ISSN: 1220-3173; E-ISSN: 2457-7618

NEW ASAP (ATMOSPHERIC SOLID ANALYSIS SAMPLE) METHOD FOR DETECTION OF MILK FAT ADULTERATION OF MILK AND MILK PRODUCTS IN ROMANIA

METODĂ NOUĂ DE ANALIZĂ ASAP (ATMOSPHERIC SOLID ANALYSIS SAMPLE) PENTRU DETECTAREA FALSIFICĂRII GRĂSIMILOR DIN LAPTE ȘI PRODUSE LACTATE DIN ROMÂNIA

Florica BĂRBUCEANU[1),4)], H. ALBU[2),*)], Rodica TĂNĂSUICĂ[2)], Laurențiu CIUPESCU[2)], Ștefania Felicia BĂRBUCEANU[3)], S. BĂRĂITĂREANU[4)], G. PREDOI[4)]

ABSTRACT | REZUMAT

Adulteration of milk and dairy products with different undeclared types of milk constitute a global food monitoring. Since this food has a large market in Romania, a study conducted by the competent authority comprise 368 milk and milk products units tested by mass spectrometry method. Adulteration often consists in cow's milk addition to costlier milk of sheep, goat or buffalo. The most adulterated milk product is the salted semi - soft cheeses in percent of 5.83%, followed by semi- soft cheeses with 4%. The mass spectrometric method has 1% precision and can be widely used in this scope. The adulteration percent found in Romania is a small one in comparison with other countries. Nevertheless, further analysis must be conducted.

Keywords: milk adulteration, cow milk, goat milk, sheep milk, buffalo milk

Falsificarea laptelui și a produselor lactate cu diferite tipuri de lapte nedeclarate este o activitate de monitorizare globală a alimentelor. Deoarece acest produs alimentar are o piață mare în România, autoritatea competentă a realizat un studiu care cuprinde 368 de unități de lapte și produse lactate testate prin metoda spectrometriei de masă. Falsificarea constă adesea în adăugarea laptelui de vacă la laptele mai scump de oaie, capră sau bivoliță. Cel mai falsificate produse din lapte sunt brânzeturile sărate semi-moi cu o pondere de 5,83%, urmate de brânzeturile semi-moi cu 4%. Metoda spectrometrică de masă are o precizie de 1% și poate fi utilizată pe scară largă în acest domeniu. Procentul de falsificare găsit în România este mic în comparație cu alte țări. Cu toate acestea, trebuie efectuată o analiză suplimentară.

Cuvinte cheie: falsificarea laptelui, lapte de vacă, lapte de capră, lapte de oaie, lapte de bivoliță

After olive oil, milk is the second food product adulterated worldwide. The adulteration of food results is sometime done for cost issues, but this may lead to a reduction of quality of the product (mixture of significantly cheaper cow's milk in high-quality sheep, goat or buffalo milk products). Also, counterfeiting may pose a health risk, making species identification an important requirement from the food safety point of view (8,11). Cow's milk addition to milk and milk products is a common practice because the production of goat's and sheep's milk is subject to seasonal fluctuations. In EU Directive no. 273/2008, the addition of cow's milk is regulated at 0.5% (EU directive 273/2008). Rigorous examination is required when the admixture of other milk is mislabelled.

1) Institute for Diagnosis and Animal Health, Bucharest, Romania
2) Institute for Hygiene and Veterinary Public Health, Bucharest, Romania
3) Carol Davila University of Medicine and Pharmacy, Faculty of Pharmacy, Bucharest, Romania
4) University of Agronomic Sciences and Veterinary Medicine, Faculty of Veterinary Medicine, Bucharest, Romania
*) *Corresponding author: horia.albu@iispv.ro*

There are no internationally or EU harmonized methods for milk species identification in dairy products. Among others, methods used for milk species identification are: chemometric technique (urea-PAGE) 1, isoelectric focusing for the detection of γ-casein, immunochromatographic techniques, chematographic and mass spectrometry techniques (RP-HPLC, MALDI-TOF, NanoESI-QTOF) (2,3,10,12), genetic techniques (PCR) (5,7), immunologic techniques (ELI SA) (6). In Romania past studies were conducted for adulteration in sheep's and goat's cheeses with cow milk using an immunochromatographic assay as method of detection and their findings were relatively high (1,13).

