CAROLINE YUMI KAYANO

Produção de albumina canina liofilizada para fins terapêuticos

São Paulo

2021

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Dissertação apresentada ao Programa de Pós-Graduação em Clínica Cirúrgica Veterinária da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo para obtenção do título de Mestre em Ciências.

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CERTIFICADO

Certificamos que a proposta intitulada "Produção de albumina canina liofilizada para fins terapêuticos", protocolada sob o CEUA nº 4000131218 (ID 006470), sob a responsabilidade de **Aline Magalhães Ambrósio** *e equipe; Caroline Yumi Kayano* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 17/04/2019.

We certify that the proposal "Production of lyophilized canine albumin for therapeutical purposes", utilizing 30 Dogs (males and females), protocol number CEUA 4000131218 (ID 006470), under the responsibility of **Aline Magalhães Ambrósio** *and team; Caroline Yumi Kayano* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 04/17/2019.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 01/2019 a 12/2020 Área: Anestesiologia

Origem:	Amostras biológicas estocadas						
Espécie:	Cães	sexo:	Machos e Fêmeas	idade:	1 a 8 anos	N:	20
Linhagem:	Diversas raças			Peso:	27 a 50 kg		
Origem:	Animais de proprietários						
Espécie:	Cães	sexo:	Machos e Fêmeas	idade:	1 a 15 anos	N:	10
Linhagem:	Diversas raças			Peso:	1 a 50 kg		

Local do experimento: Laboratório de imunobiológicos e biofármacos do departamento de tecnologia bioquímica-farmacêutica da Faculdade de Ciências Famacêuticas da Universidade de São Paulo (FCF-USP) Hospital Veterinário da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

São Paulo, 03 de junho de 2020

Prof. Dr. Marcelo Bahia Labruna
Coordenador da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Camilla Mota Mendes
Vice-Coordenador
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

São Paulo, 04 de fevereiro de 2021
CEUA N 4000131218
(ID 007422)

Ilmo(a). Sr(a).
Responsável: Aline Magalhães Ambrósio
Área: Anestesiologia

Título da proposta: "Produção de albumina canina liofilizada para fins terapêuticos".

CERTIFICADO (Emenda versão de 06/dezembro/2020)

A Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, no cumprimento das suas atribuições, analisou e **APROVOU** a Emenda (versão de 06/dezembro/2020) da proposta acima referenciada.

Resumo apresentado pelo pesquisador: "Conforme solicitado, em anexo está o novo cronograma atualizado com a nova previsão de término do projeto".

Nova previsão de término da proposta: **12/2021**

Comentário da CEUA: O Cronograma foi atualizado atendendo a solicitação desta CEUA.

Prof. Dr. Marcelo Bahia Labruna
Coordenador da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Camilla Mota Mendes
Vice-Coordenadora da Comissão de Ética no Uso de Animais
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Folha de Avaliação

Autor: KAYANO, Caroline Yumi

Título: Produção de albumina canina liofilizada para fins terapêuticos

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Data: _____/_____/_____

Banca Examinadora

Prof. Dr._____

Instituição:_____ Julgamento:_____

Prof. Dr._____

Instituição:_____ Julgamento:_____

Prof. Dr._____

Instituição:_____ Julgamento:_____

DEDICATÓRIA

Dedico este trabalho aos meus pais. Muito obrigada por sempre serem meu porto seguro e por todo o amor incondicional.

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“Aprender é a única coisa de que a mente nunca se cansa, nunca tem medo e nunca se arrepende.”

Leonardo da Vinci

RESUMO

KAYANO, C.Y. **Produção de albumina canina liofilizada para fins terapêuticos**. 2021. 103 f. Dissertação (Mestrado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2021.

A albumina é a principal proteína do sangue, exercendo diversas funções no organismo tais como: pressão oncótica, equilíbrio ácido-básico, anticoagulante, entre outras funções. A hipoalbuminemia é comumente encontrada em pacientes críticos e associada a um mau prognóstico tanto na medicina quanto na veterinária, o que torna necessário o uso de uma solução de reposição de albumina. A produção de uma solução de albumina humana já é feita há mais de 50 anos a partir de técnicas de fracionamento do plasma. Na medicina veterinária, as técnicas de fracionamento não são realizadas de rotina e há poucos trabalhos com a utilização dessas técnicas no plasma canino. Apesar de já existir uma albumina canina liofilizada produzida nos EUA, ela não está disponível no Brasil devido aos altos custos de importação. Atualmente, não há trabalhos relacionados aos parâmetros críticos de qualidade da liofilização desta albumina, sendo que há uma demanda no mercado veterinário para seu uso clínico. Sendo assim, este trabalho objetivou o estudo do fracionamento do plasma canino para a purificação da albumina e do seu processo de liofilização. Para o fracionamento do plasma, foram avaliadas três técnicas diferentes utilizando três agentes precipitantes seguidos de cromatografia, com avaliação de rendimento e pureza. Para o estudo de liofilização, foram analisadas três soluções tampão diferentes com 9 formulações cada. O estudo de liofilização englobou a definição das temperaturas críticas de liofilização, e a avaliação pós-liofilização imediata. As três técnicas de fracionamento foram eficazes para a purificação da albumina canina, com a técnica utilizando PEG 6000 combinada com filtração tangencial obtendo o maior rendimento. Na liofilização, a formulação com 5% (p/v) de glicina em tampão com arginina 50 mM foi a que obteve as maiores temperaturas críticas. Outros trabalhos para o aperfeiçoamento na técnica de fracionamento do plasma e sobre a estabilidade a longo prazo da albumina liofilizada são necessários, porém este estudo é um primeiro passo para suprir uma demanda do mercado veterinário de uma albumina canina para uso clínico.

Palavras-chave: Cão. Fracionamento. Plasma. DSC. FDM

ABSTRACT

KAYANO, C.Y. **Canine freeze-dried albumin production for therapeutical purposes**. 2021. 103 f. Dissertação (Mestrado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2021.

Albumin is the main protein of the blood, being responsible for oncotic pressure, acid-basic balance, anticoagulant function, among other functions. Hypoalbuminemia is commonly associated with critically ill patients leading to a poor outcome either in medicine and veterinary. Thus, a replacement solution is needed. Albumin solutions are already being made from human plasma using fractionation techniques for more than 50 years. In veterinary, plasma fractionation is not commonly used, having only a few studies assessing these techniques in canine plasma. Although in the USA, there is already freeze-dried canine albumin, which is not available in Brazil due to the excessive costs of import, there are no studies about the critical parameters for the freeze-drying process, thus there is a demand for canine albumin production for clinical use. Therefore, this study intended to assess the fractionation of canine plasma for albumin purification and its freeze-drying process. For the plasma fractionation, we used three different protocols, with three different precipitating agents followed by chromatography, with the evaluation of yield and purity. For the freeze-drying process, we evaluated three buffer solutions with 9 different formulations each. The freeze-drying critical temperatures were defined for each formulation and immediate post-lyophilization evaluation was performed. From the three fractionation techniques, PEG 6000 combined with tangential filtration had a better yield. For the freeze-drying process, arginine 50 mM buffer with 5% (w/v) glycine formulation presented higher critical temperatures. Further studies are necessary to improve the fractionation process for better yields and purity, and for long-term stability evaluation of the freeze-dried albumin. However, this study is a first approach to supply the demand for canine albumin for clinical use.

