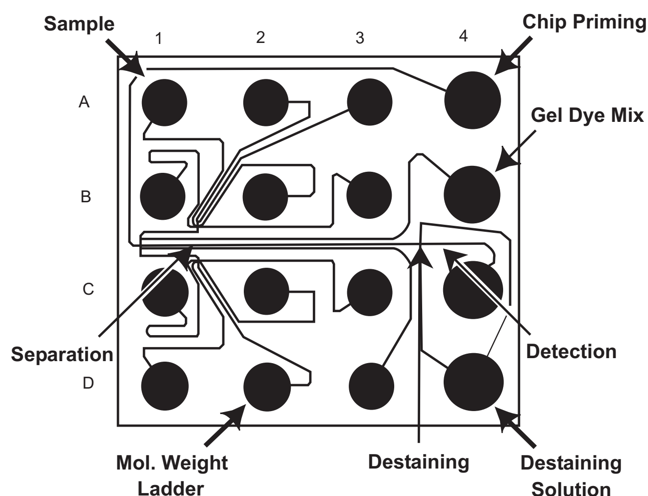

Fig. (1). Chip layout. The location of the wells is shown in black. (Picture from Poster: Vasilyeva *et al.* BIOGEN, Cambridge, MA,WCBP conference, 2002 in Forrer *et al.* 2004).

EXPERIMENTAL

Analyses were carried out on the "Violino di capra" goat raw ham, and pork dry-cured ham, that were purchased from a specialized Italian food company. Fresh goat and pork meat were purchased from a local butcher's shop. Samples were cut and immediately stored at –20°C until biochemical analyses. Each sample (10 g) was homogenized in 0.01 M phosphate buffer pH 7.00, EDTA 5mM, β-mercaptoethanol 2%; the homogenate was centrifuged under refrigeration at 4°C and 11,600 × *g* for 20 min (Biofuge Beckman, USA) according to Nazzaro *et al*. [18]. The supernatant, containing the sarcoplasmic protein fraction, was recovered and stored in 5 ml aliquots at –20°C. Protein content was evaluated according to Bradford [20].

LAB-ON-A-CHIP CAPILLARY ELECTROPHORESIS

a. Chip Technology, Sample Preparation

4 µl samples were mixed with 2 µl of a Protein 230 plus LabChip denaturing solution (3.25% polydimethylacrylamide, 120 mM tricine, 42 mMTris, pH 7.6, 0.25% SDS, 3.4 mM DTT), lower and upper markers (Agilent Technologies, Germany) [21]. Samples were incubated at 100° C for 3 minutes and mixed afterward with 84 µL of water.

The molecular weight markers were prepared according to manufacturers instructions (Protein 230 plus Assay protocol from Agilent Technologies), and 4µL of this solution was mixed with 2µL of denaturing solution, heated at 100°C for 3 min, and mixed with 84 µL of water. For the separation experiments, each channel in the glass chip was filled by loading 12 µl of a sieving matrix (soluble polymer based on polydimethylacrylamide [22] at 3.25% in a 120 mM tricine, 42 mM Tris buffer, pH 7.6, containing 0.25% SDS and the dye (Protein 230 plus LabChip Kit from Agilent Technologies) into a specific well and applying pressure for 1 min. This and other three wells were filled with this solution. The SDS dilution well contained only the sieving matrix. The molecular weight marker (6 µl) and the protein samples (6 µl) were applied to all remaining wells on the chip. Each chip, being disposable, was used only once, according to the manufacturers instructions.

b. Chip Technology, Measurement Instrument and Sample Fractionation

Separation and detection were done with an Agilent 2100 Bioanalyzer (Agilent Technologies), which uses fluorescence detection with a 10-mW semiconductor laser that emits at 630nm. The raw data were directly accumulated and stored on the same PC that controls the Bioanalyzer. Fractionation was based on protein size, and the profiles were analogous to protein separation *via* SDS-PAGE, with the smallest proteins migrating fastest. The data were analysed using the Agilent 2100 Expert software. Data were shown as electropherograms (Fig. **2**).

RESULTS AND DISCUSSION

a. Qualitative/Quantitative Analysis of Sarcoplasmic Fraction in the Fresh Goat Meat