The study aims to set up a method for discrimination and to detect the most adulterated milk product in Romania.

MATERIALS AND METHODS

The species origin of milk and milk products analysed in the study were cow, sheep, goat and buffalo (Table 2). The matrix analysed were: liquid milk (10%

buffalo, 30% goat and 60% sheep), fermented milk (10% sheep, 30% goat and 60% buffalo), hard cheeses (2% cow, 10% buffalo, 28% goat and 60% sheep), semi-hard cheeses (0,55% mixt goat-cow, 1,9% mixt goat-sheep, 2.17% mixt sheep-cow, 5% cow, 10% buffalo, 35% goat, 45,38% sheep), semi-soft cheeses (2% cow, 6% buffalo, 24% goat and 68% sheep), soft cheeses (12% cow, 17% buffalo, 21% goat and 50% sheep), butter (50% sheep and 50% goat) and cream (100% buffalo).

The samples are homogenized by mixing or stirring and are used as such without further preparation. Raw or pasteurized milk is previously centrifuged in 10 mL tubes for 20 minutes at 10 ºC, at 4000rpm. The separate fat layer at the top of the centrifuge tube is used as such. Cheese samples with low water content (hard cheese, parmesan, etc.) are hydrated beforehand by adding water in the sample fraction (4: 1 m / m) and left for hydration for 24 hours at room temperature (20 -22 ºC).

The equipment used is a Waters Synapt Q-Tof coupled with an atmospheric solid probe analysis (ASAP). Desolvation gas (nitrogen) was set at 600 L/ hour at a temperature of 250 ºC, source temperature set at 120 ºC. The time of analysis is only 1 minute. From the TIC chromatogram is extracted the mass spectrum. For the mass spectra comparison were used an open source application mMass. The final evaluation was performed by using an Excel spreadsheet to calculate the specific.

RESULTS AND DISCUSSIONS

The main idea of this work was to develop a quick method for detecting the addition of cow's milk to sheep's or goat's milk. Classical methods, including the reference method of ISO 17678: 2019, are based on chromatographic analysis of triglycerides in milk fat. In addition, there are several methods that focus on different fractions of casein, DNA, lactoglobulins, based on ELISA, PCR, RP-HPLC or electrophoretic separation (9). Compared to these, the method developed in our laboratory has the advantages of speed and very low cost. Direct or ambient analysis based on mass spectrometry involves the analysis of a sample without preparation or with a minimum preparation of the sample before analysis. The main techniques of direct analysis are DART (direct analysis in real time) and DESI (de-sorption electrospray ionization). In our laboratory was used the technique of direct analysis ASAP (at-mospheric solid analysis probe) which uses a combined ionization source APCI + ESI (ESCi). The great advantage of this technique is the low price, the source ASAP being an additional module compatible with a UPLC-Q-Tof, and which can replace the ESI source in a few minutes. The source ASAP uses a stream of heated nitrogen that vaporizes the sample and a corona discharge to ionize the sample. The sample holder is represented by a glass capillary closed at the ends which is inserted into the sample mass and then inserted into the source.

The method developed in the laboratory is based on the technique of fingerprinting the sample in the lipid profile. Animal and vegetable fats have a complex composition but from a chemical point of view fats are generally esters, meaning triglycerides which are about 95% and the rest being phospholipids and sterols. The direct analysis of milk results in a complex mass spectrum (range of interest is between 100 and 1000 u.a.m.) which is predominantly, in terms of intensity and mass, given by the glycerides in milk. From the mass spectrum analysis can be differentiated amines, diacylglycerols, triacylglycerols, glycerophosphoglycerols. Due to the complexity of the composition of milk fat, a single method is not sufficient to identify and characterize fatty acids and their derivatives. The purpose of this paper was not an attempt at structural identification and as such were identified the masses (m / z) of ions that appear constantly in all milk fat samples. The masses identified as constant in milk fat, regardless of species, are 265, 271, 299, 327, 313, 383, 411, 439, 467, 523, 577, 694, 865-884.

