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

COMPARATIVE ANALYSIS OF TOTAL RNA ISOLATION PROCEDURES FROM SMALL ADIPOSE TISSUE SAMPLES IN SHEEP

ANALIZA COMPARATIVĂ A PROCEDURILOR DE IZOLARE A ARN-ULUI TOTAL DIN PROBE MICI DE ȚESUT ADIPOS LA OVINE

Claudia-Terezia SOCOL[1),*)], C.G. ŞONEA[2)], Cristina MAERESCU[1)], F.L. CRISTE[3),*)]

ABSTRACT | REZUMAT

RNA extraction is crucial for molecular experiments, which require significant amounts of high-quality RNA. RNA isolation from adipose tissue can be challenging, being more problematic than from other tissues, due to the high lipid content. This study aims to compare the efficacy of three total RNA extraction procedures from Tsurcana sheep small adipose tissue samples, specifically of two commercially available kits and a Trizol based method. RNA quality and quantity were determined by spectrophotometric means. RNA extractions assessed in this study showed differences in total RNA quantity and quality, that should be considered when selecting the methods to be used for isolating RNA for molecular analyses in animal studies. Both kits-based methods showed high purity values of 1.9, meanwhile, the Trizol based method yielded the highest RNA quantity (718 ng/µl), but lower purity values (1.59). Our data indicate good results by using the RNAgents Total RNA Isolation System Kit yielding the satisfactory amount of high-quality RNA (157 ng/µl, 1.94), as a primary step for further downstream molecular studies still requiring to elucidate candidate gene profiles or mechanisms potentially related to adipose tissue development, production traits, and quality.

Keywords: total RNA extraction, RNA quantity, RNA purity, adipose tissue, sheep

Extracția ARN-ului este foarte importantă pentru experimentele moleculare, care necesită cantități semnificative de ARN de înaltă calitate. Izolarea ARN-ului din țesutul adipos poate fi o provocare în acest sens, fiind mai problematică decât din alte țesuturi, datorită conținutului ridicat de lipide. Acest studiu vizează compararea eficacității a trei proceduri de extracție a ARN-ului total din probe mici de țesut adipos provenit de la oi din rasa Țurcană, respectiv a două kituri comerciale și a unei metode de extracție pe bază de Trizol. Calitatea și cantitatea ARN-ului au fost determinate prin metode spectrofotometrice. Metodele de extracție ARN analizate în acest studiu au prezentat diferențe în ceea ce privește cantitatea și calitatea ARN-ului total, diferențe de care ar trebui să se țină cont la alegerea metodelor de izolare a ARN-ului utilizat pentru diferite analize moleculare în cercetările efectuate pe animale. Ambele metode bazate pe kituri au prezentat valori ridicate ale purității, de 1,9, în timp ce metoda pe bază de Trizol a condus la obținerea celei mai mari cantități de ARN (718 ng/µl), însă cu valori mai mici ale purității (1,59). Datele noastre indică rezultate bune pentru utilizarea kitului RNAgents Total RNA Isolation System Kit, conducând la obținerea unei cantități satisfăcătoare de ARN de înaltă calitate (157 ng/µl, 1,94), ca etapă primară pentru ulterioare studii moleculare, în vederea elucidării profilelor genelor candidate sau a mecanismelor potențiale cu privire la dezvoltarea țesutului adipos, caractere de producție și de calitate.

Cuvinte cheie: extracția ARN total, cantitatea ARN, puritatea ARN, țesut adipos, oaie

RNA-based technologies development and use require high quantities and quality of RNA, generally being the primary factors considered, when determining whether an RNA sample is acceptable for molecular biology analysis (25). The structural features and the composition of different animal tissues are responsible for significant differences in the isolated RNA quality and quantity (10, 11, 26), the source of RNA having the ability to affect the quantity and quality of the final product (18). Adipose tissue is an active metabolic and endocrine organ (12, 16, 19), producing a wide spectrum of hormones and factors that play crucial roles in regulating cell turnover and function (8). Due to the high content in triglycerides, RNA isolation from adipose tissue turns to be more problematic than from other tissues (2, 10, 26, 30). Moreover, the characte-