The profile of sarcoplasmic proteins present in the goat fresh meat, obtained through lab-on-chip micro-electrophoresis, is shown in Fig. (**2a**) and in Table **1**. Several of the proteins were also found in pork meat (Fig. **2c**), Table **2**) and ranged from 24 kDa to 62.5 kDa of Molecular Weight (MW). Previous works [23-26] identified these proteins as myokinase (24-24.96 kDa), triosephosphate isomerase (26.3-27.18 kDa), carbonic anhydrase (29.64-31.3 kDa), glyceraldheyde-3-phosphate dehydrogenase (34.09-35.08 kDa) and aldolase A (40.13-40.9 kDa). The heaviest protein present in both types of fresh meat exhibited MW of 57.7 kDa and 62.96 kDa, probably corresponding to pyruvate kinase muscle isozyme, and glucose-6-phosphate isomerase respectively. Goat meat also contained some of the smallest polypeptides until 8 kDa as well as various proteins with MWs of 28.8 kDa (most likely corresponding to phosphoglycerate mutase isozyme M [26]), and 38.8 kDa, as well as a protein of 143 kDa, this last probably representing an aggregate. The profile of sarcoplasmic proteins observed in fresh goat meat appeared less complex than in the pork one (Fig. **2c**, Table **2**), which exhibited a greater number of small proteins/peptides of <11 kDa, and eight proteins ranging from 11.77-22.25 kDa, 74.42-80.75 and 103.63 kDa. This last protein was also observed by Diaz *et al*. [27] during pork dry sausage ripening.

The microfluidic-based system automatically determined the relative concentration of each protein present in the sarcoplasmatic fraction (Tables **1**, **2**); this was achieved by using a single-point calibration, wherein the peak area of the protein was compared to that of an internal standard protein present at a known concentration in each sample. Inclusion of the internal standard protein had the added benefits of

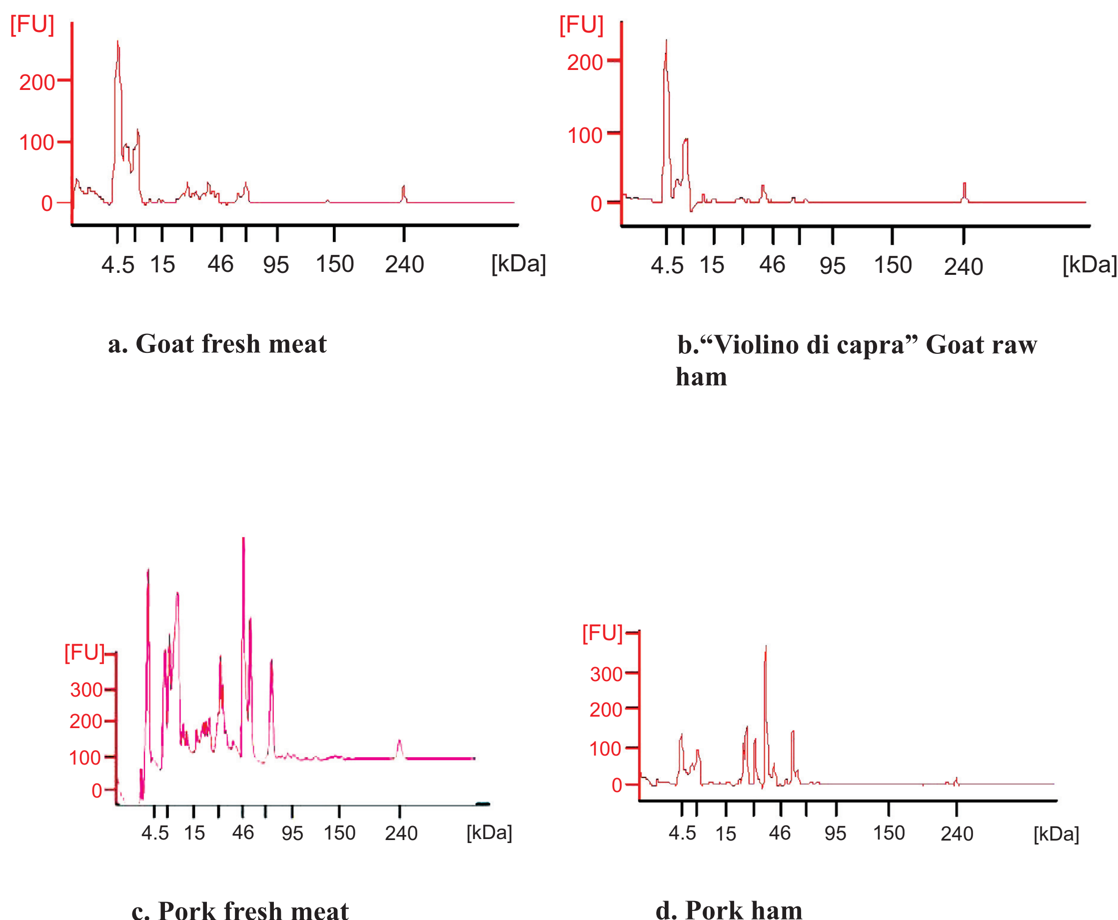

Fig. (2). Electropherograms from microfluidic capillary electrophoresis of sarcoplasmic proteins from goat and pork meat (**2a** and **2c**, respectively) and in goat and pork dry-cured hams (**2b** and **2d**, respectively). The y and x axis represent fluorescence intensity and molecular weight, respectively. For details, see: Experimentation.

allowing automatic correction of sample to-sample differences (e.g., due to injection or to the separation).

