J. Dairy Sci. 98:1–9
http://dx.doi.org/10.3168/jds.2015-9769

A rapid high-performance liquid chromatography-tandem mass spectrometry assay for unambiguous detection of different milk species employed in cheese manufacturing

Nadia Bernardi,* Giuseppe Benetti,* Naceur M. Haouet,† Manuel Sergi,‡ Lisa Grotta,‡ Sonia Marchetti,‡ Federica Castellani,‡ and Giuseppe Martino‡[1]
*Real aromi SpA, via Lungofino 3, Città S. Angelo (Pe), Italy 65013
†Department of Food Safety, Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche, Via Salvemini 1, Perugia, Italy 06126
‡Faculty of BioSciences and Technologies for Agriculture Food and Environment, University of Teramo, Italy via C. Lerici 1, Mosciano S.A., Italy 64023

ABSTRACT

The aim of the study was to investigate the possibility to differentiate the 4 most important species in Italian dairy industry (cow, buffalo, sheep, and goat), applying a bottom-up proteomic approach to assess the milk species involved in cheese production. Selective peptides were detected in milk to use as markers in cheese products. Trypsin-digested milk samples of cow, sheep, goat, and buffalo, analyzed by HPLC-tandem mass spectrometry provided species-specific peptides, some of them recognized by Mascot software (Matrix Science Ltd., Boston, MA) as derived from well-known species specific proteins. A multianalyte multiple reaction monitoring method, built with these specific peptides, was successfully applied to cheeses with different composition, showing high specificity in detection of species involved. Neither aging nor production method seemed to affect the response, demonstrating that chosen peptides well act as species markers for dairy products.
Key words: milk, cheese, animal species, HPLC-MS/MS, trypsin, peptides

INTRODUCTION

In recent years, a general trend has been observed in the food industry to characterize manufacturing products by raw material sources, geographic origins, production processes, as well as physicochemical and dietary properties of the finished products. In dairy food production, we have noted a growing number of cheese types, both new and old, once only regionally traded and now widespread in the world market as well. Likewise, besides cow and sheep milk cheeses, it is not unusual to find goat and buffalo products in markets today.

Research of selective markers to highlight species contained in foodstuffs are a challenge for food chemists; to ensure industrial quality control and assurance, official antifraud controls, and consumer safety, useful tools to assess the authenticity of food and proper labelling are needed. For dairy products, this is emphasized by allergenic characteristics of milk products, and there is great interest in developing methods able to check the species of milk used in cheesemaking. The possibility of adding cow milk to sheep or buffalo ricotta (a typical Italian dairy product) through the HPLC determination of β-carotene was investigated by Cerquaglia et al. (2011), but the results showed difficulty detecting small additions depending on the variability of β-carotene content in the various milk species. The detection of fraudulent cow milk additions in ewe milk was carried out via a stereospecific analysis of triacylglycerols by Blasi et al. (2013); a chromatographic detection of dairy product adulteration, based on sterol fractions and on concentration of *trans*-isomers of FA, was also proposed by Chmilenko et al. (2011). It is well-known that milk of different ruminant species contains closely homologous proteins with similar structures and functionality (Wal, 2004); several analytical techniques, such as capillary electrophoresis (Cartoni et al., 1998; Herreo-Martinez et al., 2000), 2-dimensional gel electrophoresis (Yang et al., 2014), PCR (Bai et al., 2009; Drummond et al., 2013), ELISA (Hurley et al., 2004), and isobaric tags for relative and absolute quantitation (Yang et al., 2013), were used to develop methods with this purpose, but the results were partial or unsatisfactory. Better performances were achieved recently with bottom-up proteomics approaches. A matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry direct analysis of milk tryptic digestion was able to assess

Received April 30, 2015.
Accepted July 28, 2015.
[1]Corresponding author: gmartino@unite.it

Table 1. Composition of cheeses made with sheep, goat, and cow milk

	Composition, %			
Item	Cheese 1	Cheese 2	Cheese 3	Cheese 4
Sheep	78	78	56	33
Goat	11	0	22	33
Cow	11	22	22	33

adulteration of sheep and goat milk (Calvano et al., 2012) and was used for milk speciation in a quaternary mixture (Cuollo et al., 2010). Liquid chromatography coupled with tandem mass spectrometry (**LC-MS/MS**; Monaci and Visconti, 2009; Guarino et al., 2010; Ansari et al., 2011) is now widely used in separating and recognizing peptides obtained by enzymatic digestion of dairy products. Guarino et al. (2010), for example, detected sheep milk in goat and cow cheeses by analyzing the tryptic digestion of isolated caseins; Ansari et al. (2011) developed a similar method able to detect milk allergens (caseins and lactoglobulins) in food products.

In our work, we investigated the possibility of differentiating the 4 most important species in Italian dairy industry (cow, buffalo, sheep, and goat milk) by detecting selective peptides in their milk that are able to act as markers for milk identification in raw materials and mainly in cheese products. For this aim, a simple and time-saving method for sample preparation was developed, applying a tryptic digestion protocol (Harvey, 2003) directly to milk and cheese. In this way, the protocol is quick and easy: the time for sample preparation depends only on the time required by digestion, few steps are involved, and the defatting solvents are saved. Trypsin-digested milk samples were analyzed by HPLC-MS/MS and the spectra were subjected to a Mascot (www.matrixscience.com; Matrix Science Inc., Boston, MA) search to evaluate, for each milk species, the possibility of detection of compounds with good specificity and sensitivity in a multiple reaction monitoring (**MRM**) experiment, with a profile (mass, charge, and fragmentation) matching a peptide with a specific AA sequence.

The method was evaluated in our laboratory by manufacturing curd cheeses by the use of single- and mixed-species milk samples, following an experimental design based on common manufacturing practices or fraudulent purposes. Commercial samples were also analyzed.

MATERIALS AND METHODS

Materials

Milk. Samples of cow, ewe, goat, and buffalo milk were collected directly from farm bulk milk. Farms were chosen from those with at least 100 animals in lactation. For all species, the tank milk samples were collected from the evening and the following morning milking. Analyses were all performed on raw milk within 72 h of collection.

Cheese. The milk samples were 10 L/species, collected directly on the farms, in the morning, and carried to laboratory using thermostatic containers set at 4 to 5°C. Once in laboratory, milk was warmed to 38 to 49°C and rennet was added at a dose of 20 mL/100 L of milk. Following a rest of 30 min, the curd was cut and transferred in cheese hoops of approximately 300 to 350 g for fresh cheeses and 1 kg for those of 60 d aging. The aging was carried out in rooms set at 10 to 12°C and at 80 to 85% moisture. The analysis portions were collected in the cheese core.

Cheeses were produced from both milk of each individual species (buffalo, cow, sheep, or goat) and from mixed milk prepared in different proportions, and analyzed after 48 to 72 h of production. For multispecies cheese, 2 different experiments were designed: one mixing sheep, goat, and cow milk (cheeses 1–4, Table 1), and one mixing buffalo and cow milk (cheeses 5–8, Table 2). In addition, cheeses from 100% of cow, ewe, and sheep milk were also analyzed after 2 mo of ag-

Table 2. Composition of cheeses made with buffalo and cow milk

	Composition, %			
Item	Cheese 5	Cheese 6	Cheese 7	Cheese 8
Buffalo	80	50	20	5
Cow	20	50	80	95