UNIVERSIDADE ESTADUAL DE MARINGÁ
CENTRO DE CIÊNCIAS AGRÁRIAS
Programa de Pós-Graduação em Ciência de Alimentos

OBTENÇÃO DE SORO DE LEITE HUMANO E CARACTERIZAÇÃO DA SUA COMPOSIÇÃO LIPÍDICA POR CG-DIC E ESI-MS

ELOIZE DA SILVA ALVES

Maringá
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Dissertação apresentada ao programa de Pós-Graduação em Ciência de Alimentos Universidade Estadual de Maringá, como parte dos requisitos para obtenção do título de mestre em Ciência de Alimentos.

Maringá

2021

Orientador

Prof. Dr. Jesui Vergilio Visentainer

Dados Internacionais de Catalogação-na-Publicação (CIP)
(Biblioteca Central - UEM, Maringá - PR, Brasil)

A474o

Alves, Eloize da Silva

Obtenção de soro de leite humano e sua caracterização por CG-DIC e ESI-MS / Eloize da Silva Alves. -- Maringá, PR, 2021.

40 f.figs., tabs.

Orientador: Prof. Dr. Jesui Vergilio Visentainer.

Dissertação (Mestrado) - Universidade Estadual de Maringá, Centro de Ciências Agrárias, Programa de Pós-Graduação em Ciência de Alimentos, 2021.

1. Leite humano - Composição lipídica. 2. Soro de leite humano. 3. Leite humano - Triacilglicerol. 4. Leite humano - Ácidos graxos. I. Visentainer, Jesui Vergilio, orient. II. Universidade Estadual de Maringá. Centro de Ciências Agrárias. Programa de Pós-Graduação em Ciência de Alimentos. III. Título.

CDD 23.ed. 663.64

Rosana de Souza Costa de Oliveira - 9/1366

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BIOGRAFIA

Eloize da Silva Alves, nascida em Umuarama – Paraná. Família residente do interior do Paraná – Icaraíma. Possui graduação em Tecnologia em Alimentos pela Universidade Estadual de Maringá – Campus Umuarama. Tem experiência na área de Ciência de Alimentos atuando principalmente nos seguintes temas: Tecnologia de alimentos, composição de alimentos, composição em ácidos graxos, interpretação de perfil lipídico de Triacilglicerol e desenvolvimento de novos produtos alimentícios. Experiência com matrizes: leite humano e leite bovino.

Dedico

A minha família em especial a meus pais Rozeney Maria da Silva Alves e Donizeti João Alves, por todo apoio concedido as minhas escolhas e sonhos. E com muito carinho aos meus amigos de pesquisa por sempre mostrarem meu melhor, além de todos ensinamentos.

AGRADECIMENTOS

Com grande satisfação, agradeço sinceramente todos àqueles que me acompanharam e contribuíram para o meu crescimento pessoal e profissional ao longo do curso de Pós-Graduação, em especial agradeço:

A Deus, e ao Universo; por me colocarem em situações que me tragam sabedoria e discernimento;

Ao meu orientador Prof. Dr. Jesui Vergilio Visentainer, pela oportunidade em me orientar, confiança e paciência;

A minha família, qual tenho amor incondicional, agradeço por todo respeito e apoio referente as minhas escolhas e sonhos;

Aos meus amigos Bruno Saqueti, Matheus Castro, Luciana Manin, quais sempre estiveram do meu lado ao decorrer do mestrado, por toda ajuda, colaboração, apoio e por serem meu "braço direito";

Aos membros do grupo de pesquisa APLE-A, pela união, e amizade; em especial ao Prof. Dr. Oscar Oliveira Santos e Ma. Patricia Daniele Santos, por sempre estarem a disposição em qualquer situação;

Ao Programa de Pós-Graduação em Ciência de Alimentos pela oportunidade de realizar esta etapa da vida acadêmica; aos docentes do programa, que sempre estiveram dispostos a ajudar e contribuir com seus ensinamentos;

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), e ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação Araucária, pelo auxílio, suporte financeiro e apoio concedido.

APRESENTAÇÃO

Esta dissertação de mestrado está apresentada na forma de um artigo científico.

Autores: Eloize Silva Alves, Matheus Campos Castro, Bruno Henrique Figueiredo Saqueti, Luciana Pelissari Manin, Roberta da Silveira, Patrícia Magalhães Souza, Oscar Oliveira Santos, Jesuí Vergílio Visentainer.

Revista: Food Research International.

Título: Human whey obtaining and characterization by GC-FID and ESI-MS

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GENERAL ABSTRACT

INTRODUCTION. Milk, by definition, originates from the mammary gland of female mammals as a source of food for newborns, aiding in the development of the organism and in survival. Human milk should be the exclusive food source during the first six months of a baby's life, and if breastfeeding is interrupted by some factors, the American Academy of Pediatrics recommends the use of pasteurized donated human milk. Human milk that does not meet the specifications must be disposed of directly in the sewage network, as stipulated in RDC / Anvisa n. 306/2004 (Brazil, 2004). In 2019, all Brazilian states presented reports of statistical data, provided by the database of the Brazilian network of human milk banks, which exposes an average disposal of 20.0 to 30.0%. The authors are not aware of any work that reuses this residual human milk for the development of co-products; however, studies on this will be promising. Whey is a derivative of milk, light yellow to greenish in color, composed of lactose, soluble proteins, vitamins, lipids and minerals. It can be obtained from the separation / sedimentation of casein from serum milk proteins, by centrifugation (Silva et al., 2007) or ultracentrifugation (Lu et al., 2018). Among several studies in the literature, studies are exposed that assess protein and immunological composition referring to human whey, however there are no reports on its lipid composition.

OBJECTIVES. In order to avoid the disposal of human whey and apply it to infant feeding, the aim of this work was to characterize the human whey derived from human milk discarded by dirt from the Human Milk Bank of Maringá (Paraná, Brazil), through analysis of fatty acid (FA) composition, triacylglycerol (TAG) lipid profile and proximate composition.

MATERIAL AND METHODS. This study was approved by the Research Ethics Committee number 2.797.476, of Universidade Estadual de Maringá. Samples of raw mature human milk discarded by dirt were collected at a cooling temperature of 4 ºC, at the Human Milk Bank of Hospital Universitário de Maringá. Subsequently, human milk was homogenized and a pool was produced. It was packed in polyethylene packaging and stored at -18 °C for further development and analysis of human whey. Obtaining human whey: the milk was centrifuged at 1500 g for 10 minutes at 10 ºC to remove the fat layer. The skimmed human milk was ultracentrifuged at 6000 g for 30 minutes at 30 ºC to sediment casein. Assessment of centesimal composition, analyzes of moisture, ash, protein and carbohydrates by calculating the difference; and lipid content. The energy food value was expressed by the sum of the macronutrients that comprise it. Fatty acid methyl esters (FAME) were prepared by total lipids methylation for composition analysis in FA. And later analyzed in a gas chromatograph (GC, Trace Ultra 3300) with flame ionization detector (FID), the split injection mode was used in the proportion of 1:100. The FAMEs were identified by comparing the retention times of the constituent samples with analytical standards. The FA compositions were expressed as relative percentage. The lipids nutritional quality was assessed by 6 indices: atherogenicity index (AI), thrombogenicity index (TI) and proportion of FA hypocholesterolemic/hypercholesterolemic (H/H). Sum of the omega-6 family due to the omega-3 family, sum of polyunsaturated fatty acids (PUFA) due to the sum of saturated fatty acids (SFA), and sum of eicosapentaenoic (EPA) FA and docosahexaenoic (DHA) FA. The triacyglycerol (TAG) profile was obtained by direct infusion into a mass spectrometry (MS) using electrospray ionization (ESI) source. TAGs were assigned and estimated (%) through the LAMES platform, which is based on the mathematical algorithm that describes the distribution of FA in TAG molecules using the

FA percentage determined by GC-FID. With the Lipid maps® database, it was possible to find a molecular formula for TAGs. The data from all other analyzes were submitted to statistical analysis of variance (ANOVA) and Tukey's test ($p < 0.05$). All analyzes were compared to mature human milk (HM).