1) University of Oradea, Romania
2) Valahia University of Târgoviște, Romania
3) National Agency for Animal Husbandry "Prof. Dr. G.K. Constantinescu", Baloteşti, Romania
*) *Corresponding authors: clausocol@yahoo.com, florinleontincriste@gmail.com*

ristics of specific adipose tissue depots depend on animal species and breeds (9). Therefore, RNA isolation from adipose tissue could be challenging when high-quality RNA is required for downstream experiments (6, 20). RNA is thermodynamically a stable molecule, rapidly digested in presence of RNase enzymes so that molecule degradation and injury potentially interfere with downstream experiments (24), requiring an adequate solution for stocking the samples of adipose tissue. The integrity and type of the RNA can vary, depending on the sampling method (3). Adequate reagents are able to stop the action of the RNases and protect the adipose tissue against their destructive action. Furthermore, a free ribonuclease environment has to be ensured (18). Moreover, the decrease of the stress factor during the biologic material sampling has to be considered. Complex transcriptome profiles in fat tissues have been proven, further studies are still required to investigate the roles of the candidate genes involved in fat deposition and meat quality (4, 13, 22, 28). Also, the difference of how strongly adipose and muscle tissues are linked to meat quality is still unknown (29). Among Romanian sheep breeds, Tsurcana satisfies the needs of small, non-professional holders, who own some 60-70% of all sheep (7), showing valuable adaptive, resistance, and production traits, showing potential to stimulate the interest in analysing the expression of specific genes associated with fat deposition. Moreover, such molecular studies are still needed and especially in this breed, which has not been yet enough studied at the molecular level.

The high lipid content of the ovine subcutaneous adipose tissue can make difficult RNA extraction. Moreover, the characteristics of specific adipose tissue depots depend on animal species and breeds (9, 17). The identification of the best suitable RNA isolation method from adipose tissue is required for achieving an adequate RNA quality and quantity; that can play a crucial role in the success of further experiments (14, 17, 21). Kits are generally ensuring sufficient RNA amounts for most downstream applications but differences in RNA quality could interfere in the ability to yield RNA acceptable for some applications (25). Such differences should be considered when selecting the methods to be used for isolating RNA designated for downstream analyses. For such purpose, three different RNA isolation procedures were assessed for extracting total RNA from sheep adipose tissue. The obtained results are directly applicable in further RNA-based molecular studies in animal science.

MATERIALS AND METHODS

Experimental design and sampling

RNA isolation was performed using three procedures: SV Total RNA Isolation System (Promega) kit, RNAgents Total RNA Isolation System (Promega) kit, and the Trizol based extraction method. RNA extractions using kits were performed based on the manufacturer instructions meanwhile, extractions using Trizol were carried out based on modified previously described protocol (5). A total of 30 samples were randomly assigned for RNA isolation by each of the kits so that 10 samples of each tissue sample were extracted by each kit. Following the RNA extraction procedures, the quantity and quality of RNA in each sample were assessed. All materials and surgical instruments used for sampling and RNA isolation were sterile or washed using bi-distilled water and autoclaved (120ºC, 20 minutes, RAYPA Stericlav SAES75). All the work surfaces were washed with the RNase Zap solution (Sigma-Aldrich) to ensure a ribonuclease-free environment. Subcutaneous adipose tissue was excised from mature sheep (Tsurcana breed) from the tail body region, according to the veterinary medical procedures for sampling. All samples were submerged immediately in a small RNA Later solution (Sigma-Aldrich) volume, for storage and to stabilize and protect cellular RNA. Samples were stored at −80°C until RNA isolation. All kits solution were prepared according to the manufacturer's guidelines. For each RNA extraction, 20 mg of tissue were homogenized in the lysis buffer using an Ultraturax IKA T8.0.

Reagents and materials

SV Total RNA Isolation System (Promega): RNA Lysis Buffer (4M guanidine-isothiocyanate, 0.01M Tris pH 7,5, 0.97% β-mercaptoethanol), RNA Dilution Buffer, β-mercaptoethanol 48.7%, DN-ase I, $MgCl_2$ 0.09 M, Yellow Core Buffer (0.0225 M Tris pH 7.5, 125 M NaCl, 0.0025% yellow dye), DNase Stop Solution (2M guanidine-isothiocyanate, 4 mM TrisHCl pH 7.5, 57% ethanol), DNase incubation mix, RNA Wash Solution (60 mM potassium acetate, 10 mM TrisHCl pH 7.5, 60% ethanol), nuclease free water, Spin Assemblies (spin columns for purification and collection microtubes), ethanol 95%. RNAgents Total RNA Isolation System (Promega): RNA denaturation solution (26 mM sodium citrate pH 4.0, 0.5% N-lauryl sarcosine, 0.125 M β-mercaptoethanol, 4 M guanidine thiocyanate), sodium acetate 2M pH 4.0, phenol: chloroform: isoamyl alcohol solution 99:24:1 pH 4.7, isopropanol, nuclease free water, ethanol 75%.

