UNIVERSIDADE FEDERAL DE MINAS GERAIS

Lívia Mayra Andrade

Captura direta de proteínas de soro de leite por líquido iônico funcional imobilizado: Desenvolvimento e uso do adsorvente magnético Fe_3O_4-IL-Ni^{2+}

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Dissertação apresentada ao Programa de Pós-Graduação em Alimentos e Saúde da Universidade Federal de Minas Gerais, como pré-requisito parcial para obtenção do título de Mestre em Alimentos e Saúde.

Orientadora: Profa. Dra. Bruna Mara Aparecida Carvalho Mesquita

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Lívia Mayra Andrade. CAPTURA DIRETA DE PROTEÍNAS DE SORO DE LEITE POR LÍQUIDO IÔNICO FUNCIONAL IMOBILIZADO: DESENVOLVIMENTO E USO DO ADSORVENTE MAGNÉTICO Fe_3O_4-IL-Ni^{2+}

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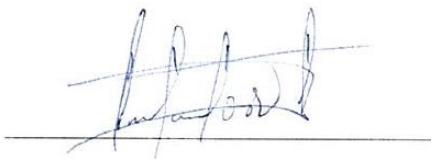

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RESUMO

O soro de leite é o subproduto mais abundante das indústrias de laticínios e, embora tenha sido descartado por muito tempo, hoje é uma das principais fontes proteicas com aplicações que vão do ramo farmacêutico ao nutricional. As proteínas do soro do leite são importantes fontes de peptídeos bioativos, que podem apresentar atividades antioxidante, anti-hipertensiva e antimicrobiana, entre outros efeitos. No entanto, para a expansão de suas aplicações farmacêuticas e nutricionais é imprescindível o desenvolvimento de métodos cada vez mais eficazes de obtenção e purificação. Dessa forma, a seção 4.1 deste trabalho apresenta alguns dos principais métodos de purificação de lactoferrina (LF), como ultrafiltração e cromatografia, juntamente com os métodos de separação magnética e extração líquido-líquido, que ainda estão em expansão. Patentes relacionadas aos métodos de purificação desenvolvidos e composições nutricionais enriquecidas com LF também são apresentadas. Tendo em vista as diversas vantagens apresentadas pela separação magnética, na seção 4.2 um novo adsorvente magnético Fe_3O_4-IL-Ni^{2+} foi produzido e sua capacidade de captura de proteínas do soro foi verificada. Através de um planejamento de experimentos, a influência do pH, da temperatura e da concentração de NaCl foi analisada na adsorção de albumina do soro bovino (BSA) e um modelo de segunda ordem foi ajustado, levando a um coeficiente de regressão de 94,51%. O processo adsortivo foi favorecido por valores de pH inferiores 5, enquanto a temperatura e a concentração de NaCl influenciaram positivamente, com melhores faixas em 30-50ºC e 200-700 mM, respectivamente. Avaliando-se a capacidade do adsorvente Fe_3O_4-IL-Ni^{2+} na captura de proteínas diretamente do soro constatou-se, por meio do perfil eletroforético em SDS-PAGE, que as proteínas alfa-lactalbumina (α-LA), beta-lactoglobulina (β-LG), imunoglobulina (IgG), albumina do soro bovino (BSA) e lactoferrina (LF) foram adsorvidas com sucesso. Portanto, com o devido escalonamento e validação do método utilizado, existe potencial para utilização industrial, dada a ampla gama de aplicações apresentadas pelas proteínas do soro e as vantagens operacionais da separação magnética com relação aos métodos tradicionais.

Palavras-chave: Separação magnética. Adsorção. Proteínas do soro de leite. Magnetita.

ABSTRACT

Whey is the most abundant by-product of the dairy industry and, although it has been discarded for a long time, today it is one of the main sources of protein with applications ranging from pharmaceutical to nutritional. Whey proteins are important sources of bioactive peptides, which may have antioxidant, antihypertensive and antimicrobial activities, among other effects. However, for the expansion of pharmaceutical and nutritional applications of whey proteins, it is essential to develop increasingly effective methods of obtaining and purifying them. Thus, section 4.1 of this work presents some of the main methods of lactoferrin (LF) purification, such as ultrafiltration and chromatography, along with the methods of magnetic separation and liquid-liquid extraction, which are still expanding. Patents related to purification methods developed and nutritional compositions enriched with LF are also presented. In view of the many advantages presented by magnetic separation, in section 4.2 a new magnetic adsorbent Fe_3O_4-IL-Ni^{2+} was produced and its ability to capture whey proteins was verified. Through a design of experiments, the influence of pH, temperature and NaCl concentration was analyzed on bovine serum albumin (BSA) adsorption and a second order model was fitted, leading to a regression coefficient of 94.51%. The adsorptive process was favored by pH values lower than 5, while temperature and NaCl concentration influenced positively, with the best ranges being, respectively, 30-50ºC and 200-700 mM. Evaluating the adsorbent Fe_3O_4-IL-Ni^{2+} ability to capture proteins directly from whey, it was found, through the electrophoretic profile in SDS-PAGE, that alpha-lactalbumin (α-LA), beta-lactoglobulin (β-LG), immunoglobulin (IgG), bovine serum albumin (BSA) and lactoferrin (LF) proteins were successfully adsorbed. Therefore, with due scaling up and validation of the introduced method, there is potential for its industrial use, given the wide range of applications presented by whey proteins and the operational advantages of magnetic separation in relation to traditional methods.

Key-words: Magnetic separation. Adsorption. Whey proteins. Magnetite.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

BSA – Albumina do soro bovino

DBO – Demanda bioquímica de oxigênio

DCCR – Delineamento composto rotacional central

DQO – Demanda química de oxigênio

GMP – Glicomacropeptídeo

Ig – Imunoglobulina

IIL – Líquido iônico imobilizado

IL – Líquido iônico

LF – Lactoferrina

MNP – Nanopartícula magnética

NP – Nanopartículas

pH – Potencial hidrogeniônico

SDS-PAGE – Eletroforese em gel de poliacrilamida com dodecil sulfato de sódio

α-La – Alfa-lactalbumina

β-Lg – Beta-lactoglobulina

SUMÁRIO

1 INTRODUÇÃO

O soro consiste no líquido remanescente da coagulação do leite durante o processo de produção de queijo e caseína (GANJU; GOGATE, 2017). Rama et al. (2019) afirma que a produção mundial de soro se encontra entre 180 e 190 milhões de toneladas por ano. Consequentemente, o descarte deste subproduto representaria um grande problema, visto que seu poder poluente é altíssimo devido à sua carga orgânica (EL-TANBOLY, 2017). Portanto, metade desta produção é processada industrialmente, seja para a fabricação de ricota, bebidas fermentadas, soro em pó ou, em menor quantidade, para a purificação de proteínas, uma área ainda passível de expansão.

As proteínas isoladas do soro possuem diversas aplicações industriais devido suas propriedades farmacêuticas, nutricionais e sua versatilidade como ingrediente (CARTER; FOEGEDING; DRAKE, 2020; CASTRO et al., 2017). As principais proteínas constituintes do soro são β-lactoglobulina (β-Lg), α-lactalbumina (α-La), imunoglobulinas (Igs), albumina do soro bovino (BSA), lactoferrina (LF) lactoperoxidase (LP), proteose-peptona e glicomacropeptídeos (GMP). Contudo, o uso de tais proteínas só é possível após sua purificação, o que, para uma qualidade satisfatória, exige métodos cada vez mais refinados (LABROU, 2014).

Por suas várias vantagens em vista dos métodos tradicionais, a separação magnética se torna uma boa alternativa. Este procedimento utiliza nanopartículas magnéticas (MNPs) que se ligam aos compostos alvo e podem ser facilmente separadas em seguida por meio de um campo magnético externo (CAO et al., 2012). Frequentemente, as MNPs necessitam ter sua superfície modificada com um ligante para assegurar sua estabilidade e aprimorar sua seletividade em relação à proteína que se deseja imobilizar (WANG et al., 2020). Com relação a esta necessidade, Zheng et al. (2014) afirma que os líquidos iônicos (IL) oferecem diversas vantagens ao processo de purificação quando imobilizados em MNPs. Além disso, como mencionado por Li, H. et al. (2012), ainda é possível agregar as peculiaridades de um metal às propriedades excepcionais do grupo MNP-IL por meio de um acoplamento posterior.

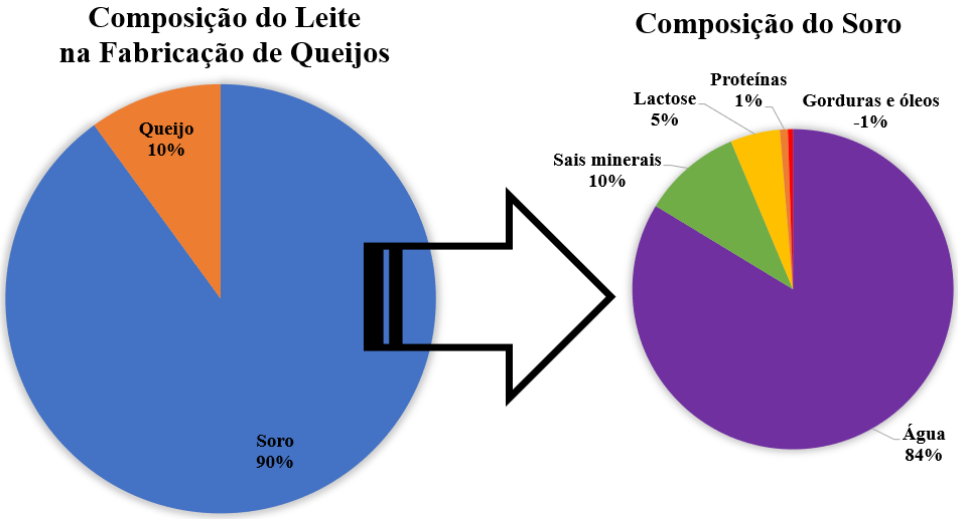
Dada a relevância das proteínas do soro de leite e as vantagens aliadas a separação magnética com relação aos outros métodos de purificação, este trabalho almejou produzir e caracterizar um novo adsorvente magnético MNPs-IL-Ni^{2+} e avaliar a sua capacidade de adsorção e dessorção de tais proteínas.

2 REVISÃO DE LITERATURA

2.1 Soro de queijo

O soro é definido por Alves, M. P. et al. (2014) como a porção aquosa do leite que se separa do coágulo durante a fabricação de queijo ou da caseína. A composição deste líquido (Figura 1) pode variar conforme a fonte do leite, alimentação dos animais, época do ano e estágio de lactação; e, consequentemente, ocasionar alterações na coloração, que variam entre amarelo, verde além de, raramente, um tom azulado (GANJU; GOGATE, 2017; RAMA et al., 2019). O soro retém cerca de 55% do conteúdo do leite e representa entre 85 e 95% de seu volume; o que corresponde a uma produção mundial de 180 a 190 milhões de toneladas por ano (YADAV et al., 2015).

Figura 1: Composição do leite e do soro de queijo



Fonte: Adaptado de Rama et al. (2019)

Devido a sua larga produção, o soro é considerado o principal subproduto das indústrias de laticínios (GANJU; GOGATE, 2017). Estima-se que a produção global de queijos alcance 26 milhões de toneladas até 2023 e, consequentemente, cerca de 230 milhões de toneladas de soro (RAMA et al., 2019). A abundância deste subproduto aliada à sua elevada carga orgânica (DBO entre 27 e 60 $g{\cdot}L^{-1}$ e DQO 50-102 $g{\cdot}L^{-1}$) o transforma numa questão preocupante do ponto de vista ambiental. Os nutrientes residuais do leite encontrados no soro são de difícil degradação e impossibilitam que seu tratamento ocorra juntamente com outros efluentes. Por outro lado, estes mesmos compostos podem ser transformados em diversos produtos de alto valor agregado quando utilizadas as tecnologias adequadas (YADAV et al., 2015).