RESULTS AND DISCUSSION. The sample with the highest moisture was HW (91.56±0.07), while HM (89.11±0.13); the HW value is due to the removal of solid matter (fat and protein) from human milk during skimming. For percentage of ash, the values were: HM (0.15±0.01) and HW (0.15±0.02), there were no significant differences between it, as both samples have the same monerals amount. In the total protein content, the HM sample showed a value of 1.29±0.10, and the HW sample, there presented a decrease in the protein concentration (1.12±0.05), due to the casein precipitation, which caused a reduction in the total crude protein amount. As for lipids, a higher value for HM (3.23±0.13) in comparison to HW (0.93±0.13) was already expected due to the creaming process. For the carbohydrate, the results were HM (6.22±0.23) and HW (6.24±0.20). In the energy value evaluation, losses of the total value were verified, due to the loss of lipids and proteins during obtaining the HW; HM obtained a value of 59.10±0.32 and HW 37.80±0.31; however, for consumption, the minimum recommended amount for feeding infants is 25 Kcal/100 mL. 32 FAs were identified by the GC-FID. Among the FAs analyzed, oleic acid (O, 18:1n-9) was the majority for both samples; HM (31.32±0.49) and HW (32.28±0.42). Then, palmitic acid (P, 16:0) with HW (28.09±0.78) and HM (22.23±0.08). For PUFAs, the FA identified in greater quantity was linoleic acid (L, 18:2n-6), considered a strictly essential FA and precursor of the arachidonic acid (AA, 20:4n-6) also found in the samples. Other long-chain PUFAs have also been obtained, such as alpha-linolenic acid (aLn, 18:3n-3), which is a precursor of the eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3). Therefore, both HM and HW maintain strictly essential FAs, its precursors and essential FAs. However, it is important to note that the essential FA content has increased in the co-product. FA C9,t11 and t10,C12, which are named conjugated linoleic acid, remained present in all samples. Once the serum was obtained, an increase in C9,t11 and a decrease in t10,C12 was identified. The HW sample showed the highest level of the saturated fatty acids sum (ΣSFA) (49.34±0.49), and HM (43.58±0.47). For monounsaturated fatty acids sum (ΣMUFA), the HW sample was predominant, with a value of 37.03±0.17%. Finally, for the polyunsaturated fatty acids sum (ΣMUFA) the HM sample stood out, obtaining a result of 22.43±0.04%. The atherogenicity index (AI) had the highest value for HM sample (0.98±0.02) and HW sample presented a value of 0.91±0.01; because of the ΣMUFA and n-3 values in the HW samples are higher in relation to HM; the 12:0 and 14:0 concentrations were also higher in the HM sample, corroborating with the result. The thrombogenicity index (TI) presented a value of 1.41±0.09 for HW sample and a lowest value for HM sample (1.07±0.01). There are no reference values in the literature for AI and TI, these indices indicate potential for platelet aggregation, therefore, low levels are desirable. The hypocholesterolemic/hypercholesterolemic (H/H) proportion, the value found were: HM sample (1.51±0.04) and a lowest value for the HW sample (1.35±0.04); the H/H proportionality indicates the FA specific effects on cholesterol metabolism, values above 2.0 are desirable, as it leads to greater health benefits, preventing cardiovascular diseases. The values found are below 2.0 for all samples, becausa the ΣSFA were higher than the ΣMUFA, both values related to the maternal diet. The ratio of Σn-6 to n-3, has an acceptable proportion for the proper functioning of the organism is between 5 and 10; HM obtained 13.01 ± 0.17, outside the acceptable proportion, while the HW sample presented values within the indicated parameter (5.95 ± 1.02). This relationship is important because

these FAs compete for the metabolic pathways of stretching and desaturation. PUFA/ SFA showed HM (0.51 ± 0.01) and HW (0.28 ± 0.01) results. Foods with an PUFA/SFA ratio below 0.45 were considered unhealthy because of their potential to induce increases in blood cholesterol; only the HM presented values above the mentioned value. Σ (EPA) + (DHA), HW (0.73 ± 0.13) and HM (0.20 ± 0.02); however, the presence of both FAs in the samples is extremely important. The Σn-6 toΣ n-3 ratio has an acceptable proportion for the proper organism functioning between 5 and 10; HM obtained 13.01±0.17, outside the acceptable proportion, while the HW sample presented values within the indicated parameter (5.95±1.02). This relationship is important because these FAs compete for the metabolic pathways of stretching and desaturation. PUFA/SFA showed HM (0.51±0.01) and HW (0.28±0.01) results. Foods with PUFA/SFA ratio below 0.45 are considered unhealthy because of its potential to induce increases in blood cholesterol; only the HM presented values above the mentioned value. Σ(EPA)+(DHA), HW (0.73±0.13) and HM (0.20±0.02); however, the presence of both FAs in the samples is extremely important. The TAG results show the HM and HW spectra obtained by direct infusion in ESI-MS in the *m/z* range from 530 to 1100, with the most intense ionic spectral peak present between *m/z* 876 and 877. In the HM spectrum, the ionic peaks are more intense compared to HW, since the lipids percentage in the HM sample is higher (3.23±0.13) than HW (0.93 ± 0.13). The 21 largest indexes *m/z* were found, with its respective TAGs, found in the region between *m/z* 792 to 916, by the LAMES Platform. Comparing the results obtained by the FAs composition, with the presented TAGs, it was possible to observe the frequency of oleic (O, 18:1n-9) and palmitic (P, 16:0) FAs in TAGs, both with highest concentrations in relation to the FAs. The TAGs percentage in the HW sample in relation to the HM sample varied due to its distribution in the fat globules, as the ultracentrifugation was performed, those present in the casein decreased its percentage $[TAG+NH_4]^+$ LaOP, MOP, PLP, MOO , PLO and OLO. While those associated with albumin, fraction soluble in serum ($[TAG+NH_4]^+$ PPP, POP, SPP, PVcO, POO, SLP, SOP, SPS, SOS, OOO, SLO, SOO, BhOP) showed a high percentage, justified because the lipids that remained in the liquid fraction had its percentage rebalanced.

CONCLUSIONS. It is possible to obtain a human milk co-product from milk discarded by the human milk banks; the human whey (HW). The results obtained revealed that the chemical composition underwent significant modifications since the HW was obtained from the HM, except for the percentage of ash and carbohydrates. Regarding the fatty acids composition, it was observed that strictly essential fatty acids, essential fatty acids and all other FAs found in HM remained present in HW, being extremely important, since these FAs are responsible for several benefits in the infants' health, as already demonstrated. Considering the lipid nutritional quality, both the AI and the TI presented adequate values for both samples, indicating a lipid food quality and its potential effects on the development of coronary diseases. Finally, the TAGs lipid profile showed variation in the samples analyzed, with higher percentage of saturated and monounsaturated fatty acids, which is important, as it assists in the infants' digestion. Therefore, HW has the potential to be applied isolated or to be used in other foods.

Keywords: Human milk; Human whey; GC-FID; Fatty acids; ESI-MS; Triacylglycerol.

RESUMO GERAL

INTRODUÇÃO. O leite, por definição, origina-se da glândula mamária de fêmeas mamíferas como fonte de alimentação para recém-nascidos, auxiliando no desenvolvimento do organismo e na sobrevivência. O leite humano deve ser a fonte alimentar exclusiva durante os primeiros seis meses de vida dos bebês, e caso a amamentação for interrompida por alguns fatores, a Academia Americana de Pediatria recomenda o uso de leite humano doado pasteurizado. O leite humano que não atender às especificações deve ser descartado diretamente na rede de esgoto, conforme estipulado na RDC/Anvisa n° 306/2004 (Brasil, 2004). Em 2019, todos os estados brasileiros apresentavam relatórios de dados estatísticos, fornecidos pelo banco de dados da rede brasileira de bancos de leite humano, que expõe um descarte médio de 20,0 a 30,0%. Os autores não têm conhecimento de nenhum trabalho que reaproveite esse leite humano residual para o desenvolvimento de co-produtos; contudo, estudos sobre isso serão promissores. O soro de leite é um derivado do leite, de cor amarelo claro a esverdeado, composto por lactose, proteínas solúveis, vitaminas, lipídios e minerais. Pode ser obtido a partir da separação/sedimentação da caseína das proteínas séricas do leite, por centrifugação (Silva et al., 2007) ou ultracentrifugação (Lu et al., 2018). Entre diversos estudos na literatura, são expostos estudos quais avaliam composição proteica e imunológicas referentes ao soro de leite humano, porém não há relatos sobre a composição lipídica do mesmo.
OBJETIVOS. A fim de evitar o descarte deste produto e aplicá-lo na alimentação infantil, o objetivo deste trabalho foi caracterizar o soro de leite humano derivado do leite descartado, por sujidade, do Banco de Leite Humano de Maringá (Paraná, Brasil), por meio de análises de composição de ácidos graxos (AG), perfil lipídico de triacilglicerol (TAG) e composição centesimal.
MATERIAL E METODOS. Este estudo foi aprovado pelo Comitê de Ética em Pesquisa número 2.797.476, da Universidade Estadual de Maringá. Amostras de leite humano maduro cru descartado por sujidade foram coletadas em temperatura de resfriamento de 4 ºC, no Banco de Leite Humano do Hospital Universitário de Maringá. Posteriormente, o leite humano foi homogeneizado e produzido um *pool*. Foi acondicionado em embalagens de polietileno e armazenado a -18 °C para posterior desenvolvimento e análise do soro de leite. Obtenção de soro de leite humano: o leite foi centrifugado a 1500 g por 10 minutos a 10 ºC para remoção da camada de gordura. E o leite humano desnatado foi ultracentrifugado a 6000 g por 30 minutos a 30 ºC para sedimentar a caseína. Avaliação da composição centesimal, análises realizadas de umidade, cinzas, proteínas e carboidratos pelo cálculo da diferença; e conteúdo lipídico. O valor energético dos alimentos foi expresso pela soma dos macronutrientes que os compõem. Ésteres metílicos de ácidos graxos (EMAG) foram preparados por metilação dos lipídios totais para análise de composição em AG. E posteriormente analisado em cromatógrafo gasoso (CG, Trace Ultra 3300) com detector de ionização de chama (DIC), foi utilizado o modo de injeção dividida na proporção de 1: 100. Os EMAGs foram identificados comparando os tempos de retenção das amostras constituintes com os padrões analíticos. As composições de AG foram expressas como porcentagem relativa. A qualidade nutricional dos lipídios foi avaliada por 6 índices: índice de aterogenicidade (IA), índice de trombogenicidade (IT) e proporção de AG hipocolesterolêmico / hipercolesterolêmico (H / H). Soma da família ômega-6 devido à família ômega-3, soma dos ácidos graxos poliinsaturados (AGPI) devido à soma dos ácidos graxos saturados (AGS), e soma dos AGs eicosapentaenóico (EPA) e AG docosahexaenóico (DHA). O perfil de Triacyglycerol (TAG) foi obtido por infusão direta

em um MS usando uma fonte de ionização por electrospray (ESI). Os TAGs foram atribuídos e estimados (%) através da plataforma LAMES, que se baseia no algoritmo matemático que descreve a distribuição de AG em moléculas de TAG usando a porcentagem de AG determinada por CG-DIC. Com o banco de dados do Lipid maps®, foi possível encontrar uma fórmula molecular para os TAGs. Os dados de todas as demais análises foram submetidos à análise estatística de variância (ANOVA) e teste de Tukey ($p < 0,05$). Todas análises comparadas a leite humano maduro (HM).