Trizol method: Trizol reagent, chloroform, isopropanol (Sigma-Aldrich).

RNA extraction using SV Total RNA Isolation System (Promega)

The tissue sample was first homogenized in 250 µl RNA lysis buffer, so easy pipetting of cell lysate was possible. 350 µl RNA dilution buffer was added, followed by incubation for 3 minutes at 70ºC using a wa-

ter bath. Centrifugation at 14000 x g for 10 minutes allowed proteins and cell detritus precipitation. Purification was carried out through spin centrifugation. The supernatant mixed with 200 µl ethanol 95% was homogenized by pipetting and transferred into the Spin Assemblies and centrifuged 14000 x g for 1 minute, enabling RNA attachment to the microcolumn membrane. 50 µl of DNase incubation mix were added to the membrane of the microcolumn and incubated for 15 minutes at room temperature. 200 µl DNase stop solution was added, followed by another centrifugation step 14000xg for 1 minute.Three washing steps using 600µl RNA wash solution and centrifugation 14000 x g for 1 min., respective 2 minutes was carried out. RNA was diluted in 100 µl nuclease-free water.

RNA extraction using RNAgents Total RNA Isolation System (Promega)

Tissue samples were mixed with 600 µl RNA denaturation solution, followed by incubation on ice for 5 minutes and homogenization step. 60 µl sodium acetate 2M and 600 µl phenol: chloroform: isoamyl alcohol solutions were added and strong shacked for 10 seconds. After a 15 minutes incubation on ice, centrifugation for 20 minutes at 10000 x g 4°C was performed, enabling the organic and aqueous phases separation. RNA precipitation was made twice, using an equal volume of isopropanol and aqueous phase recovered. After a prior homogenization, an incubation -20°C for 30 minutes, centrifugation of 10000 x g for 15 minutes at 4°C was applied. Two RNA washes were made using 1 ml ethanol 75% solution and suspended in 100 µl nuclease-free water, and also for ethanol removal samples were incubated at 37°C.

RNA extraction using the Trizol method

Tissue samples were mixed with 0,5 ml Trizol solution (Sigma-Aldrich) prior homogenized. To remove the insoluble material, the supernatant was transferred to another Eppendorf tube and additional centrifugation of 12000 x g for 10 minutes at 4°C was performed. The supernatant was transferred to another Eppendorf tube and incubated for 5 minutes at room temperature. After 200 µl chloroform (Sigma-Aldrich) addition, the mixture was vortexed, incubated 3 minutes at room temperature, and centrifuged 12000 x g for 15 minutes at 4°C, leading to three phases of separation. The aqueous phase containing the RNA was isolated in an Eppendorf tube. Two times 500 µl isopropanol (Sigma-Aldrich) was added, followed by vortexing, incubation for 10 minutes at room temperature, and centrifugation 12000 x g for 15 minutes at 4°C. The RNA pellet was diluted in 100 µl nuclease-free water.

RNA quantification

RNA quantity and quality were determined by spectral photometry. Nanodrop UV VIS ND-1000 was used at OD260 for calculation of the concentration and 260/280 ratios. Sample data measurements were collected using the specific software on a computer, being further analysed in comparison to the RNA extraction methods assessed. Protein bands were also analysed based on electrophoresis gels.

RESULTS AND DISCUSSIONS

Regardless of the method assessed, the average amount of total RNA recovered from Tsurcana small adipose tissue samples showed sufficient amount for most downstream molecular applications, but with some differences regarding the RNA yield and purity. SV Total RNA Isolation System for the total RNA isolation from subcutaneous tissue yielded relatively low amounts of total RNA of 10 ng/µl, but its purity showed high values, being around 1.91. RNA isolation using SV Total RNA Isolation System (Promega) kit relied on four essential steps: disruption of cell membranes, denaturation of nucleoprotein complexes, inactivation of endogen ribonucleases, and removal of contaminants (DNA and proteins). The DNase sample treatment, during purification, allowed substantial reduction of the contamination with genomic DNA that can interfere in further PCR amplification of specific genes.

