

STUDY ON THE SEPARATION THROUGH THIN LAYER CHROMATOGRAPHY OF THE CHOLESTEROL IN THE BUFFALO MILK ORIGINATING IN THE MESENDORF FARM

STUDIUL PRIVIND SEPARAREA PRIN CROMATOGRAFIE ÎN STRAT SUBȚIRE A COLESTEROLULUI DIN LAPTELE DE BIVOLIȚĂ PROVENIT DE LA FERMA MESENDORF

PECE AURELIA*, COROIAN C.*, GHIRILĂ BIANCA*, MUREȘAN G.*

**University Agricultural Science and Veterinary Medicine, Faculty of Animal Husbandry and Biotechnology, 3 – 5 Mănăștur Street, Cluj-Napoca, Romania*

Within the present study, we conducted a thin layer chromatographic separation of neuter fats in buffalo milk, following sodium metoxyde saponification and the exposition to $\lambda=366$ nm. The extraction of free and esterized milk cholesterol was conducted according to the Folch et al. (1957) method. The free and esterized cholesterol, as well as triglycerids appear as brown spots (iodine complexes with the double connections between the compounds under anlysis). Qualitative identification was conducted through the comparison of Rfs for free and esterized cholesterol, in the samples analyzed with existing standards. The semi-quantitative anlysis was conducted through a visual comparison of the intensity encountered in free cholesterol spots within samples under anlysis and the standard of different concentrations applied on the same board. Buffalo milk samples originated in the Mesendorf Farm, considered and certified as an ecologic farm.

Key words: cholesterol, milk, buffalo

Introduction

The quantity and composition of cholesterol are influenced in the case of milk by the genetic potential of animals, food quality, season, lactation, animal health. It was thus considered that nutrition is one of the most important factors conditioning the level of milk cholesterol. Nevertheless, cholesterol dynmics and the factors that can influence its quality and quantity in the milk have not been fully researched. The cholesterol is the most common and widespread sterol, encoun tered in the plant and animal kingdoms under free or esterized forms. The majority of cholesterol found in superior animal tissues and humna tissues doesn't have food origin and it is the result of the biosynthesis in an acetyl organism-CoA, formed during the inetrmediary fat, fatty acids and aminoacids metabolism. (Dumitru I.F., 1980; Aurora Popescu et al. 1980; Petter N.et al., 2004).

Materials and Methods

The extraction of free and esterized milk cholesterol was conducted according to the Folch et al. (1957) method with minor alterations regarding solvent or sample volumes. In this respect, 2.5 ml of milk were extracted using 48 ml chloroform/methanol/water (2:1:1, v/v/v) through intermittent stirring for 30 minutes in a separation funnel. The superior layer (methanol and water) was separated through creasing, while the middle and inferior layers (proteins and chloroform containing free and esterized cholesterol) was vacuum filtered on a Büchner funnel. The chloroform layer was filtered again on anhydrous sodium sulphate, while Na_2SO_4 is washed three times with 5 ml chloroform each. The fatty extracts were vacuum evaporated using a rotavapor on 50 °C, while the residue was recovered with 1 ml chloroform in a 2 ml vial with a teflon lid and kept at 18° C until the analysis.

Fig 1. Extraction and filtering



Fig 2. Phase separation



Fig 3. Washing with water and chloroform



Fig 4. Drying on sodium hydroxid



The sodium methoxide saponification was conducted in a Pyrex tube where the chloroform extract of free and esterized cholesterol previously obtained is added and evaporates dry through methane blow. Over the residue obtained we add

1 ml of methanolic solution of sodium metoxide (0.5M). 1 ml methanol and 1 ml toluen and it is heated to 70 °C for an hour.

After cooling, 1 ml of distilled water is added and the cholesterol is extracted with 2 x 8 ml ethylic ether in a separation funnel. Ether extracts of cholesterol are filtered through anhydrous sodium sulphate and evaporate dry.

The residue is recovered with 1 ml chloroform in a 2 ml vial. The volume applied (10 µl) was the same for all samples and standards under analysis. A chromatographic Camag camera was sued for developing, covered with a glass lid to avoid the evaporation of more volatile solvents and margn effects.

Results and Discussions

Lipid visualising was conducted through iodine vapor exposition in a chromatographic camera covered with a glass lid. Free cholesterol spots in analyzed samples can be observed after 10 minutes, but they remain visible even 3 hours after iodine vapor exposition.

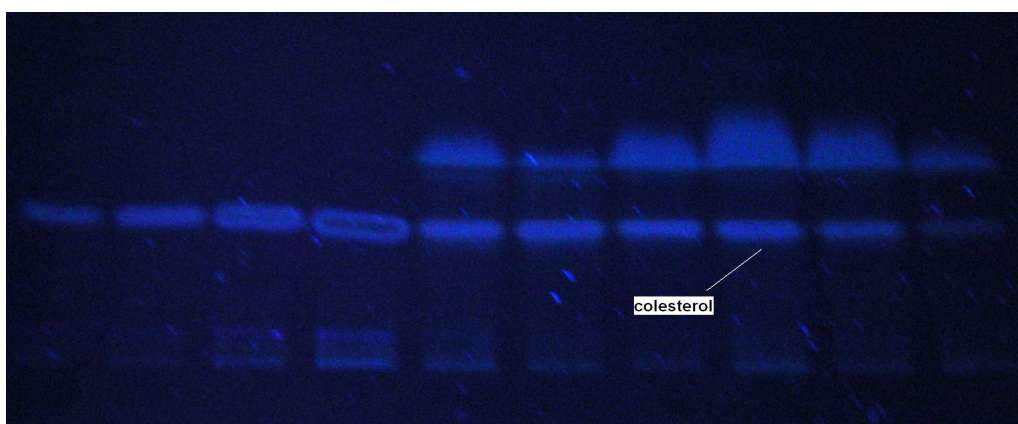


Fig. 5 The separation through thin layer chromatography of neuter lipids in buffalo milk, after sodium metoxide saponification and the exposition to $\lambda=366$ nm