Em vista de tal possibilidade, conforme Rama et al. (2019), são processadas industrialmente em torno de 90 milhões de toneladas de soro por ano, correspondentes a 50% da produção mundial . Da quantia que passa pela indústria, metade é destinada à produção de ricota e outras bebidas lácteas; 30% é utilizada como soro em pó em fórmulas infantis; 15% é vendida como lactose, após purificação e apenas 5% são utilizadas para a obtenção de proteínas isoladas (RAMA et al., 2019). As principais proteínas globulares encontradas são albumina do soro bovino (BSA), amplamente utilizada em estudos por sua alta disponibilidade, estabilidade e solubilidade em água (PHAN et al., 2015); lactoferrina (LF), conhecida por seu papel no transporte de ferro, bem como sua atividade antimicrobiana e anticarcinogênica; α-lactoalbumina (α-La), β-lactoglobulina (β-Lg), imunoglobulinas (Ig), glicomacropeptídeos (GMP), lactoperoxidase e proteose-peptonas (VASCONCELOS; BACHUR; ARAGÃO, 2018). Estas proteínas apresentam diversos aminoácidos não produzidos pelo corpo humano e que são essenciais para a síntese proteica. Desta forma, a possibilidade de enriquecimento nutricional dos alimentos pela incorporação desses aminoácidos torna tais proteínas cada vez mais requisitadas no mercado e demanda métodos de purificação ainda mais aprimorados (VASCONCELOS; BACHUR; ARAGÃO, 2018; YADAV et al., 2015).

2.2 Técnicas de purificação de proteínas

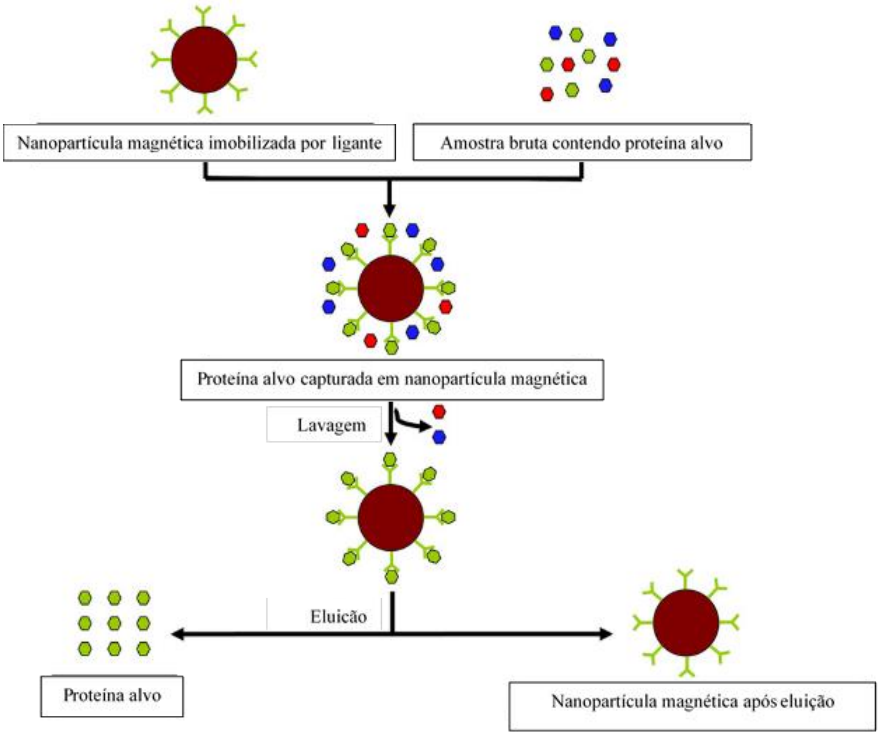
Para Amer (2019), a purificação é um objetivo intrínseco para uma representação apurada de uma proteína objetiva. Em virtude das funções nutricionais e dos benefícios das proteínas na saúde humana, existe uma crescente demanda destas substâncias para o desenvolvimento de novas aplicações, o que evidencia a necessidade de obtenção de métodos de purificação eficientes e eficazes (CAO et al., 2012; LABROU, 2014). Conforme Cao et al. (2012), as técnicas de purificação de proteínas comumente utilizadas são precipitação, centrifugação, ultrafiltração, cromatografia e diálise. Dessas, precipitação e centrifugação são etapas de pré-tratamento, de forma que exigem procedimentos posteriores para que sejam obtidos parâmetros de pureza superiores (XIAO; ZHOU, 2020). No que tange a extração de proteínas do soro de leite, as filtrações por membranas e a cromatografia, geralmente em colunas, são os procedimentos mais usuais (NICOLÁS; FERREIRA; LASSALLE, 2019). Cabe ressaltar que é imprescindível que essas técnicas de purificação sejam economicamente viáveis, visto que, conforme o produto almejado, o processo de *downstream* é ainda o mais oneroso na sua obtenção (WILKEN; NIKOLOV, 2012).

Os processos de filtração por membrana são representados pela ultrafiltração e pela diálise (LI, D. et al., 2020). Ambos têm o gradiente de concentração como força motriz, porquanto apenas moléculas de menores pesos moleculares conseguem atravessar a membrana semipermeável e compostos maiores, como as proteínas, não. A ultrafiltração se distingue pela necessidade da ação da pressão ou da força centrífuga, o que não é necessário na diálise (LIU et al., 2020). Contudo, a redução na taxa de transferência devido ao acúmulo de partículas nas membranas, que ocorre ao longo do tempo, afeta diretamente o custo e a eficiência destes processos (GAJENDRAGADKAR; GOGATE, 2016).

De acordo com Liu, S.; Li et al., (2020), embora possa apresentar algumas variações, a cromatografia consiste basicamente em fazer que uma solução contendo a proteína de interesse flua por uma coluna que contém diversas substâncias. As diferentes interações que ocorrem entre estas substâncias e as proteínas levam a tempos de retenção distintos, de forma que se consiga realizar a purificação almejada. Apesar de ser um processo altamente específico e eficaz, a cromatografia é um processo caro, de difícil aplicação e, quando aplicada convencionalmente em laboratório, demorado (NICOLÁS; FERREIRA; LASSALLE, 2019).

Em contrapartida, a separação magnética é mais rápida, passível de aumento de escala, não necessita de empacotamento em colunas e pode ser usada diretamente para remoção de compostos em amostras cruas, com materiais em suspensão (CAO et al., 2012; NICOLÁS; FERREIRA; LASSALLE, 2019). Este procedimento pode ocorrer pela metodologia direta ou pela indireta. Na primeira, os ligantes são conectados às nanopartículas magnéticas (MNPs) diretamente e então o produto é colocado em contato com as amostras brutas, onde as proteínas alvo são reconhecidas e capturadas. No método indireto, os ligantes são adicionados à amostra bruta e, em seguida, este complexo é capturado pelas MNPs. Por ser de controle mais fácil, o método direto é o mais utilizado, sendo que, os compostos indesejados são removidos após a captura das proteínas almejadas, e estas são eluídas das MNPs posteriormente, como mostra a Figura 2 (CAO et al., 2012).

Figura 2: Esquema de separação de proteínas por afinidade magnética



Fonte: Adaptado de Cao et al. (2012)

2.3 Nanopartículas magnéticas (MNPs)

Entre os diversos materiais revolucionários desenvolvidos no campo da nanotecnologia, se encontram as nanopartículas magnéticas (MNPs). MNPs são materiais particulados com dimensões menores que 100 nm e possuem superparamagnetismo, que faz com que elas possam ser manipuladas por meio de ímãs (KHAN; SAEED; KHAN, 2019). Segundo Khan, Saeed e Khan (2019), assim como as diversas nanopartículas, as MNPs são compostas de três camadas: o núcleo; a camada de casca e a superfície. O núcleo pode ser constituído de vários materiais magnéticos, como níquel, cobalto, ferro e óxidos de ferro. Estes últimos são os mais utilizados tendo em vista seus altos momentos magnéticos, facilidade de síntese e baixo custo produtivo (ALVES, M. N. et al., 2019).

Diversas vias de síntese são descritas na literatura para obtenção das MNPs de Fe_3O_4, havendo métodos físicos e químicos, onde existem variações focadas em diferentes tamanhos de partículas. Devido à sua fácil implementação e baixa nocividade, a técnica de coprecipitação é a mais comum. Este procedimento consiste na adição de uma base a uma solução de Fe^{2+} e Fe^{3+}, onde o ambiente alcalino favorece a formação de Fe_3O_4 (ADEWUNMI; KAMAL; SOLLING, 2021; LIU; YU et al., 2020).

Contudo, as MNPs de óxido de ferro puro necessitam ser revestidas com outros materiais, de forma a prevenir a agregação e aumentar sua estabilidade. Neste caso, elas podem ser revestidas com materiais inorgânicos ou podem ter sua superfície modificada por grupos funcionais, que ampliam suas aplicações (ALVES, M. N. et al., 2019; WANG et al., 2020). Consoante Zheng et al. (2014), os líquidos iônicos (IL) proporcionam alta estabilidade às MNP quando acoplados, de forma a conciliar as vantagens de ambos materiais. Além disso, a capacidade de regeneração das MNPs por meio de campos magnéticos externos e lavagens com os reagentes apropriados reduz os custos produtivos e os impactos ambientais (ADEWUNMI; KAMAL; SOLLING, 2021).

A alta biocompatibilidade das MNPs e seu amplo uso em tecnologias de separação tornam crescente o número de aplicações que vem sendo usadas em tecnologias de separação, ciências médicas, aplicações ambientais e imobilização de proteínas (LIU; YU et al., 2020). Shen et al. (2020) prepararam MNPs à base de MoS_2-Fe_3O_4 pelo método biomimético e aplicaram estes compósitos como catalisadores da reação de Fenton para degradação de corantes orgânicos, como o azul de metileno. O catalisador reagiu beneficamente com o peróxido de hidrogênio de forma a gerar radicais livres OH^- e O_2^-; consequentemente, o azul de metileno foi degradado rapidamente, em uma ampla faixa de pHs e com eficiência próxima a 100%. Além disso, as MNPs mostraram boa capacidade de reutilização, o que evidencia sua aplicabilidade na solução de problemas ambientais. No trabalho de Che et al. (2019), as MNPs foram aplicadas no processo de separação e purificação de ovalbumina da clara de ovo. Os autores produziram MNPs, cuja superfície foi modificada com polietilenoimina, e então, a revestiram com tungstotelurato (VI) por meio de interações eletrostáticas. O promissor adsorvente gerado com cerca de 0,5 mg de TeW@MNP levou a uma eficiência máxima de 91,6% na adsorção de ovalbumina 100 $\mu g\ mL^{-1}$ através de um comportamento que se ajustou ao modelo de Langmuir. Já na área das ciências médicas, Minbashi et al. (2020) mostraram por meio de simulações computacionais e comparações com resultados experimentais que a injeção de MNPs no tecido canceroso do fígado pode auxiliar no tratamento de tumores deste órgão. Conforme os autores, a potência de entrada de 35 W em uma antena de microondas aplicada no tumor quando se utiliza as MNPs leva a um efeito análogo ao obtido com 90 W na ausência de tais partículas. A hipertermia causada no tecido canceroso pode erradicar o tumor em ambos os casos, entretanto, o risco de dano a tecidos saudáveis é significativamente menor com o uso das MNPs, visto que a potência utilizada passa por uma drástica redução. Por fim, para a imobilização de enzimas, Suo et al. (2020) produziram MNPs de carboximetilcelulose modificadas por líquidos iônicos.

Os autores concluíram que os líquidos iônicos aprimoraram a estabilidade e a atividade catalítica das enzimas imobilizadas, bem como o desempenho da lipase imobilizada. O método desenvolvido também foi capaz de imobilizar a penicilina G acilase, que exige maior estabilidade. Assim, o uso de MNPs modificadas por líquidos iônicos se mostrou econômico e prático nessa área, como também uma ferramenta de aplicações promissoras.

2.4 Imobilização de Liquido iônico em MNPs

Líquidos iônicos (IL) são, de acordo com Zhao et al. (2018), sais cuja temperatura de fusão geralmente se encontra abaixo de 100ºC. A ampla gama de cátions e ânions existente proporciona a síntese de diversos líquidos iônicos, que possuem propriedades peculiares como baixa pressão de vapor, ampla janela eletroquímica, boa estabilidade térmica, boa condutividade elétrica, alta estabilidade química e forte poder de dissolução (ZHAO et al., 2018). Aliadas com uma baixa combustibilidade, estas propriedades tornam os IL, frequentemente, "mais verdes" quando comparados aos inflamáveis e voláteis solventes convencionais (SAJID, 2019).