RNAgents Total RNA Isolation System Kit yielded to satisfying RNA concentration values of 157 ng/µl in average and also its purity showed high values (1.94). The inconvenience of the vacuum desiccator's lack, device missing in the lab, for now, was overtaken by the tube incubation at 37°C, which led to the right removal of the ethanol out of the sample, a fact that is important in the previous phase of the RNA suspension.

The total RNA isolation method using RNAgents Total RNA Isolation System (Promega) shows the advantage of determining a satisfactory quantity and quality of the extracted RNA. By using organic solvents for RNA isolation, the RNA precipitation process is eliminated.

Using total RNA isolation with Trizol reagent from subcutaneous adipose tissue ensured a significant increase in the concentration of total RNA (718 ng/µl average value of concentration), albeit the purity of the sample was lower if compared with RNA samples obtained with the other methods assessed in this study (1. 59). Isolation of total RNA using the Trizol method consisted of homogenization of the adipose tissue sample, purification of total RNA using liquid-liquid extraction with organic solvents (Trizol and chloroform), and precipitation of RNA with isopropanol. In the case of total RNA isolation with Trizol, chloroform, and isopropanol, the simplicity of the method must be pointed out, the high RNA yield also, but lower values of purity were registered. The lower values achieved indicate the presence of proteins, remains of solvents (i.e., phenol/chlo-

roform) or salts in the RNA solution. The use of this extraction method might need a further purification step to remove contaminants, such as traces of organic solvents that can interfere with cDNA synthesis,being well known that all phenol-based methods are showing remaining impurities, which could compromise downstream applications (2, 6, 26).

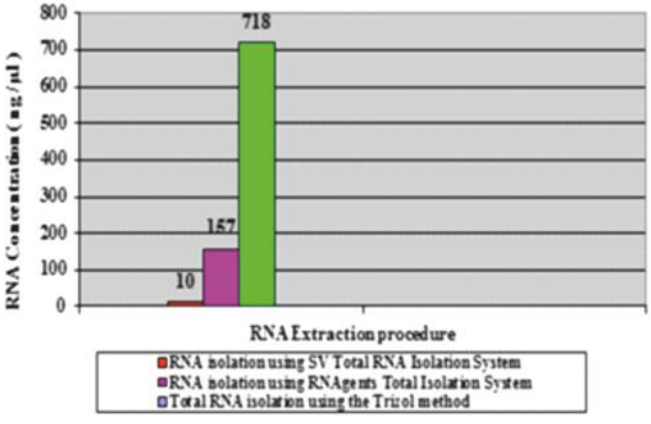

Fig. 1. Comparison of total RNA concentrations achieved using the three RNA isolation procedures

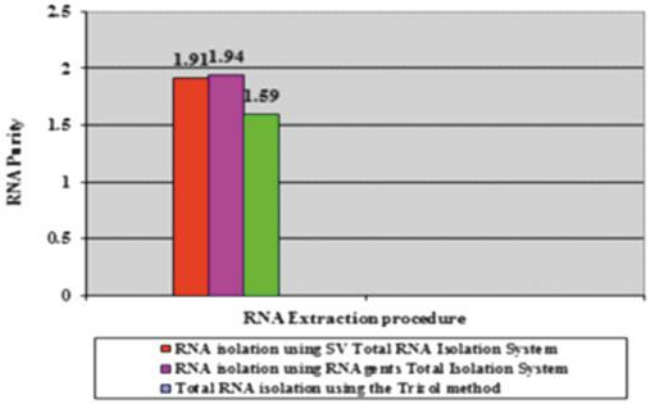

Fig. 2. Comparison of total RNA purity achieved using the three RNA isolation procedures

RNA quantification using the three methods proves that each method is suitable for isolating a qualitative total RNA (Fig. 1 and 2) being in line with statements that in general kits provide sufficient RNA amounts with some differences in RNA quality that could interfere in the ability to yield RNA acceptable for some applications (25). Also, the use of the Nanodrop UV VIS ND 1000 device allowed the quantification of the genetic material in a 1µl volume that is justified both financially and temporal. RNA quantity and quality assessment throughout spectral photometry measurements at A260/A280 ratio showing values greater than 1.8 are usually considered an acceptable indicator of good quality RNA with a low level of protein contamination for molecular analysis (1, 2, 15, 23, 31).