As iodine visualising is not satisfactory, another reactive was employed for visualising and densiometry. As such, spots were visualised with a mixture of copper sulphate/ phosphoric acid (3g copper sulphate in 10 ml phosphoric acid 10% v/v) and heated to 110 °C for 20 mintes. Spot scanning was conducted with a photodesiometer Camag TLC Scanner 3 at $\lambda = 366$ nm for an hour.

The Rfs for neuter fat standards after developing with a mixture of petroleum ether (pf 40 - 60°C) – ethylic ether- acetic acid in different proportions.

Table 1

Lipide	faza mobilă				
	A	B	C	D	E
Colesterol esterificat (CE)	0,82	0,68	0,77	0,80	0,82
Esteri ai acizilor grași (FAME)	0,76	0,61	0,67	0,72	0,76
Acizi grași liberi (FFA)	0,43	0,11	0,25	0,32	0,37
Colesterol liber (CL)	0,43	0,11	0,23	0,32	0,37

Fig. 6

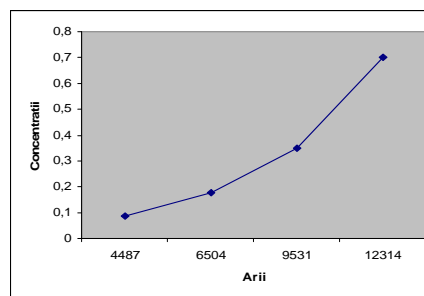


Table 1 presents the Rfs for a mixture of neuter fat standards using as a mobile phase petroleum ether- ethylic ether- acetic acid.

The solvent ratio (in volumes) in mobile phases: A: 60+ 20 + 12; B: 80 + 20 + 2; C: 80 + 20 + 3; D: 80 + 20 + 6; E: 80 + 20 + 8

As observed in table 1, the mixture of neuter fat standards was appropriately separated after developing with five mobile phases (A-E) employed, with the exception of cholesterol from free fatty acids. Consequently, we opted for mobile phase C, which ensures a satisfactory separation of the two spots (Rf FFA= 0.25 and Rf CL= 0.23).

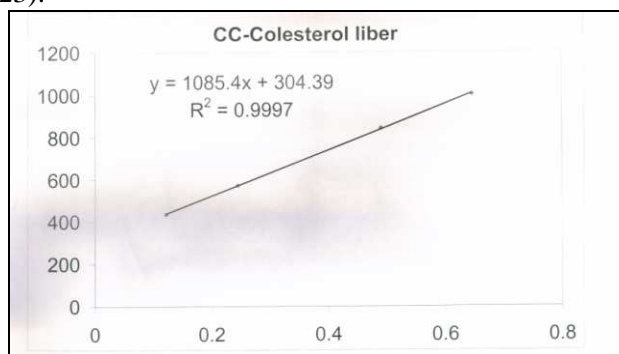


Fig. 7 Calibration curve for free cholesterol (on the abscissa, cholesterol concentration in mg/ml and on the ordinate the peak surface in u.a)

Conclusions

- As observed from the results presented above, another advantage in the employment of this system resides in the separation of neuter fats in narrow strips;
- Several mobile phases underwent analysis for obtaining a satisfactory separation especially for free cholesterol and free fatty acids. This problem emerged as a consequence of transesterification with sodium

metoxide which is not sufficient for free fatty acids;

- The determination of cholesterol in buffalo milk through thin layer chromatography coupled with a densitometry is considered as a premiere at a national level, with a high degree of sensitivity and accuracy, while the results obtained can be comparable to researches and values of specialty literature.

Bibliography

1. **Aurora Popescu, Elena Cristea, Marcela Zamfirescu**, (1980) - *Biochimie Medicală*. Ed. Medicală, București, p.324.
2. **Dylewski D.P., Dapper C.H., Valivullah H.M., Deeney J.T., Keenan T. W.**, (1995) - *Morphological and biochemical characterization of possible intracelular precursors of milk lipid globules*. Eur. J. Cell Biol. 35:99.
3. **Lee D.K., Aha J., Kwak H.S.**, (1999) - *Cholesterol removal from homogeniyed milk with β – cyclodextrin*. J.Dairy Sci. 82: 2327 -2330.
4. **Petter N., Campbell, Anthony D., Smith**, (2004) - *Biochimie ilustrată*. Ed. academiiei române. București, p.152-172
5. **Pintea Adela**, (2005) - *Biochimie structurală. Biomolecule plastice*, Editura Academic Pres, Cluj-Napoca).
6. **Sieber R.**, (1994) - *Cholesterol removal from animal food – can it be justified?* Lebensmitt. Wissenschaft Technol. 26:375 -387.