Keywords: Dog. Fractionation. Plasma. Lyophilization. DSC. FDM

Sumário

1. INTRODUÇÃO

A albumina é a principal proteína do sangue e um colóide natural. Além disso, é uma das responsáveis pela manutenção da integridade vascular, age como anticoagulante, regula o equilíbrio ácido-básico, tem ação antioxidante e transporta substâncias endógenas (íons, lipídeos, metais) e exógenas (medicamentos) (MAZZAFERRO; RUDLOFF; KIRBY, 2002; MORE; BULMER, 2013; CONNER, 2017). A hipoalbuminemia está presente em muitos pacientes graves, sendo também um preditor para mau prognóstico tanto na medicina quanto na medicina veterinária (HARDIE et al., 1995; CARR; PANCIERA; KIDD, 2002; VINCENT et al., 2003). O tratamento da hipoalbuminemia é focado majoritariamente na resolução da causa base e no suporte nutricional, uma vez que o uso de plasma para a reposição de albumina não é recomendado pela necessidade de um volume grande para este objetivo, porém no caso de doenças crônicas ou incuráveis, é necessária uma solução de reposição de albumina (CONNER, 2017).

Na medicina, técnicas de fracionamento do plasma para a produção de produtos derivados do plasma (como a albumina) já são utilizadas há mais de 50 anos e vem sendo aperfeiçoadas desde então (BURNOUF, 2007), porém, não é uma prática comum na medicina veterinária. Há relatos do uso de albumina humana em cães para reposição, porém seu uso é controverso já que não há completa homologia entre as proteínas (YAMADA et al., 2016) e, quando administrado em cães sadios, houve reações adversas graves já na primeira infusão da solução (COHN et al., 2007; FRANCIS et al., 2007; ADAMANTOS et al., 2009). Mesmo já havendo uma albumina canina liofilizada produzida nos EUA, ela não está disponível no Brasil, portanto, há uma demanda para sua produção nacional.

As soluções de albumina devem ser administradas por via parenteral devido a sua biodisponibilidade. A sua estabilização é melhor mantida quando são liofilizadas, aumentando assim seu tempo de estocagem. Porém a liofilização pode acarretar em degradação da proteína por conta dos processos de congelamento e secagem, sendo necessários estudos de formulações para haver um produto seguro, efetivo e estável (CARPENTER et al., 1997; CHANG; PATRO, 2004). Apesar disso, não há, até o

momento da escrita deste trabalho, nenhum estudo sobre formulações para a liofilização de albumina canina e avaliação de sua performance após o processo.

Portanto, visto a importância da albumina para a homeostasia e levando em conta a demanda existente por uma albumina canina para uso clínico, este trabalho englobou a purificação da albumina a partir do plasma canino e sua posterior liofilização. Sendo apresentados na forma de dois artigos relacionados a seguir.

O primeiro artigo intitulado *Canine cryopoor plasma fractionation for albumin extraction and purification* englobou a parte do uso de técnicas de fracionamento para a purificação da albumina canina, e teve como objetivo aplicar 3 protocolos diferentes de fracionamento do plasma, em escala laboratorial, e obter um protocolo simples e com bom rendimento.

O segundo artigo intitulado *Development of lyophilized canine albumin formulations: influence of buffer system and excipients on critical formulation temperatures and product performance* englobou a avaliação do processo de liofilização da albumina canina purificada oriunda do primeiro artigo, e teve como objetivo avaliar a influência do sistema tampão e dos excipientes usados nas formulações no ciclo de liofilização e performance do produto após o processo.

2. Revisão de literatura

2.1. Hipoalbuminemia

A albumina é a principal proteína no sangue, correspondendo a 50% da proteína total, responsável por aproximadamente 70% da pressão oncótica, com massa molecular de 69 KDa (MAZZAFERRO; RUDLOFF; KIRBY, 2002). Esta proteína é essencial para a homeostase não só por manter a pressão oncótica, mas também por exercer diversas funções no metabolismo tais como: manutenção da integridade vascular, anticoagulante, regulação do equilíbrio ácido-básico e ação antioxidante (MORE; BULMER, 2013). Além disso, a albumina é uma transportadora de substâncias endógenas e exógenas, sendo que alterações em sua concentração alteram a farmacocinética e farmacodinâmica de drogas que se ligam a proteínas. Ou seja, a albumina é um colóide natural que, diferente dos sintéticos, exerce diversas outras funções (VIGANÓ; PERISSINOTTO; BOSCO, 2010; CONNER, 2017).

No cão, a hipoalbuminemia é definida como albumina sérica menor que 3,0 g/dL, porém seus efeitos adversos são observados somente com níveis inferiores a 2,0 g/dL (CONNER, 2017). Os principais sinais de hipoalbuminemia são: edema periférico, ascite e efusão pleural (devido a diminuição da pressão osmótica), hipercoagulabilidade, intolerância a alimentação enteral, prejuízo na cicatrização e aumento ou diminuição da ação de medicamentos que ligam-se a albumina (MAZZAFERRO; RUDLOFF; KIRBY, 2002; SNOW; JUTKOWITZ; BROWN, 2010; CONNER, 2017).

A hipoalbuminemia pode estar associada com a síndrome da resposta inflamatória sistêmica (SRIS), sepse, peritonite, viremia, queimaduras, traumas múltiplos, intermação, neoplasia, doenças hepáticas, insuficiência renal, doença inflamatória intestinal, entre outros quadros (VIGANÓ; PERISSINOTTO; BOSCO, 2010). Ou seja, a hipoalbuminemia é associada a pacientes graves, sendo causada por perda acelerada de albumina através de sangramentos, aumento da permeabilidade vascular e a diluição pela administração de fluidos intravenosos, além da diminuição de sua produção que é associada a doenças crônicas (MAZZAFERRO; RUDLOFF; KIRBY, 2002; VINCENT et al., 2014).

A hipoalbuminemia pode piorar o quadro do paciente já grave por conta dos efeitos adversos citados previamente, além da necessidade de um ajuste de doses de

medicações que tem ligação com a proteína. Na medicina veterinária não há uma diretriz específica para esse ajuste nesses pacientes (THROOP; KERL; COHN, 2004), assim, não é por acaso que a hipoalbuminemia é um marcador de mau prognóstico em animais e em seres humanos (HARDIE et al., 1995; CARR; PANCIERA; KIDD, 2002; TROW et al., 2008; VINCENT et al., 2014; CONNER, 2017). Uma meta-análise no homem, concluiu que a cada 1,0 g/dL de diminuição no nível de albumina sérica o risco de mortalidade aumenta em 1,37 vezes (VINCENT et al., 2003).

O tratamento da hipoalbuminemia é feito principalmente com a resolução da causa de base além de um suporte nutricional adequado. Porém, pacientes com doenças crônicas incuráveis, em que o tratamento da causa base não é suficiente, podem desenvolver sinais secundários a baixa pressão oncótica e necessitam de terapia específica para aumentar a albumina (CONNER, 2017).

O uso de plasma fresco congelado não é recomendado para a reposição de albumina em cães, uma vez que a sua concentração é baixa na bolsa (em média 2,89 g/dL). Assim, um grande volume de plasma é necessário para aumentar significativamente os seus valores no receptor. Para aumentar 0,5 g/dL de albumina sérica, são necessários 20 a 25 ml/kg de plasma (CHAN et al., 2001; WARDROP; BROOKS, 2016; CULLER; IAZBIK; GUILLAUMIN, 2017). Esse grande volume, por sua vez, poderia causar um aumento na pressão hidrostática levando a uma sobrecarga volumétrica no receptor, além dos riscos inerentes de reação pós-transfusional (MATHEWS, 2008; BEER; SILVERSTEIN, 2015). Um estudo retrospectivo sobre o uso de plasma fresco congelado na rotina de um hospital veterinário feito por Snow, Jutkowitz e Brown (2010) observou que pacientes que receberam plasma na dose de 15 a 18 ml/kg não tiveram diferença significativa na concentração sérica de albumina pré e pós-transfusão.