RESULTADOS E DISCUSSÃO. A amostra com maior umidade foi HW (91,56 ± 0,07), enquanto HM (89,11 ± 0,13); o valor de HW é devido à remoção de matéria sólida (gordura e proteína) do leite humano durante o desnate. Para a porcentagem de cinzas HM (0,15 ± 0,01) e HW (0,15 ± 0,02) não apresentaram diferenças significativas entre si, pois ambos possuem a mesma quantidade de minerais. No teor de proteína total, a amostra de HM apresentou um valor de 1,29 ± 0,10, e a amostra de HW, houve uma diminuição na concentração de proteína (1,12 ± 0,05), devido à precipitação de caseína, que ocasionou uma redução na quantidade de proteína bruta total. Quanto aos lipídios, já era esperado um valor maior para HM (3,23 ± 0,13) em comparação ao HW (0,93 ± 0,13) devido ao processo de desnate para obtenção do mesmo. Os valores de carboidratos foram obtidos calculando a diferença de macronutrientes, consequentemente, os resultados foram HM (6,22 ± 0,23) e HW (6,24 ± 0,20). Na avaliação do valor energético foram verificadas perdas do valor total, devido à perda de lipídios e proteínas durante a obtenção do HW; HM obteve um valor de 59,10 ± 0,32 e HW 37,80 ± 0,31; entretanto para o consumo, a quantidade mínima recomendada para alimentação de lactentes é de 25 Kcal/100 mL. 32 AGs foram identificados pelo CG-DIC. Entre os AGs analisados, o ácido oleico (O, 18: 1n-9) foi a maioria para ambas as amostras; HM (31,32 ± 0,49) e HW (32,28 ± 0,42). Em seguida, o ácido palmítico (P, 16: 0) com HW (28,09 ± 0,78) e HM (22,23 ± 0,08). Para os AGPIs, o identificado em maior quantidade foi o ácido linoléico (L, 18: 2n-6), considerado um AG estritamente essencial e precursor do ácido araquidônico (AA, 20: 4n-6) também encontrado nas amostras. Outros AGPI de cadeia longa também foram obtidos, como o ácido alfa-linolênico (aLn, 18: 3n-3), que é um precursor de eicosapentaenóico (EPA, 20: 5n-3) e docosahexaenóico (DHA, 22: 6n- 3). Portanto, tanto HM quanto HW mantêm AGs estritamente essenciais, seus precursores e AGs essenciais. No entanto, é importante observar que o conteúdo essencial de AG aumentou no co-produto. AG C9, t11 e t10, C12, que são chamados de ácido linoléico conjugado, permaneceram presentes em todas as amostras. Uma vez obtido o soro, foi identificado aumento de C9, t11 e diminuição de t10, C12. A amostra HW apresentou o maior nível da soma dos ácidos graxos saturados (Σ AGS) (49,34 ± 0,49), e HM (43,58 ± 0,47). Para os ácidos graxos monoinsaturados (Σ AGMI), a amostra de HW foi predominante, com valor de 37,03 ± 0,17%. Por fim, para a soma dos ácidos graxos poliinsaturados (Σ AGPI) a amostra de HM se destacou, obtendo um resultado de 22,43 ± 0,04%. O índice de aterogenicidade (IA) apresentou a HM maior valor (0,98 ± 0,02), e HW apresentou o valor 0,91 ± 0,01; isso se deve ao fato de que no HW os valores de Σ AGMI e n-3 são maiores em relação ao HM; as concentrações de 12:0 e 14:0 também foram maiores na amostra HM do que na amostra HW, corroborando com o resultado. O índice de trombogenicidade (IT) teve valor obtido para HW (1,41 ± 0,09) e o menor para HM (1,07 ± 0,01). Não há valores de referência na literatura para IA e IT, esses índices indicam potencial para agregação plaquetária, portanto, níveis baixos são desejáveis. A proporção de hipocolesterolêmico/hipercolesterolêmico (H/H), valor encontrado para a amostra de HM (1,51 ± 0,04) e a menor foi para a amostra de HW (1,35 ± 0,04); a proporcionalidade de H/H indica os efeitos específicos dos AG no metabolismo

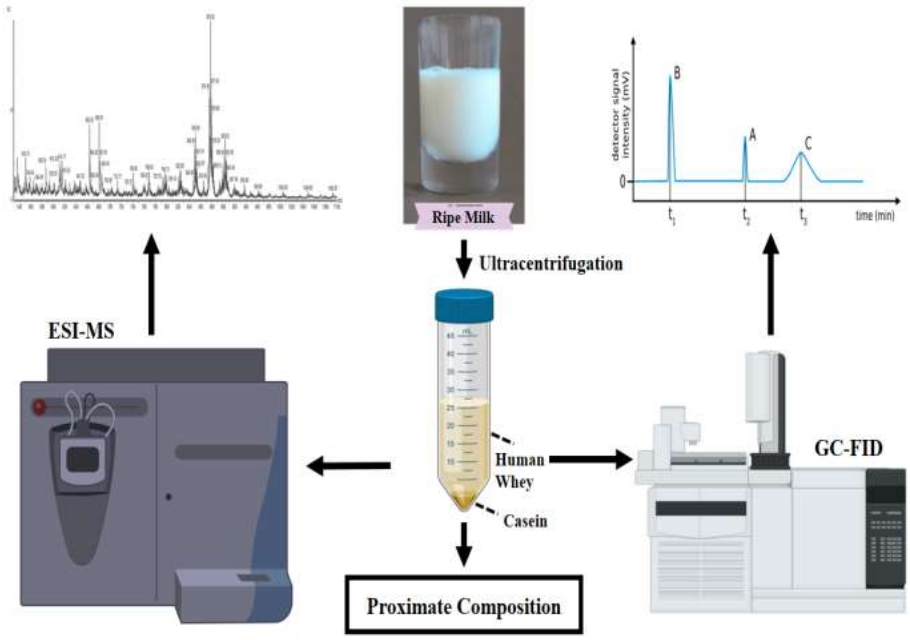
do colesterol, valores acima de 2,0 são desejáveis, pois levam a maiores benefícios à saúde, prevenindo doenças cardiovasculares. Os valores encontrados estão abaixo de 2,0 para todas as amostras, devido ao Σ AGS ser superior ao Σ AGPI, ambos relacionados à dieta materna. A proporção de Σ n-6 para Σ n-3, tem proporção aceitável para o bom funcionamento do organismo está entre 5 e 10; HM obteve 13,01 ± 0,17, fora da proporção aceitável, enquanto a amostra HW apresentou valores dentro do parâmetro indicado (5,95 ± 1,02). Essa relação é importante porque esses AGs competem pelas vias metabólicas de alongamento e dessaturação. AGPI/AGS mostrou resultados HM (0,51 ± 0,01) e HW (0,28 ± 0,01). Alimentos com uma relação AGPI/AGS abaixo de 0,45 foram considerados não saudáveis devido ao seu potencial de induzir aumentos de colesterol no sangue; apenas o HM apresentou valores acima do valor mencionado. O Σ (EPA) + (DHA), HW (0,73 ± 0,13) e o HM (0,20 ± 0,02); no entanto, a presença de ambos os AGs nas amostras é extremamente importante. Os resultados da determinação do TAG realizada por infusão direta em ESI-MS tiveram o pico espectral iônico mais intenso presente entre m / z 876 e 877. Os resultados do TAG mostram o *pool* de leite humano (HM) e soro de leite humano (HW) espectros obtidos por esta análise na faixa m / z de 530 a 1100. No espectro HM, os picos iônicos são mais intensos em comparação com HW, uma vez que a porcentagem de lipídios na amostra HM é maior (3,23 ± 0,13) do que HW (0,93 ± 0,13). Foram encontrados os 21 maiores índices m / z, com seus respectivos TAGs, encontrados na região entre 792 a 916 m / z, pela Plataforma LAMES. Comparando os resultados obtidos pela composição de AG, com os TAGs apresentados, foi possível observar a frequência dos ácidos oléico (O, 18: 1n-9) e palmítico (P, 16: 0) nos TAGs, que são aqueles com as maiores concentrações em relação ao AG. A porcentagem de TAGs na amostra HW em relação à amostra HM variou devido à sua distribuição nos glóbulos de gordura, à medida que a ultracentrifugação era realizada, os presentes na caseína diminuíam sua porcentagem [TAG + NH4] + LaOP, MOP, PLP, MOO, PLO e OLO. Enquanto aqueles associados à albumina, fração solúvel no soro ([TAG + NH4] + PPP, POP, SPP, PVcO, POO, SLP, SOP, SPS, SOS, OOO, SLO, SOO, BhOP) mostraram uma alta porcentagem, isto é justificado porque os lipídios que permaneceram na fração líquida tiveram seu percentual reequilibrado.

CONCLUSÕES. Com a realização deste trabalho pode-se concluir que foi possível obter um coproduto do leite humano descartado pelos bancos de leite; o soro de leite humano (HW). Os resultados obtidos revelaram que a composição química sofreu alterações significativas uma vez que o HW foi obtido a partir do HM, exceto para o percentual de cinzas e carboidratos. Em relação à composição dos ácidos graxos, observou-se que os ácidos graxos estritamente essenciais, ácidos graxos essenciais e todos os demais AGs encontrados no HM permaneceram presentes na HW, sendo de extrema importância, visto que esses AGs são responsáveis por diversos benefícios à saúde dos lactentes, como já demonstrado. No que se refere à qualidade nutricional lipídica, tanto o IA quanto o IT apresentaram valores adequados para ambas as amostras, indicando uma qualidade alimentar lipídica e seus potenciais efeitos no desenvolvimento de doenças coronarianas. Por fim, o perfil lipídico dos TAGs apresentou variação nas amostras analisadas, com maior percentual de ácidos graxos saturados e monoinsaturados, o que é importante, pois auxilia no processo de digestão dos lactentes. Portanto, o HW tem potencial para aplicação tanto na forma isolada quanto como utilizado em outros alimentos.