Li, H. et al. (2012) aponta que a definição de líquidos "imobilizados" deriva dos catalisadores de fase líquida que se apresentam ligados a um suporte. No processo de imobilização, as vantagens dos ILs são combinadas com as do material de suporte heterogêneos, de forma que as propriedades catalíticas dos ILs se transferem para o suporte e se aliam às previamente apresentadas por esse material. Quando imobilizados, os IL apresentam muitas vantagens sobre os IL livres, como menor lixiviação, menor geração de resíduos e melhor capacidade de recuperação e reutilização, além do fato de que a combinação dos materiais possibilita um aumento significativo do seu campo de aplicação (LI, H. et al., 2012; MOKHTARY, 2017). Além disso, em comparação com os sistemas bifásicos, não são necessárias quantidades grandes dos ILs, cujos preços são relativamente altos; de forma que a viabilidade econômica do processo é afetada positivamente (LI, H. et al., 2012). Os líquidos iônicos imobilizados (IIL) se constituem de três partes distintas: o suporte poroso, uma camada fina de IL na superfície deste suporte e os grupos funcionais que atuam como catalisadores ou co-catalisadores. Existem diversas formas para se efetuar tal imobilização, as quais se classificam pelo tipo de interação entre o IL e o suporte; são elas: imobilização via grupos de ânions ativos, ou método de imersão; imobilização via grupos catiônicos, ou método de ancoragem covalente; e imobilização via suportes sólidos, ou método de encapsulamento (LI, H. et al., 2012).

Tendo em vista a combinação das propriedades exclusivas dos IL e as singularidades dos metais, os IIL contendo metais compõem um setor promissor (LI, H. et al., 2012). No trabalho de Raut et al. (2020), óxido de grafeno serviu como suporte para a imobilização de cloreto de 1-metil-3-(3-trimetoxissililpropil) imidazólio que, por sua vez, suportou íons de rutênio. Os autores utilizaram este catalisador na aminação redutiva de ácido levulínico com diferentes aminas, comprovando sua eficácia. Já Sasaki et al. (2008) produziram e caracterizaram diversos líquidos iônicos contendo íons metálicos imobilizados em superfície de sílica; com os íons variando entre níquel, cobre, ferro, manganês, zinco, cobalto e paládio. O desempenho destes catalisadores na reação de adição de Kharasch e na reação de acoplamento cruzado de Suzuki foram analisados; apenas os compostos com a presença de ferro e de cobre foram ativos para a reação de adição enquanto os com paládio se destacaram para o acoplamento cruzado. Outra aplicação de líquidos iônicos na purificação de proteínas foi apresentada por Ren et al. (2015), que utilizou triazaciclononano na obtenção de hexahistidina marcada por meio da extração líquido-líquido. A metodologia se mostrou adequada e apta para processamento contínuo em larga escala.

2.5 Métodos de adsorção de Proteínas

Conforme Adamczyk (2019), existe uma importância vital na adsorção de proteínas que justifica seu estudo experimental e teórico. Ao mesmo tempo que o processo adsortivo é necessário para a separação e purificação de proteínas por cromatografia e filtração, para a imobilização de enzimas e para a realização de ensaios imunológicos, este procedimento pode levar a efeitos adversos quando ocorre descontroladamente, como a falha de órgãos artificiais e implantes, o bloqueio de sensores e as incrustações de unidades de filtração. O grande número de parâmetros relacionados à adsorção de proteínas a torna complexa, de forma que, como apresentado por Adamczyk (2012), este processo é frequentemente simplificado para apenas três etapas. Na primeira delas, as partículas presentes na suspensão são transferidas por forças convectivas até a vizinhança das superfícies limites. Em seguida, estas proteínas percorrem distâncias menores por meio da difusão e alcançam a adjacência da interface sólido-líquido. Por fim, as partículas se fixam no material sólido por diversas interações de naturezas distintas e formam mono ou multicamadas.

De acordo com Wahab et al. (2020), no processo adsortivo onde a fase sólida é composta por MNPs, a imobilização de proteínas pode ocorrer de forma física ou química. A primeira metodologia pode ser empregada simplesmente pela imersão do material em uma solução que

contém a proteína alvo. Entretanto, devido ao fato de que as interações envolvidas são comparativamente fracas, como forças de Van der Waals, as proteínas imobilizadas tendem a se desprender do suporte, o que compromete a eficácia do procedimento (XU et al., 2014). Na imobilização química são adicionados grupos funcionais específicos às MNPs para facilitar a ligação com as proteínas. Estes agentes de acoplamento interagem simultaneamente com o suporte e com a substância almejada por meio de ligações covalentes. Ainda que este procedimento não garanta uma alta especificidade com relação à proteína almejada, as ligações presentes são significativamente mais fortes do que as presentes no método anterior, o que evita o desprendimento das proteínas imobilizadas (XU et al., 2014). Além disso, Wahab et al. (2020) afirma que a imobilização covalente de uma proteína facilita a obtenção de uma orientação correta devido à interação com certos resíduos de aminoácidos, o que não ocorre na imobilização física. Essa adsorção ordenada de proteínas é a almejada pelos cientistas, visto que ela possibilita o exercício das funções biológicas, ao contrário das proteínas que são adsorvidas em orientações aleatórias (QUAN; LIU; ZHOU, 2019).

3 OBJETIVOS

3.1 Objetivo Geral

Desenvolver um novo adsorvente magnético MNPs-IL-Ni^{2+}, além de avaliar a sua capacidade de adsorção e dessorção das proteínas presentes no soro do leite.

3.2 Objetivos Específicos

- Revisar alguns dos principais métodos de purificação de proteínas do soro de leite;
- Sintetizar o adsorvente magnético Fe_3O_4-IL-Ni^{2+};
- Avaliar a influência da temperatura, concentração de NaCl e pH na adsorção da BSA usando o delineamento composto rotacional central (DCCR);
- Capturar proteínas diretamente do soro de queijo utilizando o adsorvente magnético Fe_3O_4-IL-Ni^{2+} produzido;
- Obter o perfil eletroforético, por SDS-PAGE, das proteínas capturadas do soro de queijo.

4 PRODUTOS CIENTÍFICOS

4.1 Lactoferrin purification methods

Abstract

Lactoferrin (LF) is a glycoprotein present in whey that plays an important role in regulating the level of free iron in body fluids, as it has the ability to bind Fe^{3+} ions. However, for the expansion of LF pharmaceutical and nutritional applications to become possible, it is essential to develop increasingly effective methods of obtaining and purifying it. This article presents some of the main methods of purification of lactoferrin, such as ultrafiltration and chromatography, along with magnetic separation and liquid-liquid extraction methods, which are still expanding in terms of optimizing the purification of this protein. Finally, patents related to the developed purification methods and nutritional compositions enriched with LF are also presented.

Keyword: Ultrafiltration. Chromatography. Magnetic separation. Liquid-liquid extraction. Proteins.

4.1.1 Introduction

Proteins are fundamental substances for human body manutention, constitution and development. These macromolecules can be decomposed into their constituent amino acids, allowing new structures to be formed to perform different functions. However, our body cannot synthesize so-called “essential amino acids” and, as we cannot store proteins without losing their original form, daily food intake is necessary (BITTENCOURT, 2018).

There are several food sources from which proteins can be obtained, including those of plant origin, such as legumes, nuts and soy; and those of animal origin, such as meat, fish, poultry, eggs and milk. Animal sources are considered complete due to the presence of all essential amino acids, the opposite occurs in vegetables, which need to be combined with each other to achieve this goal (HOFFMAN; FALVO, 2004).

Another proteic food of animal origin that is widely utilized is whey. Studies have already demonstrated its effectiveness in animals nutrition, such as pigs (ENGELENDER, 1962) and chickens (BERRY et al., 1943), since the 1940s. Since then, researches has been carried out on the nutritional value of whey in powder (RIGGS; BEATY; MALLON, 1955) and its associated factors (BAKER et al., 1963) in the addition to the intensification of humoral immune response

of mice whose diet included whey protein concentrate (BOUNOUS, Gustavo; KONGSHAVN; GOLD, 1988).

The use of whey also contribute to a environmental problem reduction, since its organic load is highly polluting (MARWAHA; KENNEDY, 1988). Bearing in mind this fact, several possibilities have emerged for its biotechnological use (GONZÁLEZ SISO, 1996), and, since the 1990s, there have been studies about the function of whey in cancer prevention (BOUNOUS, G.; BATIST; GOLD, 1991) and treatment (HAKKAK et al., 2000), antianemic preparations (DALEV, 1994), as functional food ingredient (MCINTOSH et al., 1998), applied in infant nutrition (JOST et al., 2001), in food intake and satiety regulation (LUHOVYY; AKHAVAN; ANDERSON, 2007) as well as other health benefits (SOLAK; AKIN, 2012).

When purified, whey proteins have different applications, as is the case of Lactoferrin (LF). This glycoprotein has a molecular weight of approximately 80 kDa, consists of about 690 amino acid residues and plays an important role in regulating the level of free iron in body fluids, since it has the ability to bind Fe^{3+} ions (ZHANG, Y.; LU; ZHANG, 2021). LF also stands out for its antioxidant (WANG et al., 2008), anti-inflammatory (KIM et al., 2019), antibacterial (LU, J. et al., 2021), antitumor (LI et al., 2017), antiparasitic (YOKOYAMA; ISHIKAWA; KOSHIO, 2019) e antiviral (WAARTS et al., 2005) activity, where recent studies elucidate its potential in COVID-19 prevention and treatment (CHANG; NG; SUN, 2020).

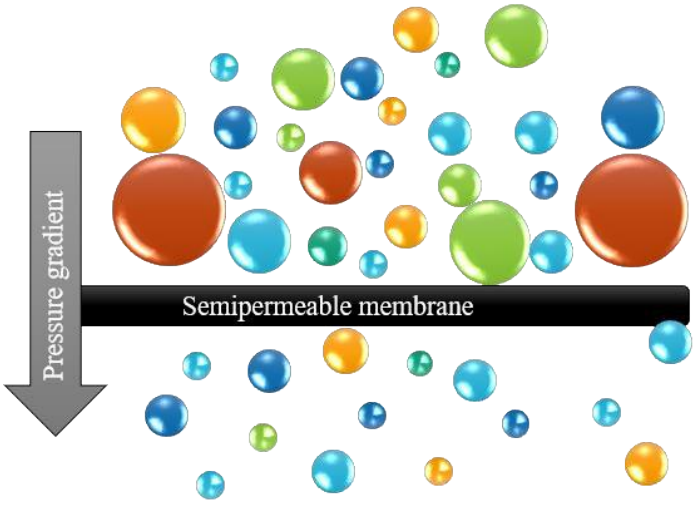
Although LF functionalities are abundant, its purification methods still constitute bottlenecks in the production process. Downstream processes, responsible for obtaining the product free of impurities, are one of the most challenging and time-consuming steps currently. According Macdonald (2018), the main problem is the downstream operations inability to keep up with the upstream operations advances. Therefore, in order to highlight the improvements in this area, the present work aims to analyze some of the possible methods for lactoferrin purification.

4.1.2 Ultrafiltration

Ultrafiltration (UF) consists of a separation process driven by pressure gradient in which macromolecular species are retained by a heterogenous interface, called membrane, according their size (Figure 1). Such steric exclusion occurs even during biological solutions filtration and makes UF have a low energetical consumption. Furthermore, the properties presented by the membrane make UF easily scalable (ROHANI; ZYDNEY, 2010; SAXENA et al., 2009).

Ultrafiltration membranes are able to retain macromolecules with a molecular weight between 10 kDa and 1 MDa due to the availability of different pore sizes, which can range from 5 to 100 nm. Although they can also be made of ceramic and metallic materials depending on the application, polymeric membranes are the most used because their high thermal stability and chemical resistivity, especially polysulfone or polyethersulfone membranes (CUI, 2005; SAXENA et al., 2009). Another important factor in the membrane's choice is molecular weight cut-off (MWCO), a factor that corresponds to the molecular weight of the smallest protein that would have around 90% rejection and is essential to estimate the selectivity that the membrane will present for a certain compound of a sample (SAXENA et al., 2009).