Um estudo retrospectivo de Culler e colaboradores (2017) avaliou o uso de plasma criopobre para a reposição de albumina, já que esse hemoderivado possui uma concentração maior de albumina comparado ao plasma fresco congelado (em média 3,17 g/dL) (CULLER; IAZBIK; GUILLAUMIN, 2017). Porém, ainda foi necessário 31 ml/kg de plasma criopobre em infusão contínua por 16 horas para a elevação de 0,5 g/dL de albumina sérica. Em um relato de caso, Ropski e colaboradores (2017) utilizaram, para um cão de 44 kg, um total de 58 unidades de plasma criopobre ao longo de 9 dias em infusão contínua para a manutenção dos níveis de albumina sérica. Portanto, ainda há o uso de um grande volume de plasma e do risco de reação pós-transfusional. Além disso,

o plasma criopobre só é produzido em bancos de sangue veterinários especializados, não sendo um produto prontamente acessível normalmente.

Soluções de albumina humana são produzidas a partir do plasma há mais de 50 anos, sendo o colóide mais utilizado de todas as soluções biofarmacêuticas produzidas do mundo (MATEJTSCHUK; DASH; GASCOIGNE, 2000). O uso dessas soluções na medicina é principalmente para a reposição volêmica além da correção de uma hipoalbuminemia importante (CAIRONI; GATTINONI, 2009; CARACENI et al., 2013; CAIRONI et al., 2014; ADAMIK; YOZOVA, 2021). Outros usos para estas soluções são: em casos de cirrose hepática, em que a albumina é indicada por conta de sua propriedade antioxidante, ação imunomoduladora, e função de proteção endotelial (ANGELI et al., 2018); em preparação de circuitos de circulação extracorpórea de cirurgias cardiopulmonares; em síndrome nefrótica; e em pancreatite (CARACENI et al., 2013). Em contraste, na medicina veterinária, o uso de soluções de albumina é feito principalmente para o aumento ou manutenção das concentrações de albumina sérica (ADAMIK; YOZOVA, 2021).

Há relatos do uso de albumina humana em cães (MATHEWS, 2008; TROW et al., 2008; VIGANÓ; PERISSINOTTO; BOSCO, 2010), porém essa prática é bastante controversa, já que a homologia estrutural entre as albuminas das duas espécies é de somente 79,8% (YAMADA et al., 2016). Isso pode levar a reações de hipersensibilidade, que inclusive já foram relatados em cães sadios que receberam infusão de solução de albumina humana, sem uma exposição prévia, que acarretou em reações adversas graves e posterior formação de anticorpos anti-albumina humana (COHN et al., 2007; FRANCIS et al., 2007; ADAMANTOS et al., 2009). Além disso, Mazzaferro e colaboradores (2020) relataram o óbito de 2 pacientes caninos secundário a reação de hipersensibilidade tipo 3 (tardia) após infusão de albumina humana para correção de hipoalbuminemia causada por peritonite séptica.

Até o momento da escrita deste trabalho, há dois estudos clínicos prospectivos, utilizando albumina canina liofilizada, produzida pelo Animal Blood Resources International, um banco de sangue veterinário particular norte-americano. Craft e Powell (2012) realizaram a infusão de albumina canina em 14 cães com peritonite séptica, sendo que ela se mostrou segura e eficaz para aumentar a pressão coloidosmótica, os níveis de albumina e a pressão diastólica após 2 horas da infusão. O segundo estudo, de Enders e colaboradores (2018), apresentado somente em forma de resumo, avaliou a infusão

repetida da albumina canina por 3 dias, em 6 cães da raça Beagle saudáveis, os quais foram acompanhados por 28 dias pós-infusão. Com este estudo, concluiu-se que as infusões repetidas foram seguras e não houve efeitos adversos. Estudos avaliando o uso dessa solução em grupos maiores de pacientes e em outros quadros clínicos ainda precisam ser realizados.

2.2. Purificação da albumina

O plasma humano coletado pode ser utilizado tanto como produto terapêutico (plasma fresco congelado), quanto como matéria-prima para a produção de produtos farmacêuticos fracionados, que são chamados de produtos do plasma ou derivados do plasma. O processo de isolamento de proteínas plasmáticas para uso terapêutico é denominado fracionamento (BURNOUF, 2007).

As técnicas por precipitação são as mais utilizadas devido ao seu baixo custo e facilidade de manuseio. A primeira preparação de uma solução intravenosa de albumina humana a partir do plasma foi desenvolvida pela técnica de precipitação por etanol frio, denominado método de Cohn, sendo essa técnica a mais utilizada em produções de larga escala e industrial atualmente (COHN et al., 1946; KISTLER; FRIEDLI, 1980; RAOUFINIA et al., 2016).

Neste método, em temperaturas de -3ºC a -5ºC, a precipitação ocorre pela interação do etanol com a água, causando um efeito de desidratação na superfície de moléculas das proteínas. Conforme a alteração de concentração de etanol e pH, há uma precipitação seletiva de proteínas. Ao longo dos anos esse método foi sendo modificado de acordo com as proteínas de interesse, sendo cada método modificado recebendo uma numeração diferente mas mantendo-se o nome original do método (KISTLER; FRIEDLI, 1980; VAN OSS, 1989).

As vantagens do método de Cohn são relacionadas ao uso do etanol que apresenta um baixo custo, é um material de fácil acesso, além de inibir o crescimento bacteriano e consequentemente, a formação de substâncias pirogênicas. Porém, suas desvantagens consistem em não ser possível obter um bom rendimento devido as várias etapas de precipitação em que é necessário um controle muito preciso da temperatura (KISTLER; FRIEDLI, 1980; RAOUFINIA et al., 2016).

Outro agente precipitante que pode ser utilizado são os sais de amônio por não causarem danos em proteínas lábeis ou enzimas. A técnica baseia-se no *salting-out*, ou

seja, os íons do sal quando solubilizados deslocam as moléculas de água da superfície das proteínas fazendo-as precipitarem. Porém esses sais são tóxicos e devem ser removidos de soluções de uso intravenoso, sendo necessário uma etapa posterior de cromatografia por troca iônica, ou de afinidade ou filtração em gel para a dessalinização. O sulfato de amônio é frequentemente usado em uma etapa inicial do fracionamento do plasma para obter uma fração rica da proteína desejada (STEINBUCH, 1980).

Há também a técnica de precipitação com polietilenoglicol (PEG), um polímero sintético não-tóxico solúvel em água usado como agente crioprotetor (HUBÁLEK, 2003). Por não apresentar efeito desnaturante nas proteínas do plasma, é utilizado para a purificação de gama-globulina, fibrinogênio e também para a purificação em larga escala de albumina (POLSON; RUIZ-BRAVO, 1972; JIMENEZ et al., 1974; SCHNEIDER et al., 1975; FALKSVEDEN; LUNDBLAD, 1980). O princípio da precipitação com PEG ainda não é completamente elucidado. É sugerido que o polímero realiza um processo de exclusão das proteínas de uma solução reduzindo a quantidade de água disponível para a sua solvatação, o que faz com que a concentração da proteína aumente e ocorra a sua precipitação (HAO; INGHAM; WICKERHAUSER, 1980).