Although direct analysis of milk fats after extraction in polar and non-polar solvents has been described in the literature (4), the attempt to reproduce them has not led to satisfactory results. In contrast, given the reactivity of fatty acids, the best results in terms of reproducibility were obtained by using NH_4OH as a dopant and the measured masses result as ammonium adducts (M+ NH4, +).Mass spectrum fingerprints

Table 1

Method performance

Performance parameters / species	Sensibility (%)	Specificity (%)	Limit of detection (LOD)*
Cow	100	100	0,2 %
Sheep	100	95	1 %
Goat	100	95	1 %
Buffalo	75	75	5 %

*) *The limit of detection is calculated as mass percent (% m/m) of product originated specifically.*
For cow milk is indicated the limit of detection from other food products as milk.
For adulteration of cow milk, the added content of sheep or goat milk is indicated as LOD in percent (m/m).

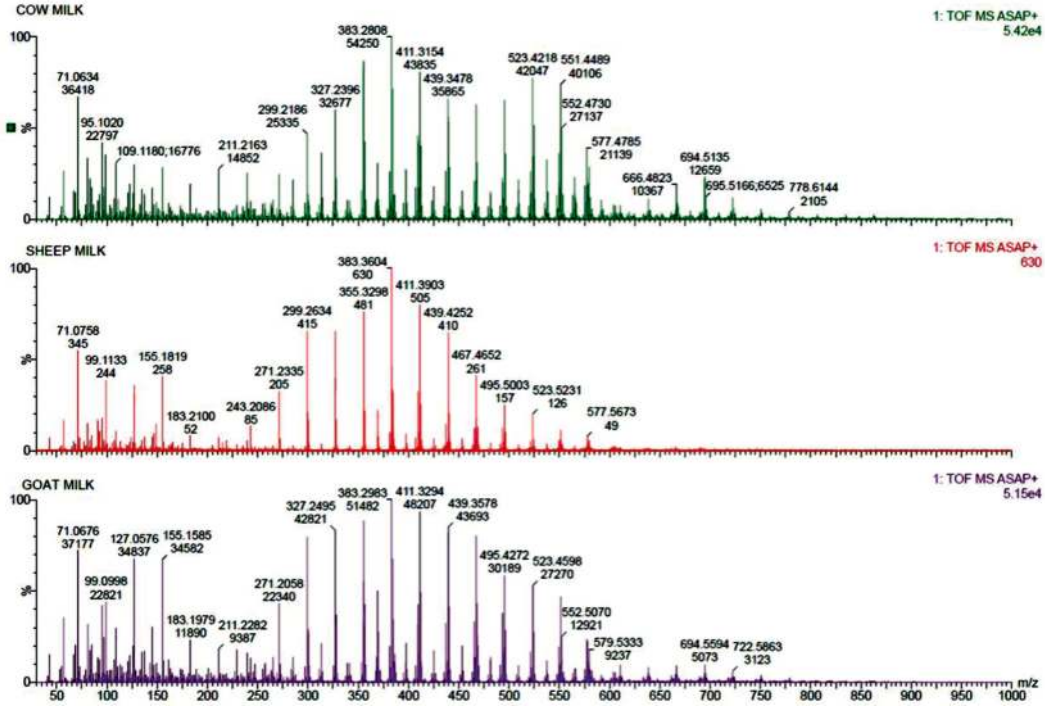

Fig. 1. Comparative mass spectrum fingerprints of cow, sheep and goat milk

R 411/467	R 439/467	R 411/523	R 383/577				RESULT 1			RESULT 2		
R1	R2	R3	R4	R3/R1	R4/R1	SumM R						
1,65	1,04	1,10	1,56	0,67	0,94	5,34	Cow			Cow		
1,69	1,06	1,16	1,56	0,69	0,92	5,46	Cow			Cow		
1,16	0,93	2,12	3,69	1,83	3,19	7,91		Goat			Goat	
1,28	1,05	1,04	1,56	0,82	1,22	4,92	Cow			Cow		
1,32	1,12	2,05	2,28	1,55	1,73	6,78		Goat			Goat	
1,93	1,57	3,96	8,04	2,06	4,16	15,53			Sheep			Sheep
1,65	1,04	1,11	1,56	0,67	0,94	5,35	Cow			Cow		
1,32	1,12	2,05	2,28	1,55	1,73	6,78		Goat			Goat	
1,93	1,57	3,96	8,04	2,06	4,16	15,53			Sheep			Sheep
1,65	1,04	1,11	1,56	0,67	0,94	5,35	Cow			Cow		
1,93	1,56	4,02	12,80	2,06	6,63	20,31			Sheep			Sheep

Fig. 2. Average coefficients for ion ratios between species

for cow's, sheep's and goat's milk result from the ratio of diacyl and triacylglycerol mass intensities. As seen in Figure 1 in the mass range of 250-750 m /z it can be seen the differences between species through the image created by the ratios between ions with masses 299, 327, 383, 411, 439, 467, 523 and 577 m/z.