Each protein present in the sample was quantified (as ng/µl of samples) relatively to the standard proteins present in the standard mixture. The quantitative analysis showed that nearly all proteins in the sarcoplasmic fractions of goat meat (978 ng/µl) and pork meat (814 ng/µl) ranged from 24 kDa to 62.5 kDa (Table **1**). The most abundant sarcoplasmic proteins present in the goat meat were probably ascribable, on the basis of their molecular weight and of some works of identification [23-27], to triosephosphate isomerase (26.3 kDa, 157 ng/µl), and fructose-bisphosphate aldolase A (40.9 kDa, 33 ng/µl); in addition, another protein of 38.2 kDa (147 ng/µl) was observed.

In terms of protein concentration, the proteins present in the sarcoplasmic fraction of pork meat were more abundant and exhibited a more regular distribution compared with the goat fresh one, except than for the fructose-bisphosphate aldolase A, which alone represented 31% of the entire protein content (by weight).

b. Analysis of Protein Patterns in Dry-Cured Hams

Table **1** and Fig. (**2b**) show the quantitative and qualitative analysis of the sarcoplasmic fraction obtained from the goat dry-cured ham, respectively.

On the whole, the most of proteins ranged in the area whose estimated MWs ranged between 24.8 and 68.5 kDa. Some of them could be identified, based on previous works [25, 26] as triose 6-phosphate isomerase (27 kDa), carbonic anhydrase (31.2 kDa), pyruvate kinase (58.3 kDa), common with the goat fresh meat.

Compared with the fresh meat, some first differences could be perceived:

i) a general quantitative diminution of the protein with an estimated MW between 24 and 62.5 kDa;

ii) the disappearance of the presumed phosphoglycerokinase (MW 28.8 kDa), aldolase (MW 40.9 kDa), piruvate kinase (MW 57.2 kDa), and glucose-6-phosphate isomerase (MW 62.5 kDa);

iii) a partial disappearance of carbonic anhydrase (MW 30.8 kDa), myokinase (MW 24 kDa) and triose-6-phosphate isomerase. This last exhibited only a partial resistance to endogenous and bacterial proteinases, as conversely observed in other dry-cured meat products [23, 24];

iv) an evident increase of the protein with an estimated weight of 38.2 kDa, which concentration was found to be of about 4 times higher than in fresh meat (578.42 ng/µl versus 146.7 ng/µl, respectively);

Table 1. Quantitative analysis of the sarcoplasmic fraction from goat meat and "Violino di capra" goat dry-cured ham performed by lab-on-chip micro capillary electrophoresis. The relative concentration of each protein present in the samples of sarcoplasmic fractions was calculated by comparing the peak area of the protein to that of two internal standard proteins (4.5 and 240 kDa) present at known concentrations in each sample. Data are reported as ng/µl in parenthesis is shown the Standard Deviation (DS). For details, see: Experimentation

Goat Fresh Meat			Violino di capra Ham		
Peak	Size (kDa)	Rel conc. ng/µl (±SD)	Peak	Size (kDa)	Rel conc. ng/µl (±SD)
1	0	0	1	0	0
2	0.7	0	2	0.3	0
3	1.8	0			
4	2.1	0			
5	4.5		3	4.5	
6	5.8	0	4	6.1	0
7	7	0	5	7.4	0
8	8	0			
			6	12.4	4.62 (±1.02)
9	24	95.9 (±12.36)	7	24.8	42.04 (±9.89)
10	26.3	157.64 (± 21.54)	8	27.3	78.3 (±11.32)
11	28.8	45.22 (± 9.74)			
12	30.8	70.98 (± 11.21)	9	31.2	56.36 (±11.14)
13	34.9	78.8 (± 12.58)			
14	38.2	146.72 (± 19.69)	10	39.3	578.42 (±31.34)
15	40.9	132.16 (± 14.78)			
16	57.2	59.2 (± 13.25)	11	58.3	97 (±10.35)
17	62.5	191.8 (± 21.21)			
			12	68.5	78.54 (±13.67)
18	143.7	14.84 (± 2.35)			
19	240		13	240	

v) the coming out of a new band with a MW of 68.5 kDa, which increasing concentration was already demonstrated during the ripening of sausage containing pork/beef and deer meats [25-28]. Such band was much more abundant in goat ham: this event was probably due to a different biochemical process compared to the pork ham, where it could be supposed as arisen essentially from the degradation of the band at MW of 103.6 kDa.