Diferente da técnica de Cohn, a precipitação com sais de amônio e com PEG ocorrem em temperatura ambiente, não sendo necessário um controle tão rígido como ocorre no primeiro. Além disso, o período de tempo entre a precipitação e a centrifugação para a separação do precipitado são menores com os sais de amônio e o PEG. Na técnica de Cohn é necessário deixar algumas horas a suspensão em homogeneização para haver a completa precipitação, enquanto que, com os outros agentes, a precipitação está completa em alguns minutos (HAO; INGHAM; WICKERHAUSER, 1980; STEINBUCH, 1980; ODUNUGA; SHAZHKO, 2013).

Atualmente, as técnicas mais utilizadas no fracionamento do plasma tem sido a combinação da precipitação com cromatografia (RAOUFINIA et al., 2016). Visto a necessidade da retirada dos agentes precipitantes da solução de albumina, a combinação com a cromatografia é vantajosa por conseguir retirar o sulfato de amônio, o etanol e o PEG da solução além de originar um produto com maior grau de pureza, sendo uma etapa de polimento no fracionamento (CURLING et al., 1977).

A cromatografia é utilizada no fracionamento do plasma desde 1960, havendo 4 motivos principais para sua utilização: melhora da pureza dos produtos, extração de

proteínas lábeis, otimização da recuperação de proteínas e remoção de agentes anti-virais, estabilizantes e sais (JOHNSTON; ADCOCK, 2000; BURNOUF, 2007). Há diversos tipos de cromatografia que podem ser utilizadas no fracionamento do plasma, a seguir são explicados brevemente duas das técnicas utilizadas neste estudo.

A cromatografia por permeação em gel separa as proteínas com base na diferença das massas moleculares e sua estrutura, sendo que não há interação com a resina cromatográfica. As proteínas maiores passam mais facilmente pela resina enquanto as menores (como sais e outras impurezas) passam mais lentamente, havendo assim uma separação. Ou seja, essa técnica é utilizada para a remoção de sais das proteínas, troca de solução tampão e separação de agregados (PORATH; FLODIN, 1959; JOHNSTON; ADCOCK, 2000).

A cromatografia por troca iônica utiliza-se do princípio de separação de íons e moléculas polares com base na afinidade com a resina da coluna trocadora de íons. Sendo que, como a maioria das proteínas do plasma fica com carga negativa em pH neutro para a proteção de atividade biológica, geralmente são usadas colunas aniônicas (BURNOUF, 1995). Após carregar a solução com as moléculas que se quer separar na coluna é feito a eluição com outra solução com quantidades crescentes de sal que competem com a ligação entre a resina e as moléculas, as com ligação mais fraca serão eluídas primeiro havendo a separação das moléculas presentes. Assim, a cromatografia por troca iônica usada sozinha não é vantajosa, uma vez que o plasma total apresenta uma alta carga iônica, o que dificulta a separação da albumina, sendo necessário a combinação de outras técnicas de fracionamento ou diferentes cromatografias. (CURLING et al., 1977).

Mesmo com as diversas técnicas de fracionamento do plasma já existentes, poucos são os trabalhos utilizando-as na medicina veterinária. Meral e colaboradores (2018) realizaram a purificação de albumina canina e felina a partir do plasma de cada espécie usando a combinação da cromatografia por troca iônica e do método de Cohn, obtendo ao final uma solução de albumina com grau de pureza maior que 95%. Além desse estudo, há 3 patentes sobre métodos usados para a purificação de albumina canina: a partir de plasma utilizando o método de Cohn (LU et al., 2004) e sulfato de amônio (LEE et al., 2012); e a partir de líquido ascítico canino utilizando PEG combinado com cromatografia por troca iônica (HASEGAWA; TAKENAKA, 2005), sendo que todas obtiveram um grau de pureza maior que 95%. Porém em nenhum desses trabalhos relatou-se o rendimento obtido ao final do fracionamento.

2.3. Liofilização

Soluções com proteínas tem uma baixa biodisponibilidade, sendo necessário a utilização de via parenteral para sua administração. Porém, formulações de proteínas em forma líquida são muito susceptíveis a degradação química (deaminação ou oxidação) e/ou física (agregação e precipitação). Por estes motivos, a liofilização é um método usado para manter a estabilidade da proteína, manter a rota parenteral de administração e aumentar o tempo de prateleira do produto (CARPENTER et al., 1997; CHANG; PATRO, 2004). A liofilização é uma operação de estabilização e secagem a baixa temperatura e pressão no qual a remoção de água ocorre por meio da sublimação, resultando assim em um material com atividade de água reduzida e alta estabilidade para estocagem (ABDELWAHED et al., 2006).

A liofilização é feita em três estágios básicos. O primeiro é o congelamento, que envolve a criação de uma matriz sólida apropriada para a secagem. O segundo é a secagem primária que envolve a remoção dá água por sublimação (do estado sólido diretamente para o gasoso), por meio da redução da pressão ambiente enquanto se mantém a baixa temperatura do produto. O terceiro é a secagem secundária, em que a água restante é removida até que a atividade de água no material alcance o nível desejado (geralmente abaixo de 2%) (CARPENTER et al., 2002; CHANG; PATRO, 2004).

A secagem primária pode ser precedida, em alguns casos, por uma etapa de *annealing*, que consiste no aquecimento acima da temperatura de transição vítrea e posterior resfriamento do material. O *annealing* tem como objetivos: a cristalização de agentes de *bulking*, como manitol e glicina que podem estar presentes na formulação; e a reorganização dos cristais de gelo na matriz, através do processo de amadurecimento de Ostwald em que cristais de gelo maiores são formados em detrimento dos cristais menores, facilitando o processo de sublimação durante a secagem primária (SEARLES; CARPENTER; RANDOLPH, 2001; CHANG; PATRO, 2004).

A liofilização pode acarretar em desnaturação da proteína, sendo a etapa de congelamento considerada a mais agressiva, podendo levar a desnaturação e agregação da proteína (WANG, 2000). Por isso, são necessários estudos de formulação para a obtenção de um bom produto final. A formulação deve ser capaz de garantir a preservação das características críticas do produto final (relacionadas à qualidade, segurança e eficácia do produto), assim como deve ser capaz de gerar uma apresentação farmacêutica elegante. Carpenter e colaboradores (2002) propuseram um modelo de formulação com 5

elementos: uma solução tampão que não se acidifica durante o congelamento, um pH específico que otimize a estabilidade termodinâmica do proteína, um estabilizante (lioprotetor), um agente de *bulking*, e um surfactante não iônico.

Cada elemento da formulação pode afetar os parâmetros críticos do processo, por isso as análises de pré-liofilização são importantes na obtenção de uma formulação ideal com os melhores parâmetros possíveis. Açúcares (principalmente os dissacarídeos como sacarose e trealose) são utilizados como estabilizantes universais por sua ação de crio e lioproteção. A própria albumina é um polímero que tem ação de crio e lioproteção sendo usada como excipiente em vários produtos farmacêuticos (ARAKAWA et al., 1993; WANG, 2000). Os agentes de *bulking* mais comuns são agentes cristalinos como a glicina e o manitol. Ambos são muito utilizados nas formulações para liofilização por formarem pastilhas mais robustas e elegantes com dissolução mais rápida (CARPENTER et al., 1997).

Nas análises pré-liofilização serão definidas as temperaturas críticas que compreendem: a temperatura de transição vítrea (T_g'), temperatura de fusão eutética (T_{eut}) e temperatura de colapso (T_{col}) de cada formulação. A seguir são definidas cada uma dessas temperaturas.