Figure 1: Schematic representation of ultrafiltration. Due a pressure gradient, the sample goes through a semipermeable membrane. Particles larger than the membrane pores are retained and the smaller ones pass through it.



Although there is the fouling possibility, due to the protein's absorption, and the lack of selectivity of the process, the improvements that have been developed expand the applicability of ultrafiltration (SAXENA et al., 2009). At first, the myth was believed that the separation between proteins was only effective when their molecular weight differed by a factor of 10, as the wide distribution of pore size and adsorption and interaction of proteins complicated the process and even led to membrane clogging. Today, it is known that is possible to minimize membrane clogging and obtain high separation selectivity by adjusting conditions such as pH and saline concentration (CUI, 2005). Higher performance membranes can be obtained by manipulating their charges, since the electrostatic effect also assists in high resolution protein separation allowing only certain solutes to pass through and retaining the rest (ROHANI; ZYDNEY, 2010). Still, ultrafiltration membranes are an area for improvement.

Applying ultrafiltration, Tsakali *et al.* (2015) explored the effect of processing conditions on lactoferrin and immunoglobulin G content in Feta cheese whey protein concentrates. All combinations analyzed by the authors led to a high protein content and a high content of the two proteins. However, the cylindrical membranes at 20ºC and with a transmembrane pressure of 4 bar combined with lyophilization generated the highest yield and better sensory characteristics.

Ultrafiltration can also be used together with another process. Maciel *et al.* (2020) purified lactoferrin from sweet whey using ultrafiltration followed by expanded bed chromatography and obtained LF at 17.4 mg/mL, resulting in a purity of 92.7% and recovery of 87.0%. Lu, R. R. *et al.* (2007) isolated LF from bovine colostrum by ultrafiltration together with production scale cation exchange chromatography. Ultrafiltration occurred in two stages: the first had its MWCO at 100 kDa, fixed transmembrane pressure (TMP) at 200 kPa, tangential flow velocity of 5 m/s and temperature of 25ºC; in the second stage, these parameters assumed, respectively, the values of 10 kDa, 150 kPa, 4 m/s and 50ºC. At the end of UF, the lactoferrin concentrate showed 30.88% (w/w) of purity and 94.04% of recovery; later, after performing cation exchange chromatography, these values changed to 94.20% and 82.46%, respectively.

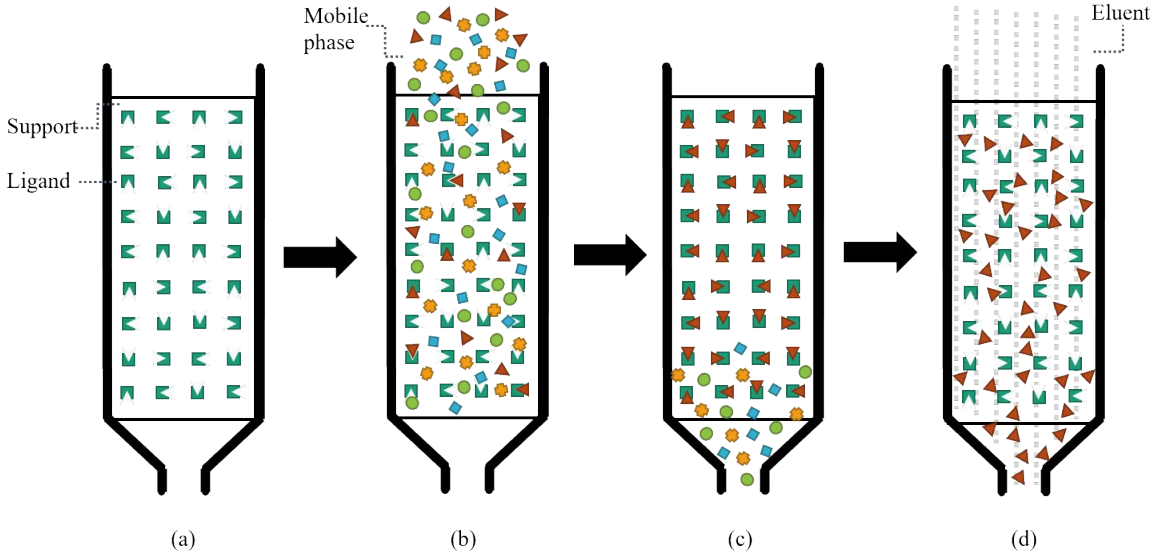
4.1.3 Chromatography

Discovered in the early 20th century by the botanist M. S. Tswett, chromatography is a method that aims to separate the various species of a mixture through the interaction and distribution of these molecules into two phases: one stationary and one mobile (LUXMINARAYAN et al., 2017). The stationary phase consists of a solid base or a layer of liquid adsorbed on the surface of a solid support, while the mobile phase is always a liquid or a gas. The properties that make the molecules separation possible may be related to adsorption, partition, affinity or molecular weight difference, causing the mobile phase compounds to interact in different ways with the matrix (COSKUN, 2016). In this way, compounds that have a greater storage preference in the stationary phase will have a longer retention time when compared to those favored by interactions with the mobile phase, resulting in the separation of solutes (COSKUN, 2016; LUXMINARAYAN et al., 2017).

Chromatography types can be named according to their mobile phase (liquid chromatography, gas chromatography), stationary phase (paper chromatography) or the form of interaction between compounds (size exclusion chromatography, ion exchange, affinity, hydrophobic

interactions). Affinity chromatography is, according to Arora, Saxena e Ayyar (2017), the most selective and versatile form of liquid chromatography. Widely used in protein purification, affinity chromatography (Figure 2) is based on the reversible and specific binding between a solute and the so-called “affinity ligand”. This ligand is immobilized on the support, or matrix, so that it is possible to selectively retain the target protein, even if it is in a complex mixture (RODRIGUEZ et al., 2020).

Figure 2: Schematic representation of affinity chromatography. With the chromatographic column already containing immobilized ligands on the support (a), the mobile phase comes through the column (b). Then, the components not bound to the solid phase are washed (c) and, finally the aimed compound is eluted (d).



After the ligand immobilization on the support, the mobile phase containing the sample comes into contact with the solid phase under the most favorable conditions (temperature, pH, ionic strength) to ligand interaction with the aimed solute. Then, the unbound components are washed and finally the target substance is eluted from the solid phase. Elution, or recovery, can occur by modifying the conditions in which the system is found or by adding a competitive agent that binds strongly with the compound of interest than the binder used on the support. Subsequently, the column containing the solid phase can be regenerated and used again (ARORA; SAXENA; AYYAR, 2017).

In addition to the need for ligand properties such as affinity with the target, specificity, immobilization feasibility and stability after washing and elutions, the choice of an appropriate matrix is also essential. These materials must be insoluble in the buffer used, adaptable to the flow conditions to which they will be subjected, have an adequate surface area and be of easy activation for the ligands coupling (ARORA; SAXENA; AYYAR, 2017).

The possibility of combining a wide range of binding agents with the most diverse supports leads affinity chromatography to high selectivity. Thus, this purification method has become an important tool in the last 50 years with applications in areas such as biochemistry, molecular biology, biotechnology, microbiology, analytical chemistry, pharmacology, biophysics and immunology (RODRIGUEZ et al., 2020). Regarding lactoferrin purification, Carvalho *et al.* (2014) used supermacroporous polyacrylamide cryogel loaded with Cu^{2+} ions via iminodiacetic acid (IDA) bonds to capture this protein from ultrafiltered whey. The authors performed an SDS-PAGE analysis that confirmed the presence of LF in the eluate and showed the absence of any contaminants.

Also using affinity chromatography, although applied to a membrane, Wolman *et al.* (2007) purified LF from whey and colostrum of bovine milk in a one-step process. Hollow fibers synthesized by grafting a hydrophilic copolymer onto polysulfone membranes exhibited a maximum adsorption capacity of 111.0 mg LF/mL of membrane. 94% purity was obtained and the only contaminants found were casein and immunoglobulin. Urtasun *et al.* (2021) purified lactoperoxidase and lactoferrin from whey using chitosan minispheres with immobilized orange triazine R-HE dye. The process performed by the authors did not require a filtration or concentration step and led to a yield of 70% for lactoperoxidase and 60% for lactoferrin and high purity for both.

4.1.4 Magnetic Separation

An old technique that has been expanding among other purification methods is magnetic separation. Among its many advantages are time savings, ease of scaling and automation (CAO et al., 2012). The magnetic nanoparticles (MNP) used in this process favor batch extraction, since such adsorbents do not need to be grouped in cartridges and magnetic decantation mediated by an external magnetic field replaces the centrifugation steps in solid-liquid separation (NICOLÁS; FERREIRA; LASSALLE, 2019). Oppositely to what happens in chromatography, magnetic separation can be performed on raw samples or samples containing particulate material, with no pre-treatment required.

The nanometric size (5-20 nm) of MNPs causes them to have superparamagnetism, suffering interference from an external magnetic field but without remaining magnetization after this field removal (NICOLÁS; FERREIRA; LASSALLE, 2019). These nanoparticles magnetism is conferred on them by the element that constitutes their core, often iron, cobalt, nickel or their

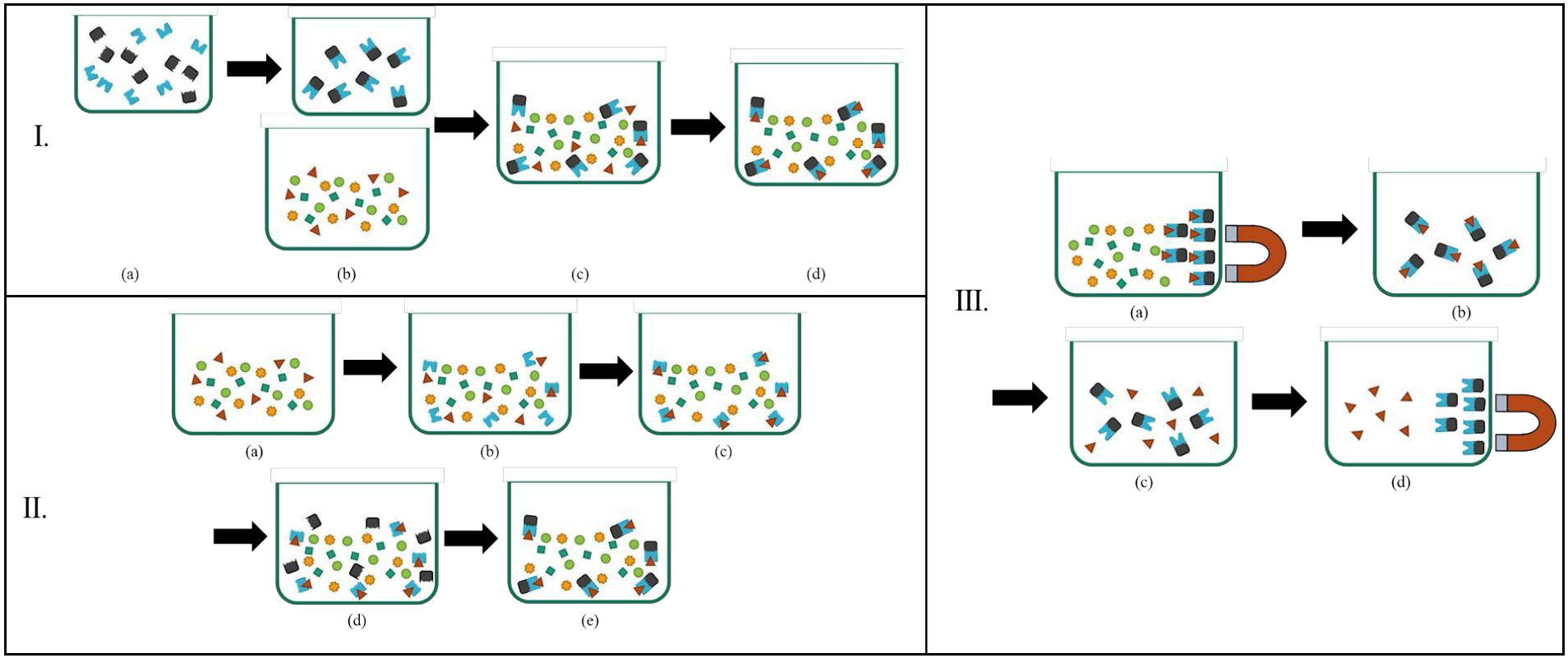
chemical compounds. In food systems, it is common to use iron oxides, such as Fe_3O_4, as they have good biocompatibility and are non-toxic, in addition, they have a large surface area (CAO et al., 2012). MNPs surface, when properly modified, can be functionalized with specific groups that grant selective affinity to the target protein through electrostatic, hydrophobic or chemical mechanisms (CAO et al., 2012; NICOLÁS; FERREIRA; LASSALLE, 2019).