Palavras-chave: Leite humano; Soro de leite humano; GC-FID; Ácidos graxos; ESI-MS; Triacilglicerol.

ARTICLE

Graphical Abstract (GA)



GA Text: Obtaining human whey by ultracentrifugation, and characterizing its proximate composition, fatty acid composition and triacylglycerol profile

HUMAN WHEY OBTAINING AND CHARACTERIZATION BY GC-FID AND ESI-MS

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Abstract

In order to avoid the excessive disposal of human milk, the present work intends to obtain and characterize a milk by-product: human whey (HW), through analyzes of chemical composition, fatty acid (FA) composition and triacylglycerol (TAG) lipid profile. The results indicated that the chemical composition changed significantly after obtaining the HW, except for the percentage of ash and carbohydrates. Regarding the FA composition, it was observed that strictly essential FA, essential FA and all other FAs found in human milk remained present in HW. For the nutritional lipids quality, the atherogenicity and the thrombogenicity indexes presented desirable values. And the TAGs lipid profile revealed a variation in the analyzed samples. Therefore, it can be concluded that the characterized HW has potential for application both in isolation and also in other foods.

Keywords: Human milk, human whey, GC-FID, fatty acids, ESI-MS, triacylglycerol.

1 Introduction

Milk, by definition, originates from the mammary gland of female mammals as a food source for newborns, assisting in the organism development and in the human survival (Franklin, & Volk, 2019). Human milk must be the exclusive food source during the infants' first six months of life, and if breastfeeding is interrupted by some factors (hypogalactia or pre-existing illnesses in the lactating mother), the American Academy of Pediatrics recommends the use of pasteurized human donor milk (Johnston et al., 2012).

In order to meet this demand, human milk banks were created, guaranteeing a safe product, free from pathogenic microorganisms, based on collection, processing, quality control and distribution (Brasil, 2008). The quality of the milk supplied to milk banks is a result of hygienic conditions, from milking to administration. Consequently, several parameters are evaluated, including nutritional, chemical and microbiological characteristics (Borges et al., 2018).

Human milk that does not meet the specifications, must be directly disposed in the sewage system, as stipulated in the RDC/Anvisa n° 306/2004 (Brasil, 2004). The

work of Grazziotin et al., (2010) on the disposal of human milk donated to a milk bank, reports that this volume is approximately 10.5% to 24.0% of the total received, with mature milk (milk with more than 15 days postpartum) of greater volume collected and discarded in the research. In 2019, all Brazilian states presented production reports from statistical data, provided by the database of the Brazilian network of human milk banks, which exposes an average disposal of 20.0 to 30.0% (Rede Brasileira de Banco de Leite, 2019). The authors are not aware of any work that reuses this residual milk for the development of co-products and studies on this development will be promising.

Whey is a milk derivative, light yellow to greenish color, composed of lactose, soluble proteins, vitamins, lipids and minerals (Guo, 2019). It can be obtained from the separation/sedimentation of casein from serum milk proteins, by centrifugation (Silva et al., 2007) or ultracentrifugation (Lu et al., 2018).

Among its extensive benefits, human whey has high concentrations of proteins and bioactive components, which are complement system proteins, regulatory proteins and antimicrobial proteins; responsible for the mucosal immune response and guarantee the growth and development of newborns (Lu et al., 2018). In addition, Gomes-Santos et al. (2015) showed in their results that the consumption of the protein present in bovine serum was able to prevent signs of inflammation and changes in the immunological characteristics typical of the food allergy pathology. The authors did not find any reports in the literature about studies involving lipidomic of human whey or its fatty acids composition.

Therefore, in order to avoid the disposal of this product and apply it to infant feeding, the objective of this work was to characterize human whey derived from discarded milk, due to dirtiness, from the Human Milk Bank of Maringá (Paraná,

Brazil), using analysis of fatty acids (FA) composition, triacylglycerol (TAG) lipid profile and proximate composition.

2 Materials and methods

2.1 Reagents

Chloroform, n-heptane, methanol, and sodium chloride (all analytical grade) were purchased from Synth (São Paulo, Brazil). Sodium hydroxide, ammonium chloride and sulfuric acid (all analytical grade) were purchased from Dinâmica (São Paulo, Brazil). Methanol and chloroform (high performance liquid chromatography (HPLC) grade) were purchased from J.T. Baker® (Philipsburg, United States) and Riedel-de Haën (Seelze, Lower Saxony, Germany), respectively. Ammonium formate (97%) was purchased from Sigma-Aldrich (Darmstadt, Germany). For gas chromatographic (GC) analyzes, the reagents and solvents used were of analytical grade and for mass spectrometry (MS), the solvents were of HPLC grade.

2.2 Sampling

This study was approved by the Research Ethics Committee (REC), number 2.797.476, of the Universidade Estadual de Maringá (UEM, Maringá, Paraná, Brazil). Samples of raw mature human milk discarded by dirtiness were collected at cooling temperature of 4 ºC, at the Human Milk Bank in the Hospital Universitário de Maringá (Maringá, Paraná, Brazil). Subsequently, the human milk was homogenized and a pool from 15 donors was carried out, obtaining a final volume of 3.0 liters. The sample of

human milk was divided in 1.0 liter to the analysis further, and 2.0 for obtaining human whey. It was packed in hermetic polyethylene packaging and stored at -18 °C for further development and analysis of the human whey.

2.3 Human Whey Obtaining

Human whey was obtained according to Lu et al. (2018); the milk was centrifuged at 1500 g for 10 minutes at 10 ºC to remove the fat layer. Skimmed human milk was ultracentrifuged at 6000 g for 30 minutes at 30 ºC to sediment casein. The Human Whey (HW) obtained was homogenized and stored in vacuum-sealed polyethylene package, subjected to freezing at -18 ºC.

2.4 Assessment of Proximate Composition

The analyzes of moisture (method 934.01), ash (method 942.05), proteins (method 990.03) and carbohydrates by calculating the difference, were performed according to AOAC (2005). The lipid content was determined according to the methodology of Folch et al. (1957). The energy value of food was expressed by the sum of macronutrients that compose it, using the nutrient conversion factors that potentially provide energy for the human body, such as lipids, carbohydrates and proteins. Each gram of carbohydrate corresponds to 4 kcal. g^{-1} (or 17 kJ. g^{-1}), proteins to 4 kcal. g^{-1} (or 17 kJ. g^{-1}) and fat to 9 kcal g^{-1} (or 37 kJ. g^{-1}) (Fao, 2003).

2.5 Fatty Acid (FA) Composition

The lipids from the human whey sample were extracted according to Folch et al. (1957), then the fatty acid methyl esters (FAME) were prepared by methylation of the total lipids according to ISO 5509 (2000). The upper phase was collected with the assistance of a Pasteur pipette, transferred to a vial and subsequently analyzed in a GC (Trace Ultra 3300, Waltham, United States) with flame ionization detector (FID), capillary column CP-7420 (100.0 m size, 0.25 mm internal diameter and 0.25 µm cyanopropyl thin film as stationary phase) and split/splitless injector. The detector and injector temperatures were 250 and 230 °C, respectively. The GC-FID oven was set at 65 °C and maintained for 4 min, then heated to 185 °C to 15 °C.min^{-1} and maintained for 12 min, then heated to 235 °C to 20 °C.min^{-1} and maintained for 14 min. The gas flow rates used were 1.4 mL.min^{-1} for carrier gas (H_2), 30 mL.min^{-1} for replacement gas (N_2), and 30 and 300 mL.min^{-1} for gas flames (H_2 and synthetic air, respectively). The split injection mode was used with a ratio of 1:100 and the sample injection volume was 2.0 µL (Simionato et al., 2010). FAMEs were identified by comparing the retention times of the constituent samples with the analytical standards (standard mixture FAME, C4-C24, Saint Louis, United States, Sigma-Aldrich). Peak areas were determined using the ChromQuest^{TM} 5.0 software and the FA compositions were expressed as relative are percentage. All samples were analyzed in triplicate.

2.6 Nutritional Lipid Quality

Nutritional quality was assessed by 6 indices: atherogenicity index (AI) (equation 1), thrombogenicity index (IT) (equation 2) and proportion of FA hypocholesterolemic/ hypercholesterolemic (H/H) (equation 3) (Ulbricht, & Southgate, 1991; Santos-Silva, Bessa, & Santos-Silva, 2002). Sum of omega-6 family due to omega-3 family (equation 4), sum of polyunsaturated fatty acids (PUFA) due to sum of saturated fatty acids (SFA) (equation 5), and sum of eicosapentaenoic (EPA) FA and docosahexaenoic (DHA) FA (equation 6).