Identification in the case of unmixed milk is easy in this case but not sufficient. From several experiments for the same species of origin, a marker for cow's milk with a mass of 694 m / z was also identified. For greater accuracy, ratios between ion intensities were established from comparative experiments. Milk-specific diglycerides and triglycerides are those with molecular weights (M + NH4 +): 265, 271, 383, 411, 439, 467, 423, 577 and acyl radicals 299, 327. Of these, the most intense ion in milk and milk derivatives is 383 ion (100%) with respect to which the intensities of the other ions were measured. To identify the species of milk origin, the area of interest is formed by molecular masses (M + NH4): 265, 271, 299, 327, 383, 411, 439, 467, 523, 577. The ratios between relative intensities of ions 411/467, 411/523, 439/467, 383/577 were used as primary criteria and the ratio between them. The ratio between relative intensity of 299/327 is an additional criterion for the differentiation between cow / sheep / goat species. Based on these reports, average coefficients were obtained that are specific to the tested species. In Fig. 2 indicates the values of the ratios

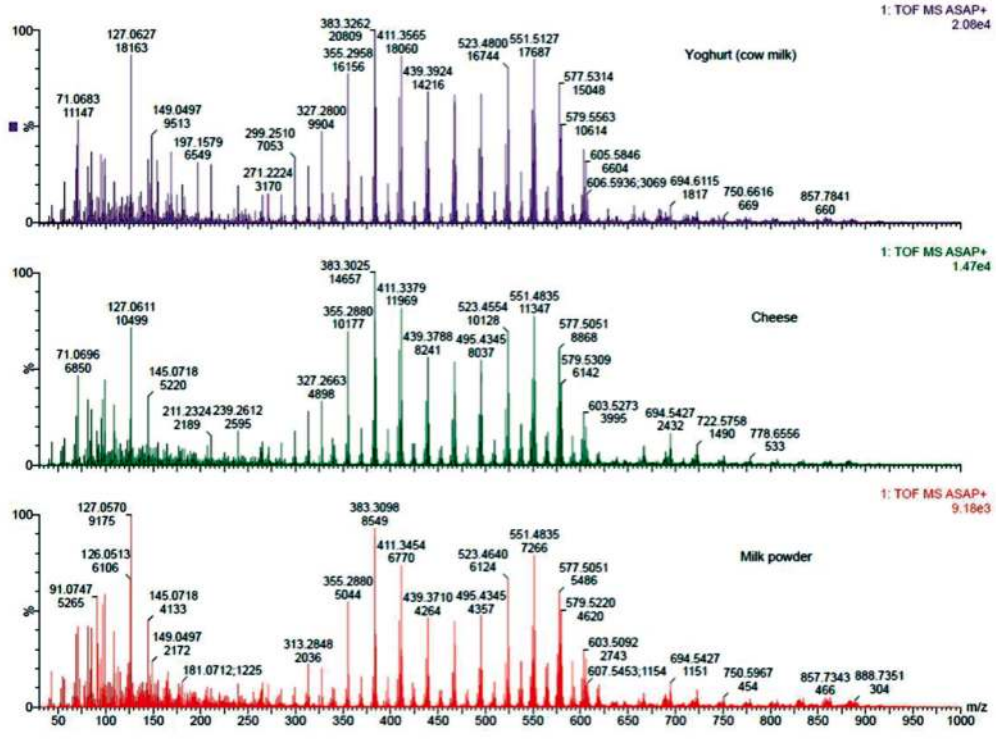

Fig. 3. Comparative mass spectrum fingerprints for cow milk in yogurt, cheese and milk powder