As stated by Nicolás, Ferreira and Lassalle (2019), MNPs can be considered as a type of stationary phase in affinity chromatography and, with the appropriate elution conditions, it is possible to recover the immobilized protein as well as reuse the MNPs. However, unlike chromatography, magnetic separation can occur by the direct or indirect method. In the direct method (Figure 3, item I), the ligands are coupled to the MNPs and, then, the particles are added to the raw samples. In the indirect methodology (Figure 3, item II), the ligands are first put in contact with the sample, reacting with the target protein and forming a complex. Then MNPs recognize the complex and capture it. In both processes there is a subsequent separation step by means of a magnetic field and the captured protein is eluted from the magnetic nanoparticles (Figure 3, item III) (CAO et al., 2012).

Concavalin A was bound to magnetite (Fe_3O_4) magnetic nanoparticles activated with carbodiimide by Lai et al. (2013) for fast selective magnetic separation of LF. The produced adsorbent reached equilibrium time in 5 min and, at pH 7 at 25ºC, the maximum adsorption capacity was 59.2 mg/g with an equilibrium constant of 0.0103 L/mg according to the Langmuir isotherm model. Adsorption selectivity was confirmed by SDS-PAGE.

Chen et al. (2007) isolated LF from acid whey by magnetic affinity separation using superparamagnetic particles of polyglycidyl methacrylate (PGMA) coupled with heparin (PGMA-heparin). The synthesized particles showed high magnetization, high selectivity for LF and a maximum capacity of 164 mg LF/g. Meyer, Berensmeier and Franzreb (2007) designed an automated process for bioproducts recovery using magnetic micro-ion exchangers (MMIX) in combination with high gradient magnetic separation (HGMS). Direct capture of LF from whey was the system chosen as a model in this work and the adsorption capacity of the target protein in MMIX was 12.6 mg/g in relation to LF in whey and 334.6 mg/g in relation to pure LF.

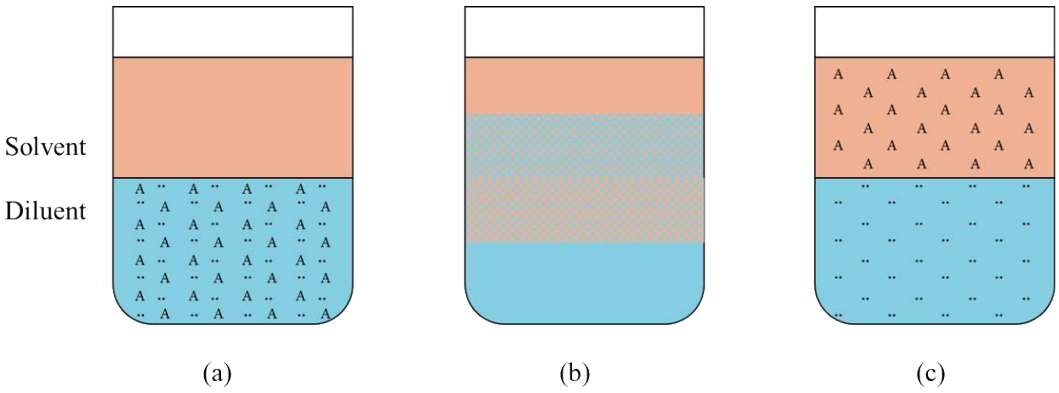
Figure 3: I. Schematic representation of magnetic separation direct method. The ligands and MNPs (a) couple (b) and are added to the sample (c), where they capture the target protein (d). II. Schematic representation of magnetic separation indirect method. Starting from the raw sample (a), ligands are added (b), forming a complex with the target protein (c). MNPs are added (d) and capture the complex (e). III. Schematic representation of the final step of magnetic separation. By means of a magnetic field (a) it is possible to separate the proteins that have not connected to the MNP-ligand system. The captured proteins (b) can then be eluted (c) and the magnetic nanoparticles are regenerated (d).



4.1.5 Liquid-Liquid Extraction

Liquid-liquid extraction (ELL) is based on the difference in solubility of a solute in two immiscible (or partially miscible) liquids at equilibrium. This method has advantages over several others due to its applicability at trace and macro concentration levels, besides that, it provides high yield, the possibility of continuous processing and reduction of operating costs (LEE; KUMAR, 2009; SILVA; FRANCO, 2000). According Silva and Franco (2000), the used phases in this extraction type are compatible with almost all known proteins and, in most systems, just a few seconds are enough to reach equilibrium. A schematic representation of liquid-liquid extraction is shown in Figure 4.

Figure 4: Schematic representation of liquid-liquid extraction. Initially (a), solute A is in the diluent and an immiscible solvent is added. After mixing, droplets improve the interface between the two phases (b). Finally (c), the solute migrates to the solvent and the impurities remain in the diluent.



Starting from a solution containing the solute of interest A in a diluent, the first step for ELL consists of adding a solvent that is immiscible in the diluent used. Then, the two phases are mixed, accelerating the transport of the solute through them. Due to surface tension, the diluent and/or solvent form droplets that significantly improve the interface between them. Finally, the system is layered, which facilitates the separation of the diluent and the solvent initially added. If the system in question is well dimensioned, solute A migrates to the solvent during the phases mixing, while the impurities remain in the diluent (ZHANG, J.; HU, 2013).

For a solvent to be considered an accurate choice in an extraction, it must meet some requirements, such as having specific selectivity for the solute, high solubility for the target solute, and low solubility for the diluent. Furthermore, it is desirable that there is a density difference between the solvent and the diluent and that there is a simple way to

dissociate them at the end of the process. If the designated solvent does not fit the system, problems such as emulsions formation and the need for excessive volumes of toxic organic solvents can occur (ZHANG, J.; HU, 2013).

Alvarez-Guerra and Irabien (2012) performed lactoferrin extraction with hydrophobic ionic liquids based on imidazolium. For the experiment, a configuration of membranes in U-shaped tubes was used. Low protein concentrations (100 mg/L), along with neutral pH (6.4-8.2) and low ionic strength (0. 03 M) favored extraction and led to higher efficiencies. Although there were no extraction efficiencies above 20%, one of the ionic liquids used (1-butyl-3-methylimidazolium bis[(trifluoromethyl) sulfonyl] imide - $BmimNTf_2$) was highly selective for LF in relation to bovine serum albumin (BSA), so the amount of BSA extracted was almost an order of magnitude smaller.

Later, in another work, Alvarez-Guerra e Irabien (2014) combined the advantages of ionic liquid-based aqueous two-phase systems (ILATPS) and three-phase partitioning (TPP) for lactoferrin recovery. The ternary system used was $BmimBF_4$ / NaH_2PO_4 / H_2O (1-butyl-3-methylimidazolium tetrafluoroborate / sodium dihydrogen phosphate / water). Varying the conditions of temperature, amount of ionic liquid and salt concentration, the authors recovered between 74% and 99% of the initial LF.

For LF selective extraction from acid whey, Pawar, Iyyaswami e Belur (2019) used the reverse micellar system with cetyltrimethylammonium bromide (CTAB) and n-heptanol. The authors were able to extract 98.7% of the LF from the acid whey used and confirmed the purity of the protein obtained by SDS PAGE analysis. In addition to being economical, the developed methodology avoids serum pre-treatment steps

4.1.6 Lactoferrin-Related Patents

The various studies developed on lactoferrin over time have yielded, in addition to numerous published articles, also several patents. Some of them are listed in tables 1 and 2, where the first deals with those that refer to the purification methods developed for LF and the second shows patents on nutritional compositions containing this protein, as well as its possible applications in human health.

Table 1: Patents referring to some LF purification methods.

Title	**Patent number**	**Method**	**Reference**
Method for making highly purified lactoferrin and lactoperoxidase from milk, colostrum and acid or sweet whey	BR 11 2021 008814 8	Monolithic chromatography with cation exchanger properties where a pH gradient is employed. Final LF purity greater than 95%.	(KETE et al., 2021)
Method for separation and purification of recombinant human lactoferrin from rice seeds	BR 11 2015 026306 2	Cation exchange chromatography at pH 6-7.5 and LF purification. Final LF purity greater than 95%.	(YANG; OU; SHI, 2017)
Expanded bed absorption methods for isolation of basic milk proteins including lactoferrin	BR 11 2016 000409 4	Expanded bed adsorption with LF purified with 0.3 to 2.0 M NaCl between 30 to 50ºC	(BANAVARA et al., 2017)
Improved process to purify lactoferrin from milk and milk products	BR 11 2015 007674 2	Milk filtration and salt treatment	(BROWN, 2014)

Title 2: Patents referring to LF applications in human health.

Title	Patent number	Application	Reference
Composition comprising lactic acid and lactoferrin	PI 0512503-0	Treatment and/or prophylaxis of conditions in the urogenital tract.	(MATTSBY-BALTZER; ANDERSCH, 2008)
Nutritional compositions based on milk containing lactoferrin and uses thereof	BR 11 2015 012105 5	Additive or synergistic beneficial effects on the health and development of a pediatric individual.	(WITTKE, 2017)
Nutritional compositions containing butyrate and/or lactoferrin and uses thereof	BR 11 2019 011802 0	Reduction of obesity and metabolic syndrome incidence.	(SCHOEMAKER et al., 2019)
Compositions comprising probiotic and prebiotic components and mineral salts, with lactoferrin	PI 1006147-9	Maintenance and/or restoration of intestinal health and prevention of the consequences of digestive tract common dysbiosis. They also have concomitant anti-inflammatory and immunomodulatory action.	(LONGOGNI; PENSI, 2010)
Inclusion of bovine or human lactoferrin in its entirety or peptide fractions in growth control of bacterial and fungal microorganisms for application in body deodorization	PI 0304024-0	Treatment and control of bad body odor that occurs due microorganisms' proliferation on skin surface.	(OKIGAMI; OKIGAMI, 2005a)
Inclusion of lactoferrin in its entirety or peptide fractions in growth control of	PI 0305874-3	Control of skin surface and open wound infections and pre-surgical decontamination procedures.	(OKIGAMI; OKIGAMI, 2005b)

bacterial and fungal microorganisms for human health application			
Lactoferrin	PI 0215682-2	Stimulation of skeletal growth and inhibition of bone resorption. Treatment of a bone disorder.	(CORNISH et al., 2005)
Lactoferrin and brain development and health in children	PI 1012211-7	Treatment or prevention of delayed brain development and/or delayed development of the nervous system.	(WANG; FAURE; SCHMITT, 2010a)
Lactoferrin and adult brain health and protection	PI 1012147-1	Brain protection, maintenance of cognitive function, prevention of cognitive decline and cognitive disorders.	(WANG; FAURE; SCHMITT, 2010b)
Lactoferrin and neuronal health and development in infant gut	PI 1010799-1	Treatment or prevention of delayed development of the enteric nervous system.	(FAURE; WANG; SCHMITT, 2010)
Lactoferrin for use in the diagnosis or prognosis of Alzheimer's disease or in the diagnosis of Parkinson's disease	BR 11 2018 010266 0	Diagnosis or prognosis of Alzheimer's or Parkinson's disease.	(DIAZ, 2017)
Peptides derived from human lactoferrin and their use	BR 11 2013 018365 9	Treatment and/or prevention of infections, inflammation, pain, wounds, scars and/or tumors.	(MAHLAPUU et al., 2016)
Neisseria Lactoferrin binding protein	PI 9811907-9	Vaccination against Neisseria disease.	(PETTERSON-FEMHOLM; TOMMASSEN, 2000)

Oral electrolyte solution containing lactoferrin and uses thereof	BR 11 2015 020864 9	Reduction of gastrointestinal irritation and duration of diarrhea symptoms.	(ALVEY; GONZALEZ; BANAVARA, 2014)
Use of nutritional compositions including lactoferrin in immune cells stimulation	BR 11 2013 016881 1	Stimulation of innate immune cells.	(WITTKE; MUNOZ; BANAVARA, 2011)
Use of lactoferrin nutritional compositions in support of resistance to disease and conditions	BR 11 2013 015458 6	Support resistance to a disease or condition caused by bacterial and viral pathogens.	(WITTKE; MUNOZ; BANAVARA, 2016)
Use of bovine lactoferrin and infant formula	PI 0611378-8	Inhibition of bacterial growth, in a patient, of a bacterial pathogen.	(MCMAHON; OCHOA; CLEARLY, 2010)
Use of lactoferrin and composition comprising it	BR 11 2014 023240 7	Prevention, improvement or treatment of diarrhea.	(WANG, 2017)

4.1.7 Final Considerations

In this work, some of the main methods used for the lactoferrin purification were reviewed, as well as articles and patents that studied and implemented it in human health. Initially, the basic principles of the operation of ultrafiltration, a simple and low energy consumption method, were presented. Then, chromatography was addressed, which, with several possibilities of combinations between ligands and solid phases, leads to high levels of selectivity.