A T_g' é definida como a temperatura na qual ocorre a vitrificação do material amorfo. Nesta temperatura, o material amorfo adquire características típicas de sólidos, havendo pouca mobilidade molecular. Em uma temperatura acima do T_g', o movimento molecular é aumentado, facilitando a deformação do material. Desta forma, há uma forte correlação com a temperatura em que a pastilha congelada colapsa. Ou seja, é um ponto crítico que, durante a secagem primária, geralmente, deve-se manter a temperatura da amostra abaixo da T_g' da formulação para evitar o colapso. Entretanto, é desejável uma formulação com um T_g' o mais elevado possível para não haver um tempo prolongado de liofilização, sendo a temperatura ideal acima de -40,0ºC (CARPENTER et al., 2002; CHANG; PATRO, 2004).

A T_{eut} é a temperatura máxima na qual o material só existe em forma sólida, ou seja, a temperatura mínima de fusão. Essa temperatura só é encontrada em formulações contendo agentes cristalinos. Deve-se manter a temperatura do produto abaixo da T_{eut} durante a secagem primária para evitar o seu *meltback* (CHANG; PATRO, 2004).

A T_{col} é definida como a temperatura em que ocorre o colapso da pastilha. O colapso é definido como o processo pelo qual a estrutura criada durante a liofilização é aniquilada com a passagem da interface de sublimação, deixando uma aparência inaceitável e resultando em propriedades indesejáveis (já que há o bloqueio de vias pelas quais a água poderia sair, causando uma menor taxa de sublimação ou inviabilizando a remoção da água). Assim, há uma retenção maior de umidade em comparação com um produto liofilizado sem colapso, sendo que a água pode estar distribuída desigualmente pela amostra. Isso pode causar também um maior tempo de reconstituição pela perda de porosidade e redução da área de contato do liofilizado, além de poder causar uma perturbação da estrutura proteica (CHANG; PATRO, 2004). A T_{col} não necessariamente é próxima da T_g', o colapso pode ocorrer em temperaturas acima da T_g'. Colandene e colaboradores (2007) relataram uma T_{col} até 15 graus maiores que a T_g' em formulações com altas concentrações de proteína.

Sendo assim, para um bom processo de liofilização, deve-se respeitar o limite das temperaturas críticas afim de não haver sinais de colapso ou *meltback* no produto final. Porém a formulação ideal deve também ter as temperaturas críticas maiores possíveis já que isso resulta em um ciclo com menor duração (CHANG; PATRO, 2004).

A combinação das análises de Calorimetria Exploratória Diferencial (DSC) e de microscopia ótica acoplada a liofilização é utilizada para a determinação das temperaturas críticas para a liofilização (SACHA; NAIL, 2009). A T_g' e a T_{eut} podem ser determinadas pela análise de DSC. Esta técnica termo analítica monitora a variação de entalpia sob uma variação controlada de temperatura entre a solução analisada e um material inerte termicamente que serve como referência, sendo possível determinar a natureza física de um evento térmico e a temperatura em que ele ocorre. A microscopia, por sua vez, possibilita uma visualização em tempo real dos eventos da liofilização e seu significado prático com definição da T_{col} (CHEN; OAKLEY, 1995; SACHA; NAIL, 2009).

Após a liofilização também devem ser observados alguns parâmetros. Neste estudo, foram avaliados a umidade residual, aparência da pastilha, tempo de reconstituição e integridade estrutural da albumina por meio de gel de eletroforese SDS-PAGE e espectroscopia Raman. A umidade residual é um parâmetro importante para a estabilidade da proteína a longo prazo, sendo que geralmente não deve exceder 2% (WANG, 2000). A aparência da pastilha deve ser avaliada pois uma perda estrutural visível pode afetar tanto a estabilidade quanto o tempo de reconstituição, podendo até

afetar a umidade residual (WARD; MATEJTSCHUK, 2010). A espectroscopia Raman é uma técnica não invasiva que auxilia na detecção de mudanças conformacionais na estrutura secundária da proteína, sendo possível comparar a estrutura antes e após a liofilização (PIETERS et al., 2013).

A liofilização é um processo que envolve muitas variáveis que podem levar a degradação da proteína por conta do estresse causado pelo congelamento e desidratação durante o processo. Assim, são necessárias análises pré e pós liofilização para haver o desenvolvimento de um ciclo de liofilização eficiente para um produto final de qualidade. Porém, mesmo havendo a comercialização nos EUA de uma albumina canina liofilizada, até o momento da escrita deste trabalho, não há nenhum estudo acerca dos parâmetros críticos para a sua liofilização.

Capítulo 1

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3. Canine cryopoor plasma fractionation for albumin extraction and purification

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Abstract

Albumin is the main protein in the plasma, having several functions that surpass its role as an oncotic pressure regulator. Hypoalbuminemia is associated with poor outcomes in critically ill patients, making a supplementing solution necessary. Even though freeze-dried canine albumin is commercially offered from Animal Blood Resources Internacional, this product is not easily accessible worldwide due to the excessive import costs and currency exchange losses. Thus, there is a demand for a canine albumin solution for veterinary use. Techniques for human plasma fractionation are available for more than 50 years to produce therapeutic derivatives (including albumin), but these techniques are rarely used for veterinary purposes. In this study, we evaluated 3 different protocols of canine plasma fractionation (precipitation technique followed by chromatography) for albumin harvest and purification from cryopoor plasma. All evaluated techniques were effective. Cohn's method had a yield of 0.11g ± 0.1 of albumin per dL with 95.1% ± 2.8 of purity. Ammonium sulfate precipitation had a yield of 1.06 ± 0.37 of albumin per dL with 79.2% ± 8.1 of purity. PEG precipitation combined with ultrafiltration had a yield of 0.56 ± 0.01 of albumin per dL with 89.4% ± 4.2 of purity, while PEG precipitation

combined with tangential flow filtration had a yield of 1.53 ± 0.1 g of albumin per dL with 81% ± 14.2 of purity. This approach represents our first step to the development of plasma derivative products for veterinary purposes which is a field not yet explored.

Keywords: dog, precipitation, ethanol, ammonium sulfate, polyethylene glycol, chromatography

3.1. Introduction

Albumin is the main protein in the plasma, and it is responsible for most of the blood's oncotic pressure. This 69 kDa protein corresponds to more than 50% of the total amount of plasmatic proteins (Mazzaferro et al., 2002). Besides oncotic pressure regulation, albumin also contributes to vascular integrity, anticoagulation, acid-basic regulation, antioxidation and is an important carrier for endogenous (e.g. ions and lipids) and exogenous substances (e.g. drugs) (Conner, 2017; More and Bulmer, 2013). Thus, it is not surprising that hypoalbuminemia is commonly associated with critically ill patients and that those patients have a poorer outcome in both human and veterinary medicine (Trow et al., 2008; Vincent et al., 2003).

In general, hypoalbuminemia can be caused by low albumin production rates, accelerated protein loss, or a combination of both. In dogs, it occurs when the serum albumin level is below 3.0 g/dL. However, the adverse events in dogs (such as peripheral edema, ascites, pleural infusion) are only perceived at a level below 2.0 g/dL. The treatment is focused on the resolution of the disease responsible for hypoalbuminemia and proper nutrition support. Although for chronic or incurable diseases a proper albumin reposition would be required (Conner, 2017). Plasma transfusion is no longer recommended for albumin reposition because high volumes of plasma are required for this purpose. For instance, in general, an increase of 0.5 g/dL of albumin in dogs requires 20 to 25 mL/Kg of plasma (Wardrop and Brooks, 2016). The total transfused volume can cause hypervolemia (volume overload) in the recipient and represent high transfusion reaction risks (Beer and Silverstein, 2015; Mathews, 2008).