RNA samples showing the highest purity values were isolated by using RNAgents Total RNA Isolation System isolation Kit and SV Total RNA Isolation System (values ranging between the limits of 1.8-2.0). The RNA isolation method based on Trizol yielded higher RNA amounts (718 ng/µl) with lower purity values (1.59). Balancing the obtained results from the isolated RNA quantity and quality point of view, using the above three methods, we find RNAgents Total RNA Isolation System Kit more suitable for RNA isolation from small adipose tissue samples, because it yielded higher amounts of RNA (157 ng/µl) of high purity (1.9), that allows its use in further experiments. Comparative analyses of the results obtained indicate that RNA extraction methods assessed in this study are all suitable for isolating total RNA from small adipose tissue samples, some differences among quantity and quality being referred. Both, RNAgents Total RNA Isolation System (Promega) and SV Total RNA Isolation System (Promega) seem to be similar in their ability to extract relatively sufficient quantities of high RNA purity, the Trizol based method yielding to the highest RNA amount, but showing lower purity values. Therefore, the practical differences between the RNA isolation kits assessed should be considered when selecting the extraction methods for extracting RNA designated for downstream molecular experiments especially associated with adiposity (17, 21, 27).

CONCLUSIONS

RNA isolation procedures from Tsurcana small adipose tissue assessed in this work could be useful for further molecular downstream studies related to this Romanian breed, which could be of interest for adiposity associated studies, but also for its adaptive and resistance traits. Our data indicate good results for using the RNAgents Total RNA Isolation System Kit (Promega) for total RNA isolation from small adipose tissue samples. The suitability for further molecular studies still requiring investigations, such as those related to gene profiles or mechanisms potentially related to adiposity, production traits, and quality.

REFERENCES

1. *Becker C.,Hammerle-Fickinger A., Riedmaier I., Pfaffl M.W.,* (2010), mRNA and microRNA quality control for RT-qPCR analysis. Methods, 50(4):237-243
2. *Braicu C., Buse M., Busuioc C., Drula R., Gulei D., Raduly L., Rusu A., Irimie A., Atanasov A.G., Slaby O, Ionescu C., Berindan-Neagoe I., (*2019), A comprehensive review on MAPK: a promising therapeutic target in cancer. Cancers (Basel), 11(10):1618
3. *Canovas A., Rincon G., Bevilacqua C., Islas-Trejo A., Brenaut P., Hove R.C., Boutinaud M., Morgenthaler C., Vanklompenberg M.K., Martin P., Medrano J.F.*, (2014), Comparison of five different RNA sources to