The values were submitted by the equations below, and all concentrations are in relative area percentage.

$$AI = \frac{[12{:}0 + (4x14{:}0) + 16{:}0]}{MUFA + n-6 + n-3} \qquad Equation\ (1)$$

$$IT = \frac{(14{:}0 + 16{:}0 + 18{:}0)}{[(0.5xMUFA) + (0.5xn-6) + (3x\,n-3) + (\frac{n-3}{n-6})]} \qquad Equation\ (2)$$

$$HH = \frac{[(18{:}1n-9 + 18{:}2n-6 + 18{:}3n-3 + 20{:}3n-6 + 20{:}4n-6 + 20{:}5n-3 + 22{:}6n-3)]}{(12{:}0 + 14{:}0 + 16{:}0)} \qquad Equation\ (3)$$

$$Proportion\ of\ omega\ family = \frac{\Sigma[n-6]}{\Sigma[n-3]} \qquad Equation\ (4)$$

$$Proportion\ of\ polyunsaturated\ and\ saturared\ fatty\ acids = \frac{\Sigma[PUFA]}{\Sigma[SFA]} \qquad Equation\ (5)$$

$$Sum\ of\ essential\ fatty\ acids = EPA + DHA \qquad Equation\ (6)$$

2.7 Triacylglycerol (TAG) Profile

The TAG profile was obtained by direct infusion in a MS using an electrospray ionization (ESI) source. The samples of human whey lipids were prepared according to Da Silveira et al. (2017); approximately 50.0 µL of lipid was added to 950.0 µL of chloroform. Then, 5.0 µL of this solution was transferred to a vial and 1.0 mL of 9:1 methanol/chloroform solution (v v^{-1}) was added. In order to obtain the ammonium adducts $[TAG+NH_4]^+$, 20.0 µL of 0.10 $mol.L^{-1}$ ammonium formate prepared in methanol were added to the final solution. The prepared solutions were infused with a flow of 10.0 $\mu L.min^{-1}$ directly into a Xevo TQ-D™ triple quadrupole MS (Waters, Milford, Massachusetts, United States) equipped with Z spray™ ESI, with source operating in positive mode (ESI+), with the following conditions: desolvation gas flow (500 L h^{-1}), source temperature (150 °C), desolvation temperature (200 °C), capillary and cone voltage (3.00 kV and 20.00 V, respectively). The TAG profile of human whey was evaluated in the mass range of *m/z* 530-1100. The results obtained were determined using the MassLynx™ software.

2.8 TAG Assignment and Estimation

TAGs were assigned and estimated (%) via LAMES Platform, which is based on the mathematical algorithm that describes the distribution of FA in TAG molecules (Antoniosi Filho, Mendes, & Lanças, 1995) using the FA percentage determined by GC-FID. With the Lipid maps® database, it was possible to find a molecular formula for TAGs.

2.9 Statistical Analysis

The data from all other analyzes were submitted to analysis of variance (ANOVA) and Tukey's test ($p<0.05$), using the software Assistat version 7.7 (Silva, & Azevedo, 2008).

3 Results and discussions

3.1 Centesimal Composition

Table 1 describes the results of the centesimal composition (%) and the calculated energy value (Kcal/100 mL) of human milk and human whey (HW).

Table 1. Centesimal composition (%) and energy value (Kcal/100 mL) of human milk and human whey.

Analysis	**HM**	**HW**
Moisture	89.11±0.13[b]	91.56±0.07[a]
Ash	0.15±0.01[a]	0.15±0.02[a]
Protein	1.29±0.10[a]	1.12±0.05[a]
Total Lipid	3.23±0.13[a]	0.93±0.13[b]
Carbohydrate	6.22±0.23[a]	6.24±0.20[a]
Energetic Value	59.10±0.32[a]	37.80±0.31[b]

Results expressed as mean ± standard deviation (SD) of triplicate. Values with different letters on the same line are significantly different ($p<0.05$) by the Tukey test. (**HM** - Human Milk; **HW** – Human Whey).

Food moisture is related to its stability and quality; in this analysis, the samples showed significantly different values according to the Tukey test ($p<0.05$). The sample with the highest moisture was HW (91.56±0.07), while HM had a value of 89.11±0.13; the HW value is due to the removal of solid matter (fat and protein) from human milk during skimming.

The ash percentage shows the total amount of minerals present in food (Cecchi, 2003). Statistically, the samples HM (0.15±0.01) and HW (0.15±0.02) did not present significant differences between it, therefore both have the same amount of minerals. The data shows similar values because the minerals are water-soluble.

In the total protein content there was no significant difference between the samples. HM sample showed a value of 1.29±0.10, corroborating Bruxel & Sica (2019) work, who mentions that the average human milk protein is 1.2 g/100 mL. In the HW sample, there was a decrease in protein concentration (1.12±0.05), due to the casein precipitation, which caused a reduction in the amount of total crude protein. According to the literature, the proportion of total protein is divided into 20% casein and 80% serum proteins.

As for lipids, the samples revealed significantly different values, a higher value for HM (3.23±0.13) in comparison to HW (0.93±0.13) was already expected due to the skimming process to obtain the HW. The HM sample showed a value close to that found by Rydlewski et al. (2019), which was 3.08±0.73 in the analysis of human milk from the mature lactation phase.

The carbohydrate values were obtained by calculating the difference in macronutrients, that is, these values are influenced by the results obtained in the analysis of moisture, ash, proteins and lipids. Consequently, the carbohydrate values did not show significant difference between samples, being HM (6.22±0.23) and HW (6.24±0.20).

In the energy value evaluation, losses of the total value were verified, due to the loss of lipids and proteins during obtaining the HW. HM obtained a value of 59.10±0.32 and HW 37.80±0.31. Since, for consumption, the minimum recommended amount for

feeding infants by RDC 171/2006 is 25 Kcal/100 mL (BRASIL, 2006), all results were higher than required.

3.2 Fatty Acid Composition by GC-FID

Table 2 describes the FA concentration as relative area percentage (%) in human milk and human whey.

Table 2. Fatty Acid Composition (%) of human milk and human whey.

Fatty Acid Composition	HM	HW
4:0	0.40±0.00[a]	0.30±0.04[b]
6:0	0.02±0.00[b]	0.20±0.03[a]
8:0	0.07±0.01[b]	0.74±0.15[a]
10:0	1.43±0.20[a]	0.34±0.01[b]
12:0	6.39±0.38[a]	1.88±0.19[b]
14:0	6.66±0.15[a]	4.05±0.08[b]
14:1n-9	0.07±0.01[a]	0.05±0.00[b]
15:0	0.27±0.00[a]	0.22±0.00[b]
16:0	22.24±0.08[b]	28.09±0.78[a]
16:1n-9	1.54±0.11[a]	1.70±0.07[a]
16:1n-7	0.21±0.03[b]	0.45±0.03[a]
17:0	0.26±0.02[b]	0.36±0.02[a]
17:1n-9	0.19±0.01[a]	0.05±0.00[b]
18:0	5.65±0.17[b]	10.90±0.47[a]
18:1n-9	31.32±0.49[a]	32.28±0.42[a]
18:1n-7	0.05±0.00[b]	1.97±0.19[a]
18:2n-6	20.25±0.04[a]	9.85±0.13[b]
18:2n-6 C9,t11 (CLA)	0.09±0.00[b]	0.27±0.02[a]

18:2n-6 t10,C12 (CLA)	0.11±0.01[a]	0.08±0.00[b]
18:3n-3	1.08±0.03[a]	0.31±0.01[b]
18:3n-6	0.09±0.00[b]	0.21±0.04[a]
20:0	0.08±0.01[b]	0.11±0.01[a]
20:1n-9	0.23±0.02[a]	0.20±0.00[a]
20:3n-6	0.21±0.04[a]	0.20±0.00[a]
20:3n-3	0.31±0.02[b]	0.98±0.18[a]
20:4n- 6 (AA)	0.06±0.02[b]	0.95±0.11[a]
22:0	0.08±0.01[b]	1.95±0.25[a]
20:5n-3 (EPA)	0.04±0.00[b]	0.36±0.07[a]
22:1n-9	0.27±0.03[a]	0.21±0.01[b]
24:0	0.05±0.00[b]	0.21±0.03[a]
24:1n-9	0.11±0.01[a]	0.11±0.01[a]
22:6n-3 (DHA)	0.16±0.02[b]	0.37±0.06[a]
Σ (n-3)	1.60±0.02[b]	2.01±0.34[a]
Σ (n-6)	20.83±0.02[a]	11.62±0.03[b]
Σ SFA	43.58±0.47[b]	49.34±0.49[a]
Σ MUFA	33.99±0.43[b]	37.03±0.17[a]
Σ PUFA	22.43±0.04[a]	13.64±0.32[b]

Results expressed as mean ± standard deviation (SD) of triplicate. Values with different letters on the same line are significantly different ($p<0.05$) by the Tukey test. (SFA - saturated fatty acids; MUFA - monounsaturated fatty acids; PUFA - polyunsaturated fatty acids). (**HM** - Human Milk; **HW** – Human Whey).

Considering Table 2, 32 FAs were identified by the GC-FID. The FA composition originates from the lactating woman's diet, and there may be changes in its concentrations (Demmelmair, & Koletzko, 2018).

Among the FA analyzed, oleic acid (O, 18:1n-9) was the majority for both samples; HM (31.32±0.49) and HW (32.28±0.42). Manin et al. (2019) performed the FA determination in human milk samples and also obtained 18:1n-9 as the majority, obtaining close values (29.47±0.75). Therefore, it can be considered that the results

found for this work are within the expected. Oleic acid is used by infant mainly as an energy source, in addition to promoting the fat absorption by the small intestine and composing the membrane structure, as well as the myelination of axons (Costa, & Sabarense, 2010; Rydlewski et al., 2020).

Afterwards, for the second highest concentration found, there is the palmitic acid (P, 16:0), with HW (28.09±0.78) and HM (22.23±0.08). SFA (P, 16:0), plays an important role for the infant, such as, the improvement of intestinal discomfort, colic reduction, as well as the influence on the levels of anadamide that has analgesic effect. These benefits are related to the central position that this FA occupies in the TAG molecule, about 70% of the palmitic acid in human milk is in this position, being easily absorbed by the body (Mehrotra, Sehgal, & Bangale, 2019; Demmelmair & Koletzko, 2018).