There are reports of albumin reposition in dogs using human serum albumin (HSA) although this use is quite controversial (Mathews, 2008; Trow et al., 2008; Viganó et al., 2010). Canine and human albumin have a structural homology of only 79.8% (Yamada et al., 2016) and severe transfusion reactions were observed in healthy dogs even after the first transfusion procedure (Adamantos et al., 2009; Cohn et al., 2007;

Francis et al., 2007). Also, death secondary to delayed-type III hypersensitivity reaction caused by HAS use in critically ill dogs was reported (Mazzaferro et al., 2020). The USA has a commercially available freeze-dried canine serum albumin (CSA) for veterinary medicine produced by Animal Blood Resources. This product is referred to in two scientific reports, one by Craft and Powell (2012) and, the other, by Enders and colleagues (2018). Currently, this product is not easily accessible worldwide, due to the excessive cost of import and currency exchange losses. Thus, there is a demand for a canine albumin solution for veterinary use.

Since World War II when Cohn and colleagues (1946) developed a method for albumin purification with ethanol (Cohn's method), the fractionation of plasma is used for the production of therapeutic plasma derivatives such as albumin, clotting factors, and immunoglobulins. Since then, different techniques were developed for the improvement of fractionation especially aiming for better yields, with the most common techniques used for albumin purification being Cohn's method and chromatography (Burnouf, 2007). Although plenty of techniques are available for plasma fractionation, they are not commonly used in veterinary medicine.

Albumin has a crucial role in the circulatory system and on homeostasis however, albumin products are scarcely available for its replacement in veterinary practice. Therefore, the present study compares 3 techniques of plasma fractionation from canine cryopoor plasma (using a precipitation agent followed by ion-exchange chromatography). We intended to evaluate those techniques for albumin purification to be used in the veterinary hemotherapy of dogs, comparing the final yield and purity of albumin in a laboratory setting, for this reason, our study did not intend to evaluate the cost of the plasma fractionation procedure.

3.2. Material and Methods

3.2.1. Ethics statement

The study was conducted under the approval of the Ethics Committee on Animal Use [Institutional Animal Care and Use Committee (IACUC)] of the School of Veterinary Medicine and Animal Science (CEUA/FMVZ) of the University of São Paulo (USP) (protocol 400013218). The biological material was kindly provided by the Laboratory of Hemotherapy of the Surgery Department of the FMVZ-USP. The laboratory procedures

were performed in the Laboratory of Immunobiologicals and Biopharmaceuticals of the Department of Pharmaceutical Technology of the School of Pharmaceutical Sciences (FCF) of the USP.

3.2.2. Cryopoor plasma bags processing

Canine fresh frozen plasma bags were provided by healthy canine volunteer donors of the Laboratory of Hemotherapy. All plasma bags were stored at -30ºC until use. The donor selection, blood collection, and blood bag processing were performed according to the standard operational procedure protocols of the Laboratory of Hemotherapy (Rodrigues et al., 2020).

To produce the cryopoor plasma, the fresh frozen plasma bags with volumes above 180 mL with no sign of hemolysis and/or lipemia were used. The bags were kept frozen for at least 2 days. After this period, the bags were thawed at 4ºC for 8 hours and centrifuged at 3100*g* for 30 minutes at 4ºC (RC-3BP model, Sorvall, Thermo Scientific, MA, USA). The supernatant (cryopoor plasma) was separated from the precipitate (cryoprecipitate) aseptically. According to the current Brazilian legislation, when stored at -30ºC the cryoprecipitate has a shelf-life of 1 year and the cryopoor plasma of 5 years from the date of the original plasma collection (Brasil, 2010).

3.2.3. Plasma fractionation

The plasma fractionation was evaluated by three different protocols: ethanol (Cohn's method), ammonium sulfate, and polyethylene glycol (PEG) precipitation. All protocols were followed by ion-exchange chromatography. The fractionation protocols were evaluated in cryopoor plasma with less than a year of storage. All fractionation techniques were first analyzed in a small-scale setup (20 mL of cryopoor plasma) to verify albumin yield and purity and to compare the technical advantages and feasibility of each protocol. After this analysis, the best-evaluated protocol was selected to be studied in a larger setup (300 mL of cryopoor plasma) using tangential flow filtration followed by ion-exchange chromatography.

After each fractionation step of the three different protocols, the precipitated material was harvested by centrifugation at 2,570 *g* for 30 minutes at 25ºC (the Cohn's method had variable centrifugation temperature as is established by the technique). Both supernatants and precipitates from each step were subjected to an SDS-PAGE

chromatographic analysis using a 12.5% polyacrylamide gel and a molecular weight marker (LMW Calibration Kit for SDS Electrophoresis, GE Healthcare, SP, Brazil). The total amount of protein was measured by Bradford protein assay. If necessary, pH adjustments were performed with 1M hydrochloric acid or 1M sodium hydroxide.

3.2.3.1. Cohn's method

The plasma fractionation was performed using a modified Cohn's method 6 (Cohn et al., 1946) as indicated in Figure 1. We opted to use a modified method of Cohn 6 starting from the 3rd fractionation step because previous steps are destined to separate proteins that were already removed from the cryopoor plasma. We intitled the last two supernatants as supernatant VI and VII for better identification.

The temperature was maintained by an ice bath and constantly measured by an electronic contact thermometer (ETS-D5 model, Ika, Staufen, Germany). Absolute ethanol (LABSYNTH Produtos para Laboratório Ltda, SP, Brazil) was used to achieve the required ethanol concentrations. In each step, the solutions were kept under adequate pH, ethanol concentration, and temperature for 30 minutes under constant stirring.

3.2.3.2. Ammonium sulfate precipitation

Ammonium sulfate fractionation was performed by adding a saturated ammonium sulfate (LABSYNTH Produtos para Laboratório Ltda, SP, Brazil) solution to the cryopoor plasma. The final ammonium sulfate concentration in the mixture was 30% (w/v). The pH of the solution was adjusted to 6.6 and the solution was kept under constant stirring for 30 minutes at room temperature. After this period, the solution was centrifuged to separate the supernatant containing the albumin from the precipitated material.

3.2.3.3. PEG precipitation

The PEG fractionation was achieved by adding a 26% (w/v) PEG 6000 (Sigma-Aldrich, MO, USA) solution to the cryopoor plasma. The final PEG 6000 concentration was 13% (w/v). The pH was adjusted to 7.0 and the solution was kept under stirring for 30 minutes at room temperature. After this period, the solution was centrifuged to separate the supernatant containing the albumin from the precipitated material.

The PEG 6000 removal was performed by ultrafiltration. To this purpose, the albumin solution was diluted in phosphate buffer (pH 8.0) to 10 times its initial volume. The solution was then ultracentrifuged using a 30 kDa filtering membrane device

(Amicon Ultra-15 Centrifugal Filter Units, Millipore, MA, USA). All the filtrate from the filtering membrane was evaluated by Bradford protein assay and SDS-PAGE electrophoresis for protein presence.

3.2.3.4. Chromatography

After each fractionation protocol, the albumin samples were desalted and buffer-exchanged by gel filtration followed by ion-exchange chromatography (IEC) using FPLC (Fast Protein Liquid Chromatography) equipment (AKTA Start, GE Healthcare, SP, Brazil). The eluted fractions were identified by UV absorption at 280 ηm using the UNICORN 7.0 software (GE Healthcare, SP, Brazil). The absorption at 280 ηm can detect mainly tryptophan and tyrosine amino acid residues (and phenylalanine to a minor extent). The salt content was identified by the change in the conductivity.