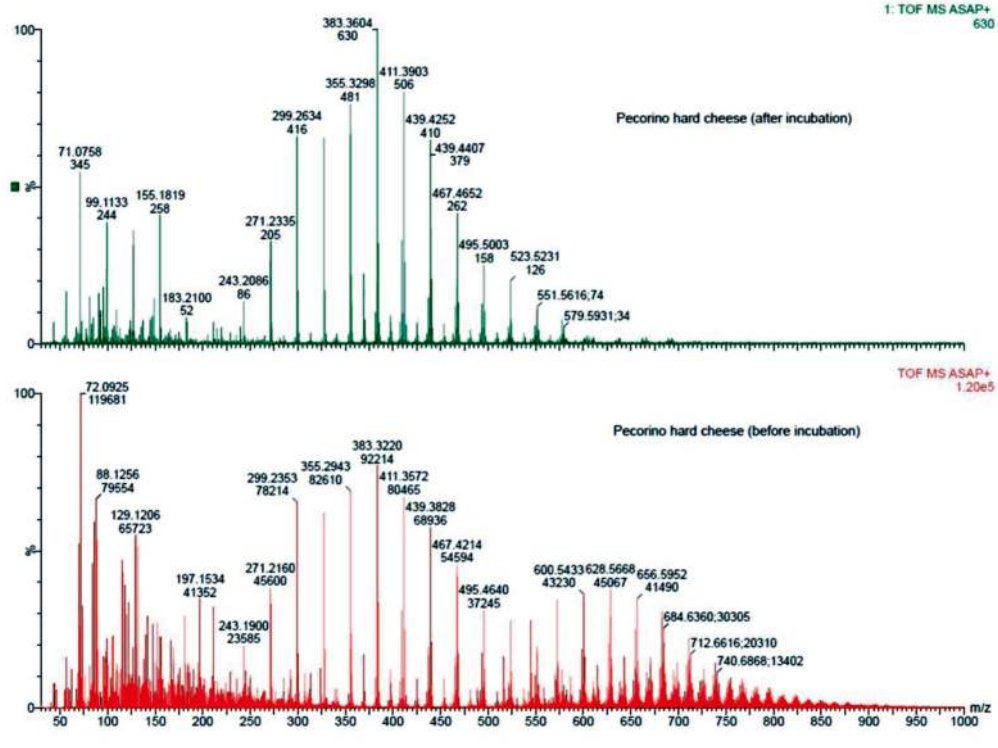

Fig. 4. The mass spectrum of Parmesan cheese before and after water addition

between ion groups for the species tested.

Marker ions and ion ratios are kept constant for different types of milk processing, the species being constantly identified in yogurt, cheese of different types, milk powder. Figure 3 show comparatively the mass spectra for cow's milk in different types of processing.

The quality of the fingerprint is instead dependent on the water content of the sample.

From the performed experiments it resulted that in the milk products with very low water content the ion ratios and as such the calculated coefficients no longer give conclusive results.

Table 2

Samples analysed in the study and results obtained

Matrix/ No. of samples	Liquid milk	Fermented milk	Hard cheeses	Semi-hard cheeses	Semi-soft cheeses	Soft cheeses	Butter	Cream
Total number of tests	8	8	4	240	55	50	1	2
Adultered samples	2*	0	0	14**	0	2***	0	0

** one sheep's milk detected positive for cow and inconclusive for sheep; one goat's detected positive cow and inconclusive for goat CA 21092/2020.*

*** 12 salt ripened sheep cheeses detected positive for sheep; one salt ripened sheep cheese detected positive for sheep and goat; one salt ripened goat cheese detected positive for goat.*

**** two curd sheep cheeses detected positive for sheep.*

In this category of products can be exemplified Parmesan cheese with long maturation, ghee butter.

The initial mass spectrum and the ion ratios are restored after a simple incubation of the sample, at 45^{0}C and with the addition of water in a ratio of 1:5 (m/m or v/v)).

In Figure 4 it can be seen the mass spectrum of Parmesan cheese before and after water addition.

The study was designed to further identify more expensive species in matrices where their properties and manufacturing process do not allow easy identification from an organoleptic point of view.

The cheese matrix has been tested more because they are more susceptible to counterfeiting because in the manufacturing process additives can be added to change the taste, color, aroma, etc., compared to less processed milk products to which the organoleptic properties can induce more or less belonging to the species.