For PUFAs, the one identified in greater quantity was linoleic acid (L, 18:2n-6), considered a strictly essential FA and precursor to arachidonic acid (AA, 20:4n-6) also found in the samples. Others long-chain PUFA have also been obtained, such as, alpha linolenic acid (aLn, 18:3n-3), which is a precursor to eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n- 3) FAs. In humans, linoleic and α-linolenic FAs are necessary to maintain, under normal conditions, cell membranes, brain functions, also participate in the transfer of atmospheric oxygen to blood plasma, hemoglobin synthesis and cell division (Yehuda et al., 2002). These essential FAs (AA, EPA, DHA) have crucial functions in the newborn's cognitive, visual, cerebral, and immune development. Furthermore, it also plays an important role in protecting against allergy, asthma, reducing inflammation rates and childhood obesity (Perini et al. 2010).

Therefore, both HM and HW maintain strictly essential FAs, its precursors and essential FAs. However, it is important to note that the essential FA content increased in the co-product.

FA C9,t11 and t10,C12, which are named conjugated linoleic acid, remained present in all samples. An increase of C9,t11 and a decrease of t10, C12 was identified once the serum was obtained, with statistical difference. This FA class is of great importance, as it is linked to several benefits to life, such as reducing body fat, immune system modulation, improving bone mineralization, antidiabetic and anticarcinogenic effects (Perini et al. 2010).

HW sample had the highest level of the sum of saturated fatty acids (Σ SFA) (49.34±0.49), in comparison to the HM sample (43.58±0.47). The longest SFA represents the circulating oxidation pool. Stearic acid (S, 18:0) can be quickly converted to oleic acid, and this pathway indicates the strict metabolic interrelationships between SFA and MUFA, respectively (Mazzocchi et al., 2018).

For monounsaturated fatty acids (Σ MUFA), the HW sample was predominant, with a value of 37.03±0.17%. Finally, for the sum of polyunsaturated fatty acids (Σ PUFA) the HM sample stood out, obtaining a result of 22.43±0.04%.

3.3 Nutritional Lipid Quality

Table 3 presents the results of the nutritional quality indices for human milk and human whey.

Table 3. Indices of lipid nutritional quality of human milk and human whey.

Indices	HM	HW
AI	0.98±0.02[a]	0.91±0.01[b]
TI	1.07±0.01[b]	1.41±0.09[a]
H/H	1.51±0.04[a]	1.35±0.04[b]
Σ (n-6)/(n-3)	13.01±0.17[a]	5.95±1.02[b]
Σ MUFA/SFA	0.51±0.01[a]	0.28±0.01[b]
Σ(EPA)+(DHA)	0.20±0.02[b]	0.73±0.13[a]

Atherogenicity index (AI), thrombogenicity index (TI), proportion of fatty acids hypocholesterolemic/hypercholesterolemic (H/H), sum of the omega-6 family due to the omega-3 Σ (n-6)/(n- 3)), sum of polyunsaturated fatty acids due to saturated fatty acids (Σ MUFA/SFA), and sum of Eicosapentaenoic and Docosahexaenoic fatty acids (Σ (EPA)+(DHA)). Results expressed as mean ± standard deviation (SD) of triplicate. Values with different letters on the same line are significantly different ($p<0.05$) by the Tukey test. (**HM** - Human Milk; **HW** - Human Whey).

The atherogenicity index (AI) showed a significant difference in relation to the Tukey test ($p<0.05$). HM presented the highest value (0.98±0.02), and HW presented the value 0.91±0.01. This is due to the fact that in the HW the values of Σ MUFA and n-3 are higher compared to HM. The 12:0 and 14:0 concentrations were also higher in the HM sample than in the HW sample, corroborating with the result.

The thrombogenicity index (TI) showed a statistical difference by the Tukey test ($p<0.05$), maintaining a behavior similar to that of the AI. The highest value obtained

was for HW (1.41±0.09) and the lowest for HM (1.07±0.01). There are no reference values in the literature for AI and TI, however, according to Ulbricht & Southgate (1991) and Santos-Silva, Bessa, & Santos-Silva (2002), these indices indicate the potential for platelet aggregation, therefore, low levels are desirable, as both indicate quality of the lipid diet and its potential effects on the development of coronary diseases.

The proportion of hypocholesterolemic/hypercholesterolemic (H/H) fatty acids showed a significant difference between the samples. The highest value found was for the HM sample (1.51±0.04) and the lowest was for HW sample (1.35±0.04). According to Santos-Silva, Bessa, & Santos-Silva (2002), the proportionality of H/H indicates the specific effects of FA on cholesterol metabolism, values above 2.0 are desirable, since it leads to greater health benefits, since hypocholesterolemic FA acts on reduction of low density lipoprotein (LDL), thus preventing cardiovascular disease. Hypercholesterolemic FA increases the level of cholesterol in the blood, which can increase the risks of coronary heart disease. Considering Table 3, it is stated that the values found are below 2.0 for all samples, due to the Σ SFA being higher than the Σ PUFA, both related to the maternal diet.

The ratio of Σ n-6 to Σ n-3 showed a statistical difference. Simopoulos (2004) reports that the acceptable proportion for the proper functioning of the organism is between 5 and 10. The HM sample obtained the highest value (13.01±0.17), outside the acceptable proportion, while the HW sample presented values within the parameter indicated (5.95±1.02). This relationship is important because these FAs compete for the metabolic pathways of stretching and desaturation. The values found in the HM are above the recommended, due to the Σn-6 being ten to twenty times greater than the Σn-3; it is significance to mention that the reason why Σn-6 is high probably comes from

the maternal diet, poor in the consumption of foods rich in n-3, characteristic of western diets.

PUFA/SFA ratio showed statistical differences, HM presented the highest value (0.51±0.01) and HW the lowest (0.28±0.01). According to the United Nations Department of Health and Social Security (Dhss, 1994), foods with a PUFA/SFA ratio below 0.45 were considered unhealthy due to its potential to induce blood cholesterol increases; only the HM presented values above the mentioned value.

The Σ (EPA)+(DHA) showed statistical differences, however the highest value of the sum corresponds to the HW (0.73±0.13) and the lowest value of the HM sample (0.20±0.02). The World Health Organization (WHO) and the United Nations Food and Agriculture Organization (FAO) recommend a daily intake of 250 mg of each FAs, in order to prevent coronary heart disease and inflammatory processes. As a result, both samples did not show the recommended values for daily intake of EPA and DHA. However, the presence of both FAs in the samples is extremely important because consumption would have a share in the intake of EPA+DHA. Long-chain n-3 PUFAs exhibit anti-inflammatory properties and can modulate immune function. Therefore, circulating DHA levels are associated with neurological, visual and IQ performance (Mazzocchi et al., 2018; Hahn-Holbrook, Fish, & Glynn, 2019).

3.4 Triacylglycerol (TAG) Determination

The results of the TAG determination performed by direct infusion in ESI-MS are described in Figure 1 and Table 4. The most intense ion spectral peak was present between *m/z* 876 and 877. This analysis is important to identify TAGs present in the HM and HW samples, and compare its correlation.

Figure 1 shows the human milk (HM) and human whey (HW) pool spectra obtained by this analysis in the *m/z* range from 530 to 1100.

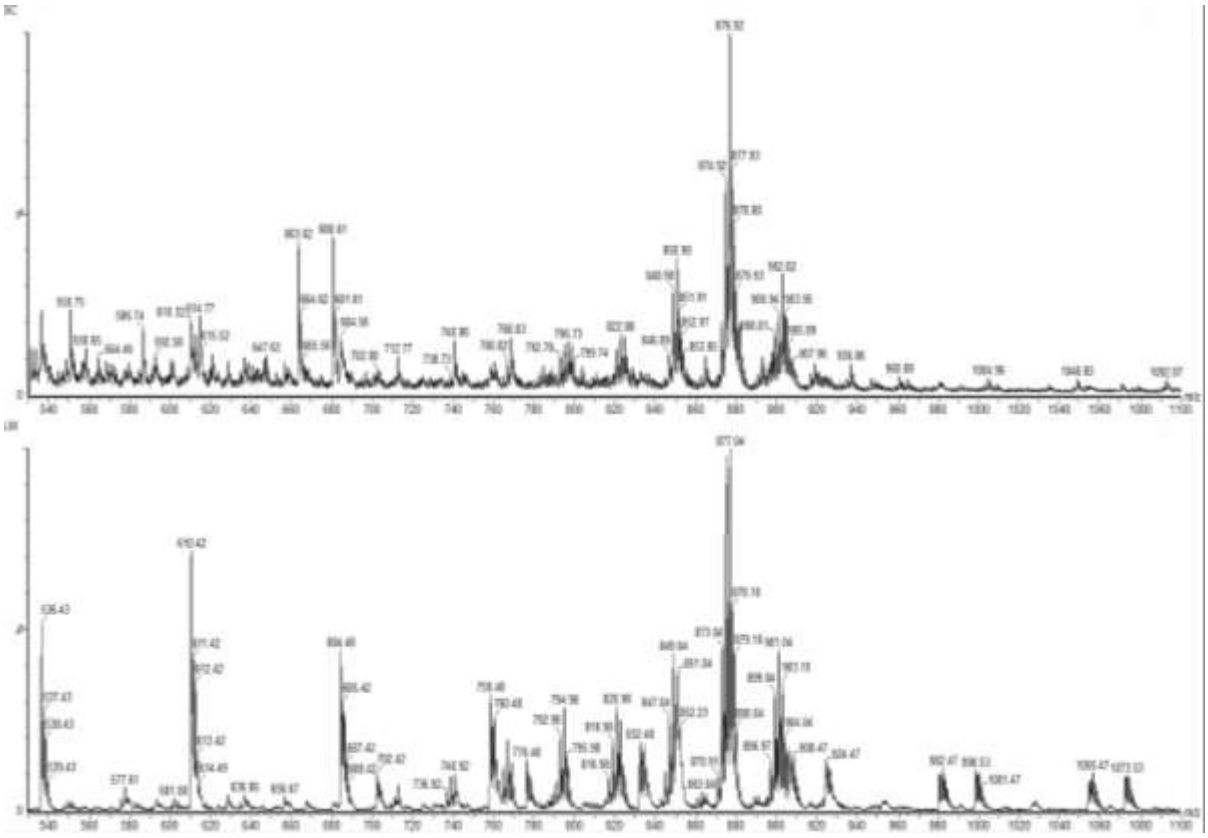

Fig. 1. Ion spectrum of $[TAG+NH_4]^+$ human milk (HM) and human whey (HW) from ESI(+)-MS

According to the Lipid maps® database in the *m/z* region 530 - 791 diacylglycerols (DAG) and monoacylglycerols (MAG) are presented, and in the *m/z* region 792 - 916 triacylglycerols (TAG) were found, being that of higher ion intensity the spectral peak $[TAG+NH_4]^+$ POO of *m/z* 877 corroborating with the same *m/z* found in the research by Rydlewski et al. (2019), who found the same result in human milk analysis. In the HM spectrum, the ionic peaks are more intense compared to HW, since the percentage of lipids in the HM sample is higher (3.23±0.13) than HW (0.93±0.13).