The desalting and buffer exchange was performed using a manually packed Sephadex G-25 column (PD-10 Desalting Columns, GE Healthcare, SP, Brazil) to remove the residual ethanol, ammonium sulfate, or PEG 6000 from the albumin samples, and exchange the buffer for 25 mM sodium phosphate buffer pH 8.0 (**buffer A**). The column was equilibrated with the elution buffer (buffer A) with 2 times the volume of the column before the injection of the sample, a volume sample of 1 mL was injected in the column at each cycle. The chromatography was performed under 2 mL/minute flow and the eluted fractions that displayed absorption at 280 ηm before the change of conductivity were collected.

After the desalting, the samples were submitted to IEC for further purification. This chromatography was performed using a manually packed Q Sepharose "*fast flow*" column (Q Sepharose Fast Flow, GE Healthcare, SP, Brazil), a strong anion exchanger, previously equilibrated with buffer A. After the injection of 1 ml of the sample, the column was washed with buffer A (with 1.5 times the volume of the column) at 5 mL/minute to remove the unbound fraction. The bound albumin fraction was then eluted with 0.5 M sodium chloride 25 mM sodium phosphate buffer pH 8.0 (**buffer B**).

To standardize the IEC, a gradient from 0% to 100% of buffer B was used. The gradient was applied at a flow rate of 5 mL/minute using 10 times the total volume of the column. The percentages of buffer B that promoted the elution of the sample fractions were annotated and used to create a standard stepwise IEC. The buffer B percentages were 42%, 54%, 65%, and 100%. This standardized stepwise chromatography using 2

times the volume of the column and 5 mL/minute flow was used to purify all samples acquired with the fractionation methods. The fractions that were recognized by absorbance at 280 ηm were intitled according to the percentage of buffer B of the elution.

3.2.3.5. Tangential flow filtration

After the evaluation of the three different fractionation protocols, the polyethylene glycol method was selected to be studied in a larger scale setting (300 mL of cryopoor plasma). For this purpose, after the precipitation with PEG and the obtention of the albumin fraction, the sample was diluted to 10 times its volume using the buffer A solution. The diluted samples were then submitted to the tangential flow filtration using a 30 kDa membrane (Sartocon Slice Cassette, Sartorius Stedim, GO, Germany) and a peristaltic pump (Sartorius Sartoflow Scilog Peristaltic Bomb Tandem model 1082, Sartorius Stedim, GO, Germany). After the filtration, the samples were further purified using IEC and concentrated with tangential flow filtration technique. Different from the previous PEG fractionation protocol, in this study, the samples were not submitted to desalting.

3.2.4. Albumin yield and purity assessment

The total protein amount in each sample was measured by Bradford assay. The SDS-PAGE electrophoresis gel images were analyzed by image densitometry (ImageJ, MD, USA) to determine albumin purity (Villela et al., 2020). Albumin purity was assessed by the ratio between albumin and total protein in the samples. The total albumin yield was calculated by dividing the final obtained mass of albumin by the initial amount of cryopoor plasma used. The purity index was assessed by the ratio of the final albumin purity compared to the initial albumin purity, the total purity index was assessed by the ratio of the final albumin purity and the cryopoor plasma percentage of albumin by the total protein. The percentage of the total protein loss and albumin loss was assessed by the ratio of the difference between the final and initial protein or albumin mass and the initial mass. Data is shown by means ± standard deviation and the graph of albumin yield was made using Prism (GraphPad, CA, USA).

3.3. Results

3.3.1. Cohn's method and chromatographic purification

The method was repeated three times in a small-scale setup. Samples were collected after each precipitation step and analyzed by SDS-PAGE (Figure 2). At the end of Cohn's method precipitation, the precipitated containing albumin was resuspended in buffer A. The protein sample was then submitted to desalting and the FPLC results are shown in Figure 3. In the figure, the collected eluted fraction is indicated by a black border rectangle.

For the first IEC, a half milliliter of the sample after desalting was submitted to a gradient ion-exchange chromatography to standardize the stepwise protocol. This standard protocol was used for all the samples. The results are shown in Figure 4.

Based on the gradient results the stepwise ion-exchange chromatography was standardized using the concentrations of 42%, 54%, 65%, and 100% of buffer B. The stepwise chromatography results are shown in Figure 5.

A total of 5 samples were collected after the ion-exchange chromatography for further analyses, those samples were eluted at: (I) 0% buffer B; (II) first peak at 42% buffer B; (III) second peak at 42% buffer B, (IV) 54% buffer B; and (V) 100% buffer B, the column was eluted with 2 times the column volume with each percentage of buffer B. No peak was found in 65% of buffer B, therefore, the stepwise used only concentrations of 42%, 54%, and 100% of buffer B. All 5 samples were analyzed by SDS-PAGE and the results are shown in Figure 6.

The SDS-PAGE electrophoresis evinced that the fraction with the highest albumin content was at the second eluted fraction of 42% of buffer B, corresponding to a NaCl concentration of 210 mM.

3.3.2. Ammonium sulfate fractionation and chromatographic purification

The method was repeated three times in a small-scale setup. After the precipitation with the ammonium salt, the supernatant and the precipitate were analyzed by SDS-PAGE (Figure 7). After the precipitation, the supernatant containing albumin was then submitted to desalting followed by IEC, using the FPLC equipment (Figure 8 and Figure 9, respectively).

A total of 5 samples were collected after IEC for further analyses, those samples were eluted at: (I) 0% buffer B; (II) first peak at 42% buffer B; (III) second peak at 42% buffer B, (IV) 54% buffer B; and (V) 100% buffer B. All 5 samples were analyzed by SDS-PAGE and the results are shown in Figure 10. The electrophoresis indicated that the second peak at 42% buffer B contained most of all albumin from the initial sample.

3.3.3. Polyethylene glycol fractionation and chromatographic purification

The method was repeated two times in a small-scale setup. After precipitation with PEG 6000, the supernatant containing albumin was submitted to centrifugation with a 30 kDa filtering membrane device to remove the PEG 6000 after the precipitation. After each step, a sample was collected and analyzed by SDS-PAGE electrophoresis (Figure 11). The Bradford assay and SDS-PAGE electrophoresis did not show a protein loss in the filtrate from the 30 kDa filtering membrane device.

After the removal of polyethylene glycol, the samples were then submitted to desalting using the FPLC equipment (Figure 12). After desalting, the eluted fraction was submitted to the IEC (Figure 13). All eluted samples from the ion-exchange chromatography were analyzed by SDS-PAGE. The results are shown in Figure 14. The electrophoresis analysis indicated that most albumin was eluted in the second peak at 42% of buffer B.

Figure 15 shows the SDS-PAGE electrophoresis of all samples after the three fractionation protocols and subsequent chromatographies.

3.3.4. Polyethylene glycol fractionation with tangential flow filtration

After the evaluation of the three different fractionation protocols, the polyethylene glycol method was selected to be studied in a larger-scale setting. This method was repeated two times using a whole bag of cryopoor plasma (300 mL) each. First, the cryopoor plasma was submitted to the PEG 6000 fractionation followed by tangential flow filtration to concentrate the sample and remove PEG. The samples were further purified by IEC and concentrated with tangential flow filtration. The supernatant of each step was collected and analyzed by SDS-PAGE electrophoresis (Figure 16).