Moreover a very little number of low molecular weight polypeptides were viewed, ranging from 0.4 kDa to 12.4 kDa, as consequence of the proteolytic events taking place during ripening of the ham. Diaz *et al*. [27] identified several polypeptides, in particular at 8, 10 and 11 kDa, at the end of 26 days ripening in pork sausage that were not present before ripening. Soriano [28] found different small polypeptides, ranging from 9 to 16 kDa, in the deer sausage.

On the whole, compared to the sarcoplasmic fraction of the pork dry-cured ham (Fig. **2d**, Table **2**), the number and concentration of the different proteins present in the goat ham led us to assume for a less effective proteolytic activity taking place in this last product, probably due also to the different time and procedures of ripening foreseen for the two products [24]. In the pork ham, some of the sarcoplasmic proteins noticeably decreased in amount or completely disappeared, almost all of them referring to a MW heavier than 59 kDa. In such extract, the most represented proteins could be supposed as enolase B (theoretical MW 52.3 kDa), creatine kinase (41.6 kDa), and glyceraldehyde 3-phosphate dehydrogenase (36 kDa), as therefore demonstrated by Picariello *et al.* [24].

A general impression of the two proteic profiles in pork and goat samples let us hypothesise a different overview. The number of proteins found in the pork fresh meat,

Table 2. Quantitative analysis of the sarcoplasmic fraction from fresh pork meat and pork dry-cured ham performed by lab-on-chip micro capillary electrophoresis. The relative concentration of each protein was calculated by comparing the peak area of the protein to that of two internal standard proteins (4.5 and 240 kDa) present at known concentrations in each sample. Data are reported as ng/µl in parenthesis is shown the Standard Deviation (DS). For details, see: Experimentation

Pork Fresh Meat			Pork Raw Ham		
Peak	Size (kDa)	Rel conc. ng/µl (±SD)	Peak	Size (kDa)	Rel conc. ng/µl (±SD)
1	0	0	1	0	0
2	0	0	2	0.5	0
3	1.2	0	3		
			4	2.1	0
4	4.5		5	4.5	0
5	5.14	0	6	6.1	0
6	6.47	0	7	7	0
7	7.5	0			
8	8.67	0			
9	11.77	17.7 (±1.89)	8	11	0
10	14.01	22.32 (±2.35)			
11	14.97	18.09 (±3.63)			
12	16.26	16.22 (±3.59)	9	16.1	8.79 (±2.65)
13	17.86	23.76 (±3.98)			
14	22.25	44.31 (±6.54)	10	21.7	8.78 (±3.11)
15	23.41	93.92 (±7.12)	11	23.7	103.72 (±10.1)
16	24.96	75.28 (±16.25)	12	25.2	150.42 (±19.57)
17	27.18	53.05 (±4.65)			
18	29.64	42.91 (±6.96)	13	29.5	121.69 (±20.65)
19	32.11	25.49 (±3.74)			
20	35.08	19.67 (±4.12)	14	36	380.37 (±25.45)
21	40.13	307.82 (±27.56)	15	41.6	51.2 (±9.52)
22	45.43	174 (±24.31)			
			16	49.5	8.89 (±1.31)
			17	54.2	145.28 (±17.86)
23	59.69	48.57 (±12.36)			
24	62.96	67.2 (±12.36)			
			19	68	6.94 (±1.05)
25	74.42	0.88 (±12.36)	20	75.8	6.32 (±1.11)
26	80.75	0.71 (±12.36)			
27	103.63	7.6 (±12.36)			
			21	227.3	6.59 (±0.99)
28	240		22	240	

extracted by using the same procedures of goat meat, was clearly superior than in the goat one, with more bands having a MW ranging from 11 to 18 kDa; in addition, a band of MW 45 was absent in the goat meat and very abundant in the pork fresh meat. In the goat ham, the number of proteins observed after the analysis was smaller than in pork ham. We found 4 bands in the range between 21 and 36 kDa in the sarcoplasmic fraction of pork ham and, on the other hand, only three bands, ranging from 21 to 31, appeared in the protein profile of sarcoplasmic fraction of goat ham.

The lab-on-a-chip system can be taken into account an ideal tool for the analysis of food proteins, providing information on their size and concentration in a single assay. Advantages include user-friendliness, automated separation, good reproducibility and digitized data output. The miniaturization of analysis ensures shorter times of run and avoids the use and handling of great quantities of hazardous wastes (i.e., polydimethylacrylamide, SDS-PAGE running buffer, etc.). The automatic staining/destaining of samples eliminates the manual steps required for the SDS-PAGE and the relative consumption of considerable volumes of methanol and acetic acid. Indeed, the hardware and software of lab-on-chip systems can be used with other chemicals and chips for many applications in food analysis, such as the analysis of different lactic acid bacteria [29] of food pathogens [30].

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