Table 4 presents the 21 largest ratios *m/z*, with its respective TAGs, found in the region between 792 to 916 *m/z*, the LAMES Platform, which was used, was developed for the random configuration of TAGs for vegetable oils (Antoniosi Filho, Mendes, & Lanças, 1995), but it can also be used for animal tissues. However, for human milk the order of FAs in the TAG (Sn-1, Sn-2, Sn-3) is not in accordance with the literature. Since palmitic acid (16:0) for this matrix (human milk) is mostly in the Sn-2 position, however this fact does not interfere with the final result of this work (Koletzko, 2016).

Comparing the results obtained by the FA composition (Table 2), with the TAGs presented in Table 4, it was possible to observe the frequency of oleic (O, 18:1n-9) and palmitic (P, 16:0) acids in TAGs, which are the ones with the highest concentrations in relation to FA, results similar to the studies by Manin et al. (2019) and Rydlewski et al. (2019).

Table 4. Estimation of TAG ions (%) determined by ESI(+)-MS of human milk (HM) and human whey (HW) defined by the LAMES platform.

				TAG	TAG Estimate (%)	
Molecular Formula	**Shorthand**	**Ionization**	***m/z***	**Assignment**	**HM**	**HW**
$C_{49}H_{90}O_6$	46:2	$[M+NH_4]^+$	792	LaLP	2.663	-
$C_{49}H_{92}O_6$	46:1	$[M+NH_4]^+$	794	LaOP	4.107	1.804
$C_{51}H_{92}O_6$	48:3	$[M+NH_4]^+$	818	LaLO	3.755	-
$C_{51}H_{94}O_6$	48:2	$[M+NH_4]^+$	820	LaOO	2.895	-
$C_{51}H_{94}O_6$	48:2	$[M+NH_4]^+$	820	MLP	2.788	-
$C_{51}H_{96}O_6$	48:1	$[M+NH_4]^+$	822	MOP	4.299	3.798
$C_{51}H_{98}O_6$	48:0	$[M+NH_4]^+$	824	PPP	1.684	3.868
$C_{53}H_{96}O_6$	50:3	$[M+NH_4]^+$	846	MLO	3,931	-
$C_{53}H_{98}O_6$	50:2	$[M+NH_4]^+$	848	PLP	4.620	4.047
$C_{53}H_{98}O_6$	50:2	$[M+NH_4]^+$	848	MOO	3.031	2.183

$C_{53}H_{100}O_6$	50:1	$[M+NH_4]^+$	850	POP	7.123	13.340
$C_{53}H_{102}O_6$	50:0	$[M+NH_4]^+$	852	SPP	-	4.502
$C_{55}H_{98}O_6$	52:4	$[M+NH_4]^+$	872	PLL	4.224	-
$C_{55}H_{100}O_6$	52:3	$[M+NH_4]^+$	874	PLO	13.026	9.305
$C_{55}H_{100}O_6$	52:3	$[M+NH_4]^+$	874	PVcO	-	1.899
$C_{55}H_{104}O_6$	52:2	$[M+NH_4]^+$	877	POO	10.042	15.334
$C_{55}H_{104}O_6$	52:2	$[M+NH_4]^+$	877	SLP	2.372	3.140
$C_{55}H_{104}O_6$	52:1	$[M+NH_4]^+$	878	SOP	3.658	10.349
$C_{55}H_{106}O_6$	52:0	$[M+NH_4]^+$	880	SPS	-	1.746
$C_{57}H_{86}O_6$	54:1	$[M+NH_4]^+$	884	SOS	-	2.007
$C_{57}H_{100}O_6$	54:5	$[M+NH_4]^+$	898	OLL	5.956	-
$C_{57}H_{103}O_6$	54:4	$[M+NH_4]^+$	900	OLO	9.183	5.348
$C_{57}H_{104}O_6$	54:3	$[M+NH_4]^+$	902	OOO	4.720	5.875
$C_{57}H_{104}O_6$	54:3	$[M+NH_4]^+$	902	SLO	3.345	3.609
$C_{57}H_{106}O_6$	54:2	$[M+NH_4]^+$	904	SOO	2.578	5.948
$C_{59}H_{94}O_6$	56:1	$[M+NH_4]^+$	916	BhOP	-	1.899

Results expressed as an average of three spectral repetitions. La: lauric acid (12:0); M: myristic acid (14:0); P: palmitic acid (16:0); S: stearic acid (18:0); O: oleic acid (18 1n-9); Vc: vaccenic acid (18:1n-7); L: linoleic acid (18:2n-6); Bh: docosanoic acid (22:0). (**HM** - Human Milk; **HW** – Human Whey).

The highest estimates in the percentage of TAGs are present in *m/z* 874 $[TAG+NH_4]^+$ PLO with values in HM (13.026) and HW (9.305), *m/z* 876 $[TAG+NH_4]^+$ POO in HM (10.042) and HW (15.334), *m/z* 900 $[TAG+NH_4]^+$ OLO in HM (9.183) and HW (5.348), *m/z* 850 $[TAG+NH_4]^+$ POP in HM (7.123) and HW (13.340), *m/z* 902 $[TAG+NH_4]^+$ OOO in HM (4.720) and HW (5.875), *m/z* 848 $[TAG+NH_4]^+$ PLP in HM (4.620) and HW (4.047). Similar results to most TAGs found in the study by Manin et al. (2019) analyzing pasteurized and lyophilized human milk during six months of storage.

Comparing the samples of raw human milk from the mature phase (HM) before performing the ultracentrifugation to obtain the human whey (HW), five TAGs were observed, which do not appear in its percentage in Table 4 for the HM sample, but for HW ($[TAG+NH_4]^+$ SPP, PVcO, SPS, SOS, BhOP). This is due to its percentage below 1% of the estimate in the HM sample, a factor used to select the main TAGs of the LAMES Platform.

The percentage of TAGs in the HW sample in relation to the MM sample varied due to its distribution in the fat globules, as the ultracentrifugation was performed, those present in the casein decreased its percentage $[TAG+NH_4]^+$ LaOP, MOP, PLP, MOO, PLO and OLO. While those associated with albumin, fraction soluble in serum ($[TAG+NH_4]^+$ PPP, POP, SPP, PVcO, POO, SLP, SOP, SPS, SOS, OOO, SLO, SOO, BhOP) showed a high percentage, this is justified because the lipids that remained in the liquid fraction had its percentage rebalanced.

According to Mazzocchi et al. (2018), TAGs containing SFA, MUFA are rapidly hydrolyzed by gastrointestinal lipases, without the need for bile salts, making its products more easily absorbed and taken to the liver, assisting in the infants' digestion process. These characteristics are present in HW compared to HM.

4 Conclusion

From the above, it can be concluded that it was possible to obtain a co-product of human milk discarded by milk banks; the human whey (HW). For its characterization, analyzes of the proximate composition, fatty acid composition, evaluation of the nutritional quality of the lipids, as well as the triacylglycerols profile were performed. The results obtained revealed that the chemical composition underwent

significant changes since the HW was obtained from the HM, except for the percentage of ash and carbohydrates. Regarding the fatty acids composition, it was observed that strictly essential fatty acids, essential fatty acids, and all other FAs found in HM, remained present in HW, being extremely important, as these FAs are responsible for several health benefits of the infants, as already demonstrated.

Referring the lipid nutritional quality, both the atherogenicity index and the thrombogenicity index showed adequate values for both samples, indicating a lipidic food quality and its potential effects on the development of coronary diseases. Finally, the triacylglycerols lipid profile showed variation in the samples analyzed, with a higher percentage of saturated and monounsaturated fatty acids, which is important, as it helps in the digestion process of infants. Therefore, HW has potential for application both in isolated form and as used in other foods.

CRediT authorship contribution statement

Eloize Silva Alves: Formal analysis. Writing- original draft. Conceptualization. **Matheus Campos Castro**: Formal analysis. Writing- original draft. Conceptualization. **Bruno Henrique Figueiredo Saquetti**: Formal analysis. Investigation. **Luciana Pelissari Manin**: Data curation. Visualization. **Roberta da Silveira**: Review e editing. Visualization. **Patricia Magalhães Souza**: Formal analysis. Investigation. **Oscar Oliveira Santos Junior**: Conceptualization. Supervision. **Jesuí Vergílio Visentainer**: Funding acquisition. Supervision

Declaration of competing interest

The authors declare absence of conflict of interest.