Magnetic separation was also exposed and it was shown that, unlike other methods, it can be applied directly to raw samples and favors batch extraction. Liquid-liquid extraction, on the other hand, stands out for the possibility of continuous processing, as well as for its wide coverage at trace and macro concentration levels. Finally, patents were listed that refer to purification methods developed for lactoferrin and nutritional compositions that contain it and generate several benefits when applied to human health.

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4.2 Direct capture of whey protein by immobilized functional ionic liquid: A new magnetic adsorbent Fe_3O_4-IL-Ni^{2+}

Abstract

Whey proteins are important sources of bioactive peptides, which can present antioxidant, anti-hypertensive and antimicrobial activities among other effects. However, for profound and exact representation of a target protein as well as its specific application on human health is essential its purification, where magnetic nanotechnology constitutes an interesting alternative. In this work, a new magnetic adsorbent Fe_3O_4-IL-Ni^{2+} was developed and its ability in whey proteins capture was checked. The influence of pH, temperature and NaCl concentration on bovine serum albumin (BSA) adsorption was analyzed through a design of experiments and a second order model was adjusted with a regression coefficient of 94.51%. Increasing of temperature and NaCl concentration affected positively this adsorption. Otherwise, pH values lower than 5 were more favorable to the BSA adsorption on the magnetic adsorbent. Studying the direct capture of whey proteins in Fe_3O_4-IL-Ni^{2+}, it was found, through SDS-PAGE, that alpha-lactalbumin (α-LA), beta-lactoglobulin (β-LG), immunoglobulin (IgG), bovine serum albumin (BSA) and lactoferrin (LF) were successfully adsorbed. Therefore, with due scaling and validation of the presented method, its possibility of industrial use is evident, given the whey proteins wide range of applications and the operational advantages of magnetic separation in relation to traditional methods.

Keywords: Ionic liquid, magnetic separation, adsorption.

4.2.1 Introduction

Isolated proteins from whey are popular due their functional and nutritional quality (CARTER; FOEGEDING; DRAKE, 2020). For this reason, they have application in different fields, covering sectors such as food and dairy industry and also the pharmaceutical industry (MASOTTI et al., 2017; YADAV et al., 2015). Whey proteins are important sources of bioactive peptides, which can present antioxidant, anti-hypertensive and antimicrobial activities among other effects (CASTRO et al., 2017). Moreover, whey proteins aid in greater muscle protein synthesis than casein or soy protein and prevent sarcopenia, which lead to muscle mass losses between 3 to 8% per decade after the age of 30 (CARTER; FOEGEDING; DRAKE, 2020). The main constituent proteins from whey are β-lactoglobulin (β-Lg), α-lactalbumin (α-La), immunoglobulins (Igs), bovine serum albumin (BSA), lactoferrin (LF) lactoperoxidase (LP), proteose-peptone and glycomacropeptide (GMP) (YADAV et al., 2015).

Although all whey proteins are used industrially, each has its particularities. β-lactoglobulin represents about 50-60% of total whey protein and its unique tridimensional structure favors its use in nutraceutical delivery strategies. According Radomirovic et al. (2022) phycocyanobilin-modified β-lactoglobulin exhibits increased antioxidant properties and stability to digestion and heating. When associated with selenium, β-Lg induces the apoptosis of human lung cancer A549 cells via an intrinsic mitochondrial pathway, as stated by Zheng, G. Q. et al. (2018). α-lactalbumin is the only calcium-binding protein and, due to its binding site, α-La is suitable to transport active materials as kaempferol, which was used by Diao et al. (2021) to enhance the cytotoxicity on HeLa cells via endocytosis. The authors also noted that antioxidant capacity of the complex was higher than kaempferol alone. Anticancer activity was also observed by Yarramala et al., (2019), who used apo bovine α-lactalbumin complexed with La^{3+}. The complex is preferentially more toxic against breast cancer cells when compared to oral cancer and cervical cells, while it was almost non-toxic to the healthy cells. Immunoglobulins are categorized into different classes based on their mechanism action, charge and size (MEHRA et al., 2021). Immunoglobulins G (IgG), for example, possess immune-modulatory properties but recent studies show that, in addition, a whey fraction rich in IgG combined with *Bifidobacterium longum* subsp. *infantis* ATCC 15697 exhibits synergistic effects against *Campylobacter jejuni* (QUINN et al., 2020). Bovine serum albumin is a carrier protein *in vivo* and, due to its homology with human serum albumin, it is the preferred model protein to a wide variety of studies. Zang et al. (2022) found that BSA at different concentrations, specifically at 0.15 mg/mL, inhibited the degradation of blueberry anthocyanins and the loss of antioxidant capacity during processing and *in vitro* simulated digestion. BSA can also be applied in nutrition, and studies have shown that, when utilized as the dominant form of dietary protein, it reduces subcutaneous fat mass, plasma leptin and plasma corticosterone in high fat-fed C57/BL6J mice (MCMANUS et al., 2015). Lactoferrin is a highly active glycoprotein which has characteristics against many microbes, viruses and other pathogens besides its antibacterial, anti-inflammatory and immunomodulatory properties. Li, W. et al. (2015) pointed out LF activity in suppression of lipopolysaccharide-induced endometritis in mice. It is also evident LF potential for the prevention or treatment of emerging diseases, such as COVID-19, since LF strongly inhibits SARS-CoV-2 variants of concern in vitro through direct entry inhibition and immunomodulatory mechanisms (WOTRING et al., 2022).

However, these proteins need to be purified before its specific applications on human health.

Purification is, according Amer (2019), an intrinsic objective for profound and exact portrayal for a target protein. Unfortunately, the purification techniques commonly used, for instance chromatography and ultrafiltration, are still complex, expensive and have time-consuming pre-treatments (CAO et al., 2012). Thus, magnetic nanotechnology appears as an alternative to those process, since magnetic nanoparticles (MNPs) are easily and economically prepared and can bind proteins selectively. MNPs apply in adsorption process can lead to substitution of centrifugation steps by the magnetic decantation mediated by an external magnetic field and counts with the possibility to regenerate and reuse the magnetic material (NICOLÁS; FERREIRA; LASSALLE, 2019b). Thereby, magnetic separation possesses several advantages, in other words, it is time-saving, scalable, easy automated and can be directly used to remove target proteins from crude samples, with suspended material (CAO et al., 2012). The MNP can be simple and fast isolated through using a magnet due its superparamagnetism and nanosize (NICOLÁS; FERREIRA; LASSALLE, 2019b). However, to prevent aggregation caused by surface charges and to increase selectivity for aimed proteins, Alves et al. (2019) points out that, usually, the magnetic cores are coated with inorganic materials, polymers and/or functionalized with organic and biological molecules.

Ionic liquids (IL), which are salts with melting temperature often below 100ºC and peculiar characteristics, can be anchored on magnetic materials and, then, the properties of IL are combined with the magnetic materials ones (ZHAO et al., 2018; ZHENG, X. et al., 2014). After ligands immobilization on MNPs, these particles are added into crude samples where the ligand-bound magnetic nanoparticles recognize and capture the target proteins (CAO et al., 2012). Li, H. et al. (2012) says that a promising area is represented by metal-containing immobilized ILs, since they combine the unique properties of ILs with the peculiar properties of the incorporated metals. Metals ions such as Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, when incorporated in ILs, improved catalytic reactivity of the silica-grafted ILs towards cycloaddition reactions of CO_2 and epoxides (HAN et al., 2012). Besides catalytical activities, immobilized ionic liquids has been showing their importance in separation science, being applied as sorbents in liquid chromatography and also as recyclable ligands (LI, H. et al., 2012). Furthermore, some proteins contain amino acids residues capable of coordinating with transition metals, where we can quote histidine (His), which is present in BSA and lactoferrin surface (NICOLÁS; FERREIRA; LASSALLE, 2019b).

This work aimed to produce a new magnetic adsorbent Fe_3O_4-IL-Ni^{2+}, and analyze its ability in BSA and LF whey proteins capture. In addition, the effects of pH, temperature and NaCl concentration on BSA adsorption were investigated through a design of experiments. Finally, the adsorbent ability to bind to other whey proteins was analyzed.

4.2.2 Materials and methods

4.2.2.1 Material

Ferric chloride hexahydrate ($FeCl_3.6H_2O$), ferrous chloride tetrahydrate ($FeCl_2.4H_2O$), 3-chloropropyltrimetoxysilane, toluene, ethanolamine, nickel chloride, imidazole, bovine serum albumin (lyophilized powder, ≥ 96%), HEPES (2-[4-(2-hydroxyethyl) piperazin-1-yl] ethane sulfonic acid), SDS buffer: Tris-hydrochloride (Tris-HCl), glycerol, sodium dodecyl sulfate (SDS), bromophenol, 1,4-dithiothreitol (DTT); coomassie brilliant blue, methanol and acetic acid were purchased from Sigma-Aldrich. Sodium hydroxide (NaOH) and sodium chloride (NaCl) were purchased from Merck. Proteic pattern was purchased from GE Healthcare. Whey was kindly provided by "Laticínios Vida" company. Reverse osmosis purified water was used for all experiments and all chemical reagents were of high analytical degree.

4.2.2.2 Synthesis of magnetic adsorbent

Magnetic adsorbent was produced according to the method described by Safari e Zarnegar (2013), Sobhani, Nasseri e Zarifi (2018) with some modifications. Fe_3O_4 was synthetized by coprecipitation in a typical procedure. 20 mM of $FeCl_3.6H_2O$ and 10 mM of $FeCl_2.4H_2O$ were dissolved in 150 mL of water followed by the dropwise addition of 50 mL of NaOH (4M) with mechanical stirring by 1 h under 60ºC. The black precipitate was isolated by magnetic separation, washed with water and dried at 60ºC. 3-chloropropyltrimetoxysilane (1 mL) was added to Fe_3O_4 (1 g) previously diluted in toluene and, then, refluxed for 24 h. The resulting chloro-functionalized Fe_3O_4 was magnetically separated, washed with toluene and water and dried at 60ºC. Subsequently, ethanolamine (1 mL) and chloro-functionalized Fe_3O_4 (1 g) dispersed in toluene were refluxed for 24 h. Fe_3O_4-IL synthetized was separated by magnetic separation, washed with ethanol and water and dried at 60ºC. Finally, Fe_3O_4-IL (1g) was refluxed with $NiCl_2$ (0.2 g) earlier diluted in water

for 24 h. The modified MNPs obtained were magnetically separated and washed with ethanol and water, proceeding to drying at 60ºC.