examine the lactating bovine mammary gland transcriptome using RNA-Sequencing. Sc.Reports, 4:5297
4. *Choi J.Y., Shin D., Lee H.J., Oh J.D.,* (2018), Comparison of long noncoding RNA between muscles and adipose tissues in Hanwoo beef cattle. Anim Cells Syst (Seoul), 23(1):50-58
5. *Chomczynski P. and Sacchi N.,* (1987), Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem, 162(1):156-159
6. *Cirera S.,* (2013), Highly efficient method for isolation of total RNA. BMC Res Notes, 6:472
7. *FAO,*(2003),Romanian strategy for a sustainable management of farm animal genetic resources. Romania Country Report for SoW-An-GR. Available at: http://www.fao.org/docrep/pdf/010/a1250e/annexes/CountryReports/Romania.pdf (Accessed: June, 1, 2021)
8. *Guan H.,Yang K.*,(2008), RNA isolation and real-time quantitative RT-PCR. Methods Mol Biol. 456:259-270
9. *Hausman G.J.,Basu U.,Wei S.,Hausman D.B, Dodson M.V.,* (2014), Preadipocyte and adipose tissue differentiation in meat animals: influence of species and anatomical location. Annu Rev An Biosci. 2:323-351
10. *Hemmrich K., Denecke B., Paul N.E., Hoffmeister D.,* (2010), RNA Isolation from Adipose Tissue: An Optimized Procedure for High RNA Yield and Integrity. Lab Medicine, 41(2):104-106
11. *Johnson M*., (2012), RNA extraction. United States Synatom Research, Princeton, New Jersey, USA
12. *Kershaw E.E., Flier J.S.,* (2004), Adipose tissue as an endocrine organ. J Clin Endocr Metab. 89:2548-2556
13. *Li B., Qiao L., An L., Wang W., Liu J., Ren Y., Pan Y., Jing J., Liu W.,* (2018), Transcriptome analysis of adipose tissues from two fat-tailed sheep breeds reveals key genes involved in fat deposition. BMC Genomics, 19(1):338
14. *Méndez V., Avelar E.,Morales A.,Cervantes M., Araiza A., González D.,* (2011), A rapid protocol for purification of total RNA for tissues collected from pigs at a slaughterhouse. Gen Mol Res, 10(4):3251-3255
15. *Mishra A., Reddy I.J., Gupta P.S.P., Mondal S.,* (2018), Total RNA content in sheep oocytes and developing embryos produced in vitro, a comparative study between spectrophotometric and fluorometric assay, Cytol Genet, 52(1):62-74
16. *Mohamed-Ali V., Pinkney J.H., Coppack S.W.,* (1998), Adipose tissue as an endocrine and paracrine organ. Int J Obes, 22(12):1145-1158
17. *Nagpal R., Mishra S.P., Yadav H.,* (2020), Unique gut microbiome signatures depict diet-versus genetically induced obesity in mice. Int J Mol Sci, 21(10):3434
18. *Norollah S.A., Kokhaee P., Rashidy-Pour A., Hojati V., Norollahi S.E., Larijani L.V., Samadanivol A.A.,* (2018), Comparison of methods of RNA extraction from breast and gastric cancer tissues. Crescent J Med Biol Sci, 5(1):25-28
19. *Palmquist D.L., St-Pierre N., Mcclure E.,* (2004), Tissue fatty acid profiles can be used to quantify exogenous rumenic acid synthesis in lamb. J Nutr, 134 (9):2407-2414
20. *Pratt S.L., Burns T.A., Owens M.D., Duckett S.K.,* (2013), Isolation of total RNA and detection procedures for miRNA present in bovine-cultured adipocytes and adipose tissues.Meth Mol Biol,936:181-194
21. *Primo D., Izaola O., De Luis D*., (2021), Leptin gene polymorphism (rs 7799039; G2548A) is associated with changes in lipid profile during a partial meal-replacement hypocaloric diet. J Hum Nutr Diet, 34(2): 456-463
22. *Rusu A.V., Criste F.L., Mierliţă D., Socol C.T., Trif M.,* (2020), Formulation of lipoprotein microencapsulated beadlets by ionic complexes in algae-based carbohydrates. Coatings, 10(3):302
23. *Sambrook J., Fritsch E.F., Maniatis T.,* (2001), Molecular Cloning: A Laboratory Manual, (Ed.) Cold Spring Harbor Press, New York, New York, USA
24. *Schroeder A., Mueller O., Stocker S., Salowsky R., Leiber M., Gassmann M., Lightfoot S., Menzel W., Granzow M., Ragg T.,* (2006), The RIN: an RNA integrity number for assigning integrity values to RNA measurements. BMC Mol Biol, 7(3):1-14
25. *Sellin Jeffries M.K., Kiss A.J. Smith A.W., Oris J.T.,* (2014), A comparison of commercially-available automated and manual extraction kits for the isolation of total RNA from small tissue samples, BMC Biotechnol, 14:94
26. *Socol C.T., Trif M., Criste F.L., Mierliţă D., Şonea C.G., Rusu A.V.,* (2020), Optimization of expression and purification of recombinant protein in *E. coli* based on a leptin model. Rom Biotechnol Lett., 5(4): 1810-1815
27. *Şonea C.G., Socol C.T., Criste F.L.,* (2020), Current perspectives for Mangalica and Bazna pigs breeding for efficient biodiversity management, genetic conservation and animal improvement in Romania, Revista Romana de Medicina Veterinara, 30 (1):29-33
28. *Struţi D.I., Mierlita D., Simeanu D., Pop I.M., Socol C.T., Papuc T., Macri A.M.,* (2020), The effect of dehulling lupine seeds (*Lupinus albus* L.) from low-alkaloid varieties on the chemical composition and fatty acids content. Rev Chim, 71(4):59-70
29. *Tao X., Liang Y., Yang X., Pang J., Zhong Z., Chen X., Yang Y., Zeng K., Kang R., Lei Y., Ying S., Gong J., Gu Y., Lv X.,* (2017), Transcriptomic profiling in muscle and adipose tissue identifies genes related to growth and lipid deposition. PLoS One, 12(9):e0184120
30. *Tavangar K., Hoffman A.R., Kraemer F.B.,* (1990), A micromethod for the isolation of total RNA from adipose tissue. Anal Biochem, 186:60-63
31. *Trif M., Vodnar D.C., Mitrea L., Rusu A.V., Socol C.T.,* (2019), Design and development of oleoresins rich in carotenoids coated microbeads. Coatings, 9(4):235.