No falsifications were registered for fermented milk products, hard pasta, semi-frozen pasta, butter and sour cream. There were also no falsifications of mixed semi-finished paste products. The most counterfeits were registered for semi-sheep cheese products based on sheep's milk, and the fewest for sheep and goat's milk. For the two falsified milk products the results were clearly positive for the cow and inconclusive for the species of origin.

By using an immunochromatographic assay in Romania past studies were conducted for sheep's and goat's cheeses adulteration with approx. 60% counterfeiting percent (13). May be because of the method used (immunological type) or may be, in time, the producers were becoming more consciences of their business, our findings suggest that this food is not that high adulterated.

CONCLUSIONS

The method developed is suitable and reliable for the scope of species milk discrimination. Fast result obtained in routine analysis and the relative low cost of the analysis are the main advantages.

This technique applied as routine analysis could be used in currently control programme for food fraud detection. The results obtained in the study indicate a relative low percent of milk fat adulteration in Romania.

REFERENCES

1. *Vernile A., Beresford T.P., Spano G., Massa S., Fox P.F.,* (2007), Chemical studies of Pecorino Siciliano cheese throughout ripening. Milchwissenschaft, 62:280-284
2. *Hayaloglu A.A.; Cakmakci S.; Brechany E.Y.; Deegan K.C.; McSweeney P.L.H.*, (2007), Microbiology, biochemistry, and volatile composition of Tulum cheese ripened in goat's skin or plastic bags. J Dairy Sci, 90:1102-1121
3. *Roncada P., Gaviraghi A., Liberatori S., Canas B., Bini L., Greppi G.F.*, (2002), Identification of caseins in goat milk. Proteomics, 2:723-726
4. *Pohlentz G., Kölbl S., Peter-Katalinic J.*, (2005), High sequence coverage by in-capillary proteolysis of native proteins and simultaneous analysis of the resulting peptides by nanoelectrospray ionization-mass spectrometry and tandem mass spectrometry. Proteomics, 5:1758-1763
5. *Cunsolo V., Galliano F., Muccilli V., Saletti R., Marletta D., Bordonaro S., Foti S.*, (2005), Detection and characterization by high-performance liquid chromatography and mass spectrometry of a goat β-casein associated with a CSN2 null allele. Rapid Commun Mass Spectrom, 19:2943-2949
6. *Klotz A., Einspanier R.,* (2001), Development of a DNA-based screening method to detect cow milk in ewe, goat and buffalo milk and dairy products using PCR-LCR-EIA-technique. Milchwissenschaft, 56:67-69
7. *Lopparelli R.M., Cardazzo B., Balzan S., Giaccone V., Novelli E.,* (2007), Real-time TaqMan polymerase chain reaction detection and quantification of cow DNA in pure water buffalo mozzarella cheese: Method validation and its application on commer-

cial samples. J Agric Food Chem, 55:3429-3434
8. *Levieux D., Venien A.*, (1994), Rapid, sensitive two -site ELISA for detection of cows' milk in goats' or ewes' milk using monoclonal antibodies. J Dairy Res, 61:91-99
9. *Poonia A., Jha A., Sharma R., Singh H., Rai A., Sharma N.*, (2016), Detection of adulteration in milk: A review. International Journal of Dairy Technology, 70(1):23-42
10. *Osman A.M.G., Chittiboyina A.G., Khan I.A.*, (2013), Chapter 32: Plant Toxins. In: Foodborne Infections and Intoxications. 4. Auflage, S. 435-451
11. *Stănciuc (Sava) N., Râpeanu G.,* (2010), Identification of adulterated sheep and goat cheeses marketed in Romania by immunocromatographic assay, Food and Agricultural Immunology, 21(2): 157-164
12. *Bulancea M., Rapeanu G.,* (2009), Authentication and identification of food product adulteration, Teaching and Pedagogical Publishing, Galati, Romania
13. *Peter Z.,* (2011), Identification of milk and milk products, Mljerkarstvo, 61(3):199-207
14. *Hrbek V., Vaclavik L., Elich O., Hajslova J.,* (2014), Authentication of milk and milk-based foods by direct analysis in real time ionization–high resolution mass spectrometry (DART–HRMS) technique: A critical assessment. Food Control, 36(1):138-145.