4.2.2.3 Adsorption studies of the influence of pH, NaCl concentration and temperature

The methodology applied for adsorption isotherms obtainment was based on Carvalho et al. (2013), with some modifications. Approximately 0.5 mg of Fe_3O_4-IL-Ni^{2+} were added into eppendorf tubes, followed by the addition of a NaCl solution and different volumes of a 1 mg/mL BSA solution in order that the protein final concentrations reached the values of 0 to 0.100 mg/mL. Then, equilibration imidazole buffer was added to make the volume up to 1000 µL and the tubes were kept under constant agitation and controlled temperature for 12 h. Afterwards, the tubes were submitted for magnetic separation for obtainment of the supernatant from where the protein concentration was measured using the Bradford method (BRADFORD, 1976). The quantity of protein adsorbed by the MNPs was determined according to Eq. (1):

$$q = \frac{V(C_0 - C)}{m} \quad (1)$$

where q (mg/g) is the protein concentration in solid phase, V (mL) is the liquid phase volume, m (g) is the solid phase mass, C_0 (mg/mL) is the initial protein concentration in the liquid phase and C (mg/mL) is the final protein concentration in liquid phase, after equilibrium establishment.

4.2.2.4 Design of experiments (DOE)

A central composite rotational design (CCRD) was used in the optimization of temperature (ºC), pH conditions and NaCl content on Langmuir-Freundlich (mg/g) isotherm model (Eq. 2). Table 1 shows these independent variables and its experimental range and levels investigated.

$$q = \frac{Q_m C^n}{K_d + C^n} \quad (2)$$

where q (mg/g) is the protein concentration in solid phase, Q_m represents the maximum adsorption capacity of the monolayer, K_d is the apparent dissociation constant and n is the Langmuir-Freundlich coefficient (SALA et al., 2014).

4.2.2.5 Modeling the equilibrium data

The software Statistica 8.0 was used for the experimental design analysis and for calculation of predicted data.

4.2.2.6 Whey proteins adsorption studies in Fe_3O_4-IL-Ni^{2+}

Whey was centrifuged to remove fat and 20 mL were stirred with 0.5 g de Fe_3O_4-IL-Ni^{2+} for 2 h at ambient temperature. Afterwards, the adsorbent nanoparticles were magnetically separated and washed with a solution composed by NaCl (300 mM) and 5 mL of pH 7.4 HEPES buffer under constant mechanical stirring for 2 h. Subsequently, a sample was taken for analysis of the electrophoretic profile by SDS-PAGE to proteins separation and, afterwards, their identification.

4.2.2.7 SDS-PAGE

The obtained electrophoretic profile was compared with GE Healthcare proteic pattern. 100 µL sample were added to an eppendorf tube which contained 100 µL of SDS buffer (Tris-HCl 0.125 M, 20% glycerol, 10% SDS, 0.02% bromophenol and 0,2 M DTT blue) and, after heating at 95ºC for 10 min, the sample was submitted to SDS-PAGE electrophoresis in 0.75 mm thickness gels (4% stacking and 12% gel resolution) according Santos et al. (2020) methodology with some modifications. The run occurred during about 3 h at 120 V. Thereafter, gels were stained with coomassie brilliant blue (0,01% p/v) and destained with methanol, acetic acid and water solutions (450:100:450 v/v) until complete bands revelation.

4.2.3 Results and discussion

4.2.3.1 Magnetic adsorbent produced

After all synthesis steps, the magnetic adsorbent obtained was a finely distributed particulate material, dark in color and highly magnetizable (Figure 1). Figure 2 reveals that the synthesized Fe_3O_4-IL-Ni^{2+} is attracted by an external permanent magnet quickly, while the magnet removal leads to the dispersion of nanoparticles.

4.2.3.2 Adsorption adjustment to Langmuir-Freundlich model: Statistical analysis

The adsorption parameters adjusted for the Langmuir-Freundlich model on the respective conditions generated at CCRD runs are presented on Table 2. Then, statistical analyses were performed and regression parameters adjusted (Table 3). Considering 90% of confidence ($p > 0.10$), non-statistically significant variables were removed, generating the reduced model shown in Eq. 3. As can also be seen in Pareto's chart (Figure 3), pH was the most influencing factor, followed by temperature and NaCl concentration. All of these three mentioned coefficients had a negative effect and, except pH, it was quadratic. Concerning the interaction of two factors, pH and temperature interaction had a negative effect while temperature and NaCl concentration interacted positively.

$$Y_1 = 54.81 - 9.70X_1 + 2.69X_2 - 9.34X_2^2 + 4.26X_3 - 7.91X_3^2 - 4.16X_1X_2 + 4.11X_2X_3 \qquad \text{(Eq. 3)}$$

It is remarkable the nearness between the experimental values of q_{max} and those estimated by Equation 1, as demonstrated in Figure 4. Indeed, the second order model was validated by analyses of variance (ANOVA – Table 4) where $F_{calculated}$ was bigger than $F_{tabulated}$ and the p value was lower than 0.01%, corroborating the regression significance. Furthermore, the lack of fit was not statistically significant ($p < 10\%$) and the regression coefficient was 94.51%.

4.2.3.3 Influence of pH, NaCl concentration and temperature on maximum adsorption capacity

Contour curves evaluation were carried out in relation statistically significant interactions: temperature (X_2) *versus* pH (X_1) and NaCl concentration (X_3) *versus* temperature (X_2), which correspond to Figure 5 a and b items, respectively. It was possible to note that BSA adsorption is favored by lowers pH values, mainly when it is below 5. With regard to temperatures, the best results were found with increasing temperatures, with the optimal range being between 30 and 50ºC. The same behavior was observed by Kopac, Bozgeyik and Yener (2008) when studying the effect of pH and temperature on the adsorption of bovine serum albumin onto titanium dioxide. According these authors, BSA isoelectric point is about pH 5, and its water solubility is less at this pH value. Thus, BSA interactions with aqueous phase decrease and, consequently, more interactions with the hydrophobic surface arise, increasing adsorption. Kopac, Bozgeyik and Yener (2008) state that association of high temperatures with greatest adsorbent capacities may be

associated to increased protein activity, which leads to higher diffusion in the adsorbent. In agreement with what is reported in literature, the elevation in saline concentration favored the adsorptive process, highlighting the range of 200-700 mM. Hydrophobic surfaces stay more exposed in presence of salts, since water molecules are repelled, which facilitates adsorption (CHEN; SUN, 2003).

4.2.3.4 Identification of whey proteins adsorbed by Fe_3O_4-IL-Ni^{2+}

After contact of whey with magnetic adsorbent under the appropriate conditions, adsorbed proteins were eluted and analyzed by SDS-PAGE (Figure 6) before (c) and after (d) concentration. For comparison purposes, a whey sample (b) was also analyzed, as well as the molecular weight markers (a). It is possible to observe characteristic molecular weight bands of alpha-lactalbumin (α-LA), beta-lactoglobulin (β-LG), immunoglobulin (IgG), bovine serum albumin (BSA) and lactoferrin (LF) in column d, relative to concentrated eluate, which shows that the used adsorbent has captured all of these proteins. Worth to mention that IgG appears with a light fraction (25 kDa) and a heavy fraction (50 kDa).

The magnetic adsorbent produced has an immobilized Ni^{2+} ion, in order to attract amino acids with electron donating groups, such as histidine, cysteine and tryptophan which contain, respectively, imidazole, thiol and indole groups (ÇIMEN; DENIZLI, 2012). Moreover, pH also exerts a significant influence in the process. Bresolin, Miranda, Bueno (2009, apud CARVALHO et al., 2013) assert that binding with metallic ions such Cu^{2+}, Ni^{2+}, Zn^{2+} or Co^{2+} is favored when electron donating groups are deprotonated (pH > pKa); and for proteins containing histidine and cysteine residues, pH values between 6 and 8 are more favorable. The used whey presented pH close to 6 and all captured proteins from it has one or more amino acid aforementioned, as can be seen in Table 5.

The capture of several proteins evidences the possibility of applying this magnetic separation method to obtain proteins for application in human nutrition, since, according Castro et al. (2017), whey protein is a versatile and active ingredient due to their associated properties as emulsifying, thickening, gelation, foaming, and water-binding agents which leads to manufactured products with similar and desired characteristics compared to those produced with classical ingredients. In

addition, the reported method is time-saving, scalable, gentle, easily automated and can be directly used to remove target compounds from crude samples, without the inconvenience of fouling or the need to be packed into cartridges like traditional chromatography or ultrafiltration methods (CAO et al., 2012; NICOLÁS; FERREIRA; LASSALLE, 2019a).

4.2.4 Conclusion

A new magnetic adsorbent was successfully synthesized in this work and proved its applicability in whey protein purification. BSA adsorption on this adsorbent was well fitted to Langmuir-Freundlich isotherm model, as stated by the analysis of variance (ANOVA), with a $R^2 = 94.51\%$. Influence of pH, NaCl concentration and temperature on the BSA adsorption was analyzed through a design of experiments. Temperature and NaCl concentration affected positively the process, with the best ranges being, respectively, 30-50ºC and 200-700 mM. Otherwise, the mentioned protein was better adsorbed on pH values lower than 5.

Whey proteins adsorption studies in Fe_3O_4-IL-Ni^{2+} evidenced, through SDS-PAGE analysis, the adsorption of alpha-lactalbumin (α-LA), beta-lactoglobulin (β-LG), immunoglobulin (IgG), bovine serum albumin (BSA) and lactoferrin (LF). All of these proteins have electron donating groups which, under appropriate conditions, interact with the adsorbent metallic ion. With the successful capture of these proteins by the synthesized adsorbent, there is potential for industrial application of this method with appropriate scaling up and validation, since whey protein is a versatile and active ingredient and magnetic separation has several operational advantages over traditional methods.

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Figures captions

Figure 1: Magnetic adsorbent synthesized (Fe_3O_4-IL-Ni^{2+})

Figure 2: Adsorbent magnetization: a) Fe_3O_4-IL-Ni^{2+} water solution b) Fe_3O_4-IL-Ni^{2+} water solution near a permanent magnet, where can be seen the displacement of nanoparticles attracted by the magnetic field.

(a) (b)

Figure 3: Pareto's chart with standardized effects of pH (X_1), temperature (X_2) and NaCl concentration (X_3). The vertical line refers to t value corresponding to $p = 0.05$.

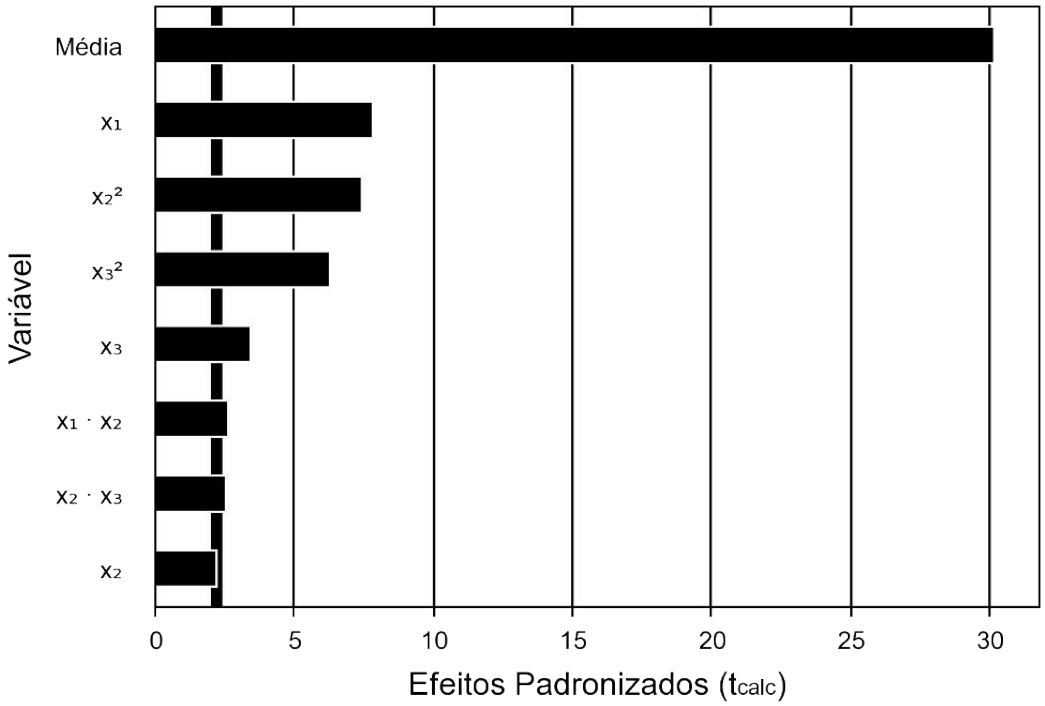


Figure 4: Predicted and experimental values of q_{max}

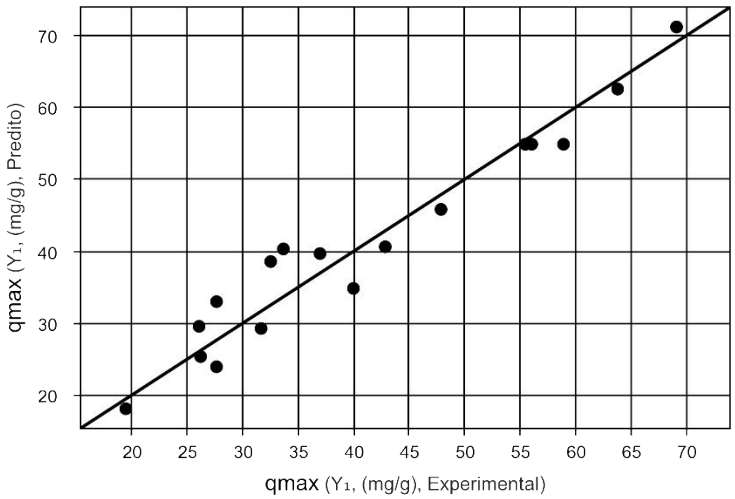


Figure 5: Contour curves: $q_{máx}$ values according (a) temperature (X_2) *versus* pH (X_1) and (b) NaCl concentration (X_3) *versus* temperature (X_2).