Acknowledgment

The authors would like to thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for financial assistance, to the research group APLE-A; and to the Human Milk Bank of University Hospital of Maringá for its partnership and donation of human milk samples.

5 REFERENCES

Antoniosi Filho, N. R., Mendes, O. L., & Lanças, F. M. (1995). Computer prediction of triacylglycerol composition of vegetable oils by HRGC. *Chromatographia*, 40, 9-10, 557-562.

Association of Official Analytical Chemists – AOAC. (2005). *Official Methods of analysis of the Association of Official Analytical Chemists*. (18th ed). Gaithersburg: AOAC.

Borges, Mayla S., Oliveira, Angela M. de M., Hattori, Wallisen T., & Abdallah, Vânia O.S. (2018). Quality of human milk expressed in a human milk bank and at home. *J. Pediatria*, 94, 4, 399-403.

Brasil. Ministério da Saúde. Agência Nacional de Vigilância Sanitária. (2008). *Banco de Leite Humano: funcionamento, prevenção e controle de riscos.* (1st ed.). Brasília: Diário Oficial da União.

Brasil. Ministério da Saúde. Agência Nacional de Vigilância Sanitária. (2004). *Regulamento Técnico para o gerenciamento de resíduos de serviços de saúde, Resolução da diretoria colegiada - RDC nº 306, de 7 de Dezembro de 2004.* Brasília: Diário Oficial da União.

Brasil. Ministério da Saúde. Agência Nacional de Vigilância Sanitária. (2006). *Regulamento Técnico para o funcionamento de Bancos de Leite Humano. Resolução da diretoria colegiada-RDC nº 171, de 4 de setembro de 2006.* Brasília: Diário Oficial da União.

Bruxel, R., & Sica, C. D. A. (2019). Análise de proteina e micronutrientes em amostra de leite humano. *RBONE*, 13, 78, 194-201.

Carta, G., Murru, E., Lisai, S., Sirigu, A., Piras, A., Collu, M., Batetta, B., Gambelli, L., & Banni, S. (2015). Dietary Triacylglycerols with Palmitic Acid in the sn-2 Position Modulate Levels of N-Acylethanolamides in Rat Tissues. *PLoS ONE*, 10, 3.

Cecchi, H. M. (2003). *Fundamentos teóricos e práticos em análise de alimentos*. Editora da UNICAMP.

Costa, A. G. V., & Sabarense, C. M. (2010). Modulation and composition of fatty acids in human milk. *Rev. de Nutr.*, 23, 3, 445-457.

Da Silveira, R., Vágula, J. M., De Lima Figueiredo, I., Claus, T., Galuch, M. B., Santos Junior, O. O., & Visentainer, J. V. (2017). Rapid methodology via mass spectrometry to quantify addition of soybean oil in extra virgin olive oil: A comparison with traditional methods adopted by food industry to identify fraud. *Food Res. Int.*, 102, 43–50.

Demmelmair, H., & Koletzko, B. (2018). Lipids in human milk. *Best Practice & Research Clinical Endocrinology & Metabolism*, 32, 1, 57-68.

Folch, J., Lees, M., & Sloane Stanley, G. H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.*, 226, 1, 497–509.

Food and Agriculture Organization – FAO. (2003). Food Energy – Methods of analysis and conversion factors. In *Report of a technical workshop. FAO Food and Nutrition Paper*. (77th ed). Rome: FAO.

FRANKLIN, Prarthana; VOLK, Anthony A. Breast Feeding. 2019.

Gomes-Santos, A. C., Fonseca, R. C., Lemos, L., Reis, D. S., Moreira, T. G., Souza, A. L., Silva, M. R., Silvestre, M. P., Cara, D. C., & Faria, A. M. (2015). Hydrolyzed whey protein prevents the development of food allergy to β-lactoglobulin in sensitized mice. *Cell. Immunol.*, 298, 1-2, 47–53.

Grazziotin, A. L., Grazziotin, M. C. B., & Letti, L. A. J. (2010). Descarte de leite humano doado a Banco de Leite antes e após medidas para reduzir a quantidade de leite imprópria para consumo. *J. Pediatria*, 86, 4, 290-294.

Guo, M. (2019). *Whey Protein Production, Chemistry, Functionality, and Applications*. Hoboken, NJ:John Wiley & Sons.

Hahn-Holbrook, J., Fish, A., & Glynn, L. M. (2019). Human Milk Omega-3 Fatty Acid Composition is Associated with Infant Temperament. *Nutrients*, 11, 12, 2964.

International Fishmeal and Fish Oil Organisation – IFFO. (2017). *Codex Nutrient Reference Value (NRV) for EPA and DHA for Non-Communicable Diseases (NCDs).* Retrieved from https://www.iffo.net/codex-nutrient-reference-value-nrv-epa-and-dha-non. Accessed April 29, 2020.

International Organization for Standardization – ISO. 5509. Animal and vegetable fats and oils – preparation of methyl esters of fatty acids. 2000.

Johnston, M., Landers, S., Noble, L., Szucs, K., & Viehmanm, L. (2012). Breastfeeding and the use of human milk. *Pediatrics*, 129, 3.

Koletzko, B. (2016). Human milk lipids. *Ann. Nutr. Metab.*, 69, 2, 27-40.

Lu, J., Wang, X., Zhang, W., Liu, L., Pang, X., Zhang, S., & Lv, J. (2016). Comparative proteomics of milk fat globule membrane in different species reveals variations in lactation and nutrition. *Food Chem.*, 196, 665–672.

Lu, J., Zhang, S., Liu, L., Pang, X., Ma, C., Jiang, S., & Lv, J. (2018). Comparative proteomics analysis of human and ruminant milk serum reveals variation in protection and nutrition. *Food Chem.*, 261, 274–282.

Manin, L. P., Rydlewski, A. A., Galuch, M. B., Pizzo, J. S., Zappielo, C. D., Senes, C. E. R., Santos, O. O., & Visentainer, J. V. (2019). Evaluation of the Lipid Quality of Lyophilized Pasteurized Human Milk for Six Months by GC-FID and ESI-MS. *J. Braz. Chem. Soc.*, 30, 8, 1579-1586

Mazzocchi, A., D'Oria, V., De Cosmi, V., Bettocchi, S., Milani, G. P., Silano, M., & Agostoni, C. (2018). The Role of Lipids in Human Milk and Infant Formula. *Nutrients*, 10, 5, 567.

Mehrotra, V., Sehgal, S. K., & Bangale, N. R. (2019). Fat structure and composition in human milk and infant formulas: Implications in infant health. *Clin. Epidemiology Glob. Health*, 7, 2, 153-159.

Perini, J. Â. D. L., Stevanato, F. B., Sargi, S. C., Visentainer, J. E. L., Dalalio, M. M. D. O., Matshushita, M., Souza, N. E. D., & Visentainer, J. V. (2010). Ácidos graxos poli-insaturados n-3 e n-6: metabolismo em mamíferos e resposta imune. *Rev. de Nutr.*, 23, 6, 1075-1086.

Rede Brasileira de Banco De Leite. (2019). Retrieved from https://producao.redeblh.icict.fiocruz.br/portal_blh/blh_brasil.php. Accessed Septembet 09, 2020.

Rydlewski, A. A. (2020). Métodos analíticos utilizados para a determinação de lipídios em leite humano: uma revisão. *Rev. Virtual de Química*, 12, 1.

Rydlewski, A. A., Silva, P. D., Manin, L. P., Tavares, C. B. G., Paula, M. G., Figueiredo, I. L., Neia, V. B. M. J. C., Santos, O. O., & Visentainer, J. V. (2019). Lipid Profile Determination by Direct Infusion ESI-MS and Fatty Acid Composition by GC-FID in Human Milk Pools by Folch and Creamatocrit Methods. *J. Braz. Chem. Soc.*, 30, 5, 1063-1073.

Santos-Silva, J., Bessa, R. J. B., & Santos-Silva, F. (2002). Effect of genotype, feeding system and slaughter weight on the quality of light lambs: II. Fatty acid composition of meat. *Livest. Prod. Sci.*, 77, 2-3, 187-194.

Silva, F. D. A. S., & Azevedo, C. A. V. (2016). The Assistat Software Version 7.7 and its use in the analysis of experimental data. *Afr. J. Agric. Res.*, 11, 39, 3733-3740.

Silva, R. C., Escobedo, J. P., Gioielli, L. A., Quintal, V. S., Ibidi, S. M., & Albuquerque, E. M. (2007). Composição centesimal do leite humano e caracterização das propriedades físico-químicas de sua gordura. *Quím. Nova*, 30, 7, 1535-1538.

Simionato, J. I., Garcia, J. C., Santos, G. T., Oliveira, C. C., Visentainer, J. V., & Souza, N. E. (2010). Validation of the determination of fatty acids in milk by gas chromatography. *J. Braz. Chem. Soc.*, 21, 3, 520-524.

Ulbricht, T. L. V., & Southgate, D. A. T. (1991). Coronary heart disease: seven dietary factors. *Lancet*, 338, 8773, 985-992.

Visentainer, J. V. (2012). Aspectos analíticos da resposta do detector de ionização em chama para ésteres de ácidos graxos em biodiesel e alimentos. *Quím. Nova*, 35, 2, 274-279.

World Health Organisation – WHO. (2003). *Population nutrient intake goals for preventing diet-related chronic diseases.* Retrieved from https://www.who.int/dietphysicalactivity/publications/trs916/en/gsfao_overall.pdf?ua=1>. Accessed April 29, 2020.

Yehuda, S., Rabinovitz, S., Carasso, R. L., & Mostofsky, D. I. (2002). The role of polyunsaturated fatty acids in restoring the aging neuronal membrane. *Neurobiol. Aging*, 23, 5, 843–853.