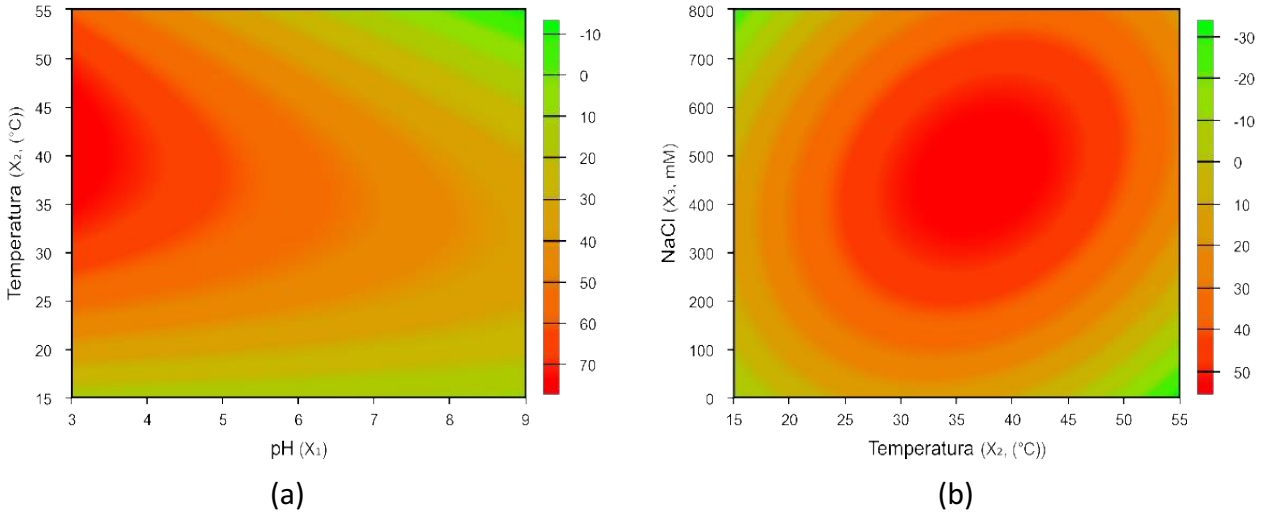
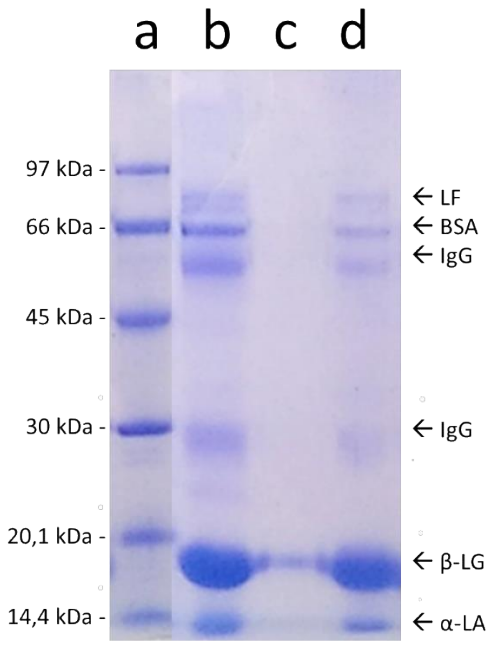


Figure 6: SDS-PAGE analysis: Molecular weight markers (a), centrifugated whey (b), eluted before (c) and after (d) concentration.



*LF – Lactoferrin, BSA – bovine serum albumin, IgG – Immunoglobulin G, β-LG – β Lactoglobulin, α-LA – α Lactalbumin.

Tables

Table 1. CCRD codified parameters

	-1.68	**-1**	**0**	**1**	**1.68**
pH (X_1)	3.48	4.50	6.00	7.50	8.52
Temperatura ºC (X_2)	18.20	25.00	35.00	45.00	51.80
NaCl (mM) (X_3)	64.00	200.00	400.00	600.00	736.00

Table 2: Obtained apparent dissociation constant (K_d), maximum adsorption capacity (q_{max}) and the coeficient n for Langmuir-Freundlich isotherm model in each run

Run	**pH (X_1)**	**Temperature ºC (X_2)**	**NaCl (mM) (X_3)**	**K_d**	**q_{max} (mg/g)**	**n**	**R^2**
1	4.50 (-1)	200 (-1)	25.0 (-1)	0.01246	33.68	1.08	0.99
2	7.50 (1)	200 (-1)	25.0 (-1)	0.00344	31.610	0.92	0.99
3	4.50 (-1)	200 (-1)	45.0 (1)	0.000001	47.82	2.50	0.99
4	7.50 (1)	200 (-1)	45.0 (1)	0.00017	19.43	1.78	0.99
5	4.50 (-1)	600 (1)	25.0 (-1)	0.05501	42.82	0.55	1.00
6	7.50 (1)	600 (1)	25.0 (-1)	0.00045	26.04	1.61	0.98
7	4.50 (-1)	600 (1)	45.0 (1)	0.00121	63.73	1.18	0.97
8	7.50 (1)	600 (1)	45.0 (1)	0.00089	39.95	1.25	0.98
9	3.48 (-1.68)	400 (0)	35.0 (0)	0.00442	69.08	0.80	0.97
10	8.52 (1.68)	400 (0)	35.0 (0)	0.00005	32.57	1.87	1.00
11	6.00 (0)	400 (0)	18.2 (-1.68)	0.00002	27.59	2.00	1.00
12	6.00 (0)	400 (0)	51.8 (1.68)	0.03658	27.58	0.67	1.00
13	6.00 (0)	64 (-1.68)	35.0 (0)	0.00012	26.24	1.56	0.99
14	6.00 (0)	736 (1.68)	35.0 (0)	0.00008	37.04	1.65	0.97
15	6.00 (0)	400 (0)	35.0 (0)	0.00238	56.01	1.18	1.00
16	6.00 (0)	400 (0)	35.0 (0)	0.08149	58.89	0.47	0.97
17	6.00 (0)	400 (0)	35.0 (0)	0.02981	56.69	0.92	0.98
18	6.00 (0)	400 (0)	35.0 (0)	0.00585	55.48	1.12	0.98

Table 3: Data obtained from statistical analyses. X_1 refers to pH, X_2 to temperature and X_3 to NaCl concentration

	Coefficient	Standard Error	t calc	p-value
Average	56.34	2.31	24.42	0.0000
X_1	-9.70	1.25	-7.76	0.0001
X_1^2	-1.42	1.30	-1.09	0.3062
X_2	2.69	1.25	2.15	0.0634
X_2^2	-9.64	1.30	-7.42	0.0001
X_3	4.26	1.25	3.41	0.0093
X_3^2	-8.20	1.30	-6.31	0.0002
$X_1.X_2$	-4.16	1.63	-2.55	0.0342
$X_1.X_3$	-1.26	1.63	-0.77	0.4619
$X_2.X_3$	4.11	1.63	2.52	0.0361

Table 4. Analysis of variance - ANOVA

Fonte de variação	**Sums of Squares**	**Degrees of Freedom**	**Mean Square**	**$F_{calculated}$**	**$F_{tabulated}$**	**p-value**
Regression*	3597.5	7	513.9	24.6	3.14	0.00002
Residual	209.0	10	20.9			
Lack of fit	201.0	7	28.7	10.8	8.89	0.03837
Pure error	8.0	3	2.7			
Total	3806.5	17				

*R^2 = 94.51%

Table 5: Whey proteins and some of their properties

Proteins	Molecular weight (kDa)[1]	Isoelectric point (IP)[1]	Amino acid with electron donating group
Lactoferrin (LF)	80	9.6	Histidine[2]
Lactoperoxidase	77	8.0	-
Bovine serum albumin (BSA)	66	4.9 - 5.1	Tryptophan, cysteine[3]
Immunoglobulin (IgG)	150*	5.8 - 7.3	Cysteine[4]
Beta-lactoglobulin (β-LG)	18.3	5.2 - 5.4	Cysteine[5]
Alpha-lactalbumin (α-LA)	14	4.7 - 5.1	Tryptophan, cysteine[5]
Glycomacropeptide (GMP)	4 – 6.7	-	-

*Immunoglobulin molecule consists of two light chains (25 kDa) and two heavy chains (50 kDa) linked by inter-chain disulfide bonds (VIDARSSON; DEKKERS; RISPENS, 2014).

[1] (INGLE; LALI, 2018)

[2] (CARVALHO et al., 2013)

[3] (TOPALĂ et al., 2014)

[4] (VIDARSSON; DEKKERS; RISPENS, 2014)

[5] (TAVARES; MALCATA, 2013)

5 CONCLUSÕES

- Foram revisados os principais métodos de purificação de proteína do soro de leite; ultrafiltração, cromatografia, separação magnética e extração líquido-líquido; em um artigo de revisão bibliográfica
- O novo adsorvente magnético MNPs-IL-Ni^{2+} foi sintetizado com sucesso e teve sua capacidade de capturar as proteínas do soro comprovada;
- Os parâmetros temperatura e concentração de NaCl afetaram positivamente a adsorção de BSA, com melhores faixas em 30-50ºC e 200-700 mM, respectivamente. A adsorção foi favorecida por valores de pH menores que 5.
- O adsorvente produzido conseguiu capturar diretamente do soro de leite as proteínas alfa-lactalbumina (α-LA), beta-lactoglobulina (β-LG), imunoglobulina (IgG), albumina do soro bovino (BSA) e lactoferrina (LF), como foi evidenciado pelo perfil eletroforético, por SDS-PAGE.

6 CONSIDERAÇÕES FINAIS

Após a revisão dos principais métodos de purificação de proteína do soro de leite e tendo em vista as diversas vantagens apresentadas pela separação magnética, o adsorvente magnético Fe_3O_4-IL-Ni^{2+} foi produzido com sucesso. A capacidade adsortiva do material produzido foi comprovada e estudou-se também a influência dos parâmetros temperatura, concentração de NaCl e pH no processo adsortivo. Além disso, o adsorvente sintetizado capturou diversas proteínas quando colocado em contato direto com o soro de leite; todas elas contendo grupos elétron-doadores que interagem com o íon metálico presente; como foi evidenciado pelo perfil eletroforético obtido por SDS-PAGE. A metodologia adotada se mostrou eficiente em todas as etapas, levando a uma maior compreensão do processo como um todo e confirmando a efetividade da separação magnética na purificação de proteínas do soro de leite. Este trabalho evidencia a possibilidade de aplicação industrial do processo apresentado, visto que foram exibidas diversas vantagens com relação aos métodos tradicionais de purificação. Entretanto, é indispensável o desenvolvimento de estudos que tratem do devido escalonamento do método apresentado.

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