3.3.5. Albumin yield and purity assessment

The evaluated parameters were: the initial and final albumin concentrations, the total protein ratio from the cryopoor plasmas, albumin purity, purity index, total process purity index, albumin loss, and protein loss. All the parameters before and after each step are displayed in Figure 17 and Tables 1 to 3.

The yield for Cohn's method combined with chromatography was 0.11 ± 0.1 g of albumin per dL of plasma, for ammonium sulfate precipitation with chromatography was 1.06 ± 0.37 g of albumin per dL of plasma, for PEG 6000 combined with filtering membrane device and chromatography was 0.56 ± 0.01 g of albumin per dL of plasma, and for PEG 6000 combined with tangential filtration and chromatography was 1.53 ± 0.1 g of albumin per dL of plasma (Figure 17).

From Figure 17, it is observed an important loss in albumin concentration by Cohn's method after the precipitation. This loss can be seen in Table 1 data that shows an albumin loss of more than 90%. This was not observed with the ammonium sulfate and PEG methods, with 9.1% and 29.6% albumin loss, respectively. The ammonium sulfate method had the least albumin loss; however, the purity was the lowest (60.1%) of the three precipitation techniques.

The ultrafiltration step with filtering membrane device led to an albumin loss of almost 50%, meanwhile, tangential flow filtration had a loss of only 9.1%. Overall, PEG desalting step with the filtering membrane device method had the least albumin loss (1.88%). On the IEC step, PEG combined with tangential filtration had the least albumin loss, but the purity did not improve, unlike with other methods. The highest purity was achieved with Cohn's method (95.1%), followed by PEG combined with filtering membrane device (89.4%), PEG combined with tangential filtration (81%), and ammonium sulfate (79.2%).

Cohn's method combined with chromatography had the higher total purity index and the PEG method combined with tangential filtration and chromatography had the lowest.

3.4. Discussion

All the canine cryopoor plasma bags used in our study had an albumin concentration that corroborates with the study of Culler, Iazbik, and Guillaumin (2017),

which concluded that canine cryopoor plasma bags have an albumin concentration of 3.17 ± 0.6 g/dL.

Veterinary plasma fractionation has only a few scientific reports, Meral and colleagues (2018) report the obtention of canine and feline albumin with more than 95% purity by using IEC followed by precipitation with ethanol, however, the yield was not assessed. The same goes for three different patents for canine albumin fractionation, one using Cohn's method (Lu et al., 2004), one using ammonium sulfate precipitation (Lee et al., 2012), and the third using PEG fractionation with anion-exchange chromatography in canine ascitic fluid (Hasegawa and Takenaka, 2005); all three reports indicate a purity above 95% with no expected albumin yield. Our study did not achieve the same purity (with exception of Cohn's method) however the final yield was assessed for all the techniques.

Large-scale human plasma fractionation is currently performed with Cohn's method with a current mean albumin yield of 25 to 28 grams per liter of plasma and with purity ranging from 96% to 98% (Burnouf, 2007; Tanaka et al., 1998). This technique has several advantages over the available methods. For instance, it has lower costs, readily available reagents and, the use of ethanol inhibits bacterial growth and therefore inhibits the formation of pyrogens. However, this technique has the disadvantage of requiring precise control of the precipitation parameters (such as pH and temperature). It also usually achieves low yields due to the many steps of the method although it results in a high albumin purity (Kistler and Friedli, 1980).

Our work confirms that under a laboratorial setting the Cohn's method tends to display low yields due to both the difficulty of controlling the critical parameters and the many numbers of steps required. This implied an important albumin loss occurring on precipitate IV-IV, as it is seen in the SDS-PAGE gel. In this sense, a multipurpose reactor or a dedicated system will undoubtfully lead to better results, increasing the yield and purity of the final product. This fact is seen when comparing laboratorial and industrial fractionation results and by the fact that Cohn's method is the current method used for large-scale human plasma fractionation (Burnouf, 2007; Raoufinia et al., 2016). However, canine cryopoor plasma is not a readily available blood component, as the blood collection relies on canine volunteer donors and a veterinary blood bank with instrumentation for blood component production. For this reason, our study aimed for a purification process suitable for the amount of raw material available, as large-scale

human plasma fractionation technology is designed for processing typically 2000-4000 liters of plasma pools (Burnouf et al., 2020).

Tanaka and colleagues (1998) report that with Cohn's method combined with liquid chromatography they could achieve 2.5 ± 0.05 g of albumin per dL of plasma with 99% purity, considered an excellent result for both techniques. This yield, however, could not be achieved under our current setting. Our results were of an only average of 0.11 g of albumin per dL of plasma, with a purity of 95.1%. However, in Tanaka's report, there were only two precipitation steps, and a size exclusion chromatography was performed after the ion-exchange chromatography which may have influenced their yield.

Odunuga and Shazhko (2013) evaluated ammonium sulfate precipitation combined with liquid chromatography for the purification of bovine serum albumin (BSA) for laboratory applications. The amount of precipitated BSA had a peak between 50% and 60% saturation of ammonium sulfate. In our study, we maintained the CSA in the supernatant with 30% saturation of ammonium sulfate with an average albumin loss of 9.9%. Odunuga and Shazhko achieved a final yield of 2.8 g of albumin per dL of plasma, the purity was not evaluated in this study. For the IEC, BSA started eluting significantly from the column at a NaCl concentration of 250 mM, which was similar to our study where the fraction with higher CSA concentration was eluted with a NaCl concentration of 210 mM. Our work demonstrated that CSA has similar behavior with BSA in terms of concentration of ammonium sulfate for precipitation and ion-exchange chromatography, with the lower yield (1.06 g of albumin per dL of plasma) achieved in our work due to the loss on the chromatographic step.

Hosseini and colleagues (2014) report the obtention of supernatant with 85.1% of albumin after four different PEG 4000 precipitations. By varying the PEG 4000 concentration they could separate different proteins from the plasma. The technique had the advantage of being a faster method than Cohn's method. Our polyethylene glycol protocol was conceived based on the work of Falksveden e Lundblad (1980), which applied a similar procedure for the obtention of IgG. In our protocol, albumin is kept in the supernatant after the precipitation and the final albumin concentration (80.6% ± 4.9) is similar to the reported with PEG 4000 fractionation.

In our laboratorial scale, the PEG fractionation had the most of the protein loss happened during the concentration of the samples by ultrafiltration with the membrane

device. We inferred that this loss was a result of protein retention in the filter membrane due to the centrifugal force, as protein was not detected in the filtrated by Bradford assay and SDS-PAGE electrophoresis. Because of that, we chose to explore the technique further with a larger volume of cryopoor plasma and using tangential flow filtration. The PEG precipitation technique was chosen as it had an albumin purity greater than sulfate ammonium precipitation and a greater yield than Cohn's method.

The tangential flow filtration is a commonly applied technique in pharmaceutical industries to perform buffer exchanges, protein concentration, and inorganic salt removal among other purposes. The technique was also efficiently used for PEG removal from solutions (Burnouf, 2007; Del Hoyo et al., 2007; Garidel and Presser, 2018; Hao et al., 1980). When applied to plasma fractionation processes, the tangential flow filtration allows the removal of the precipitating agent while concentrating the solution (Martinache and Henon, 1980). In our work, the tangential flow filtration made it possible to avoid the need for desalting steps during the purification of albumin. It was also possible to achieve the highest yield (1.53 g/dL) in comparison with the other methods without tangential filtration. Although further improvements in the purification protocol with tangential filtration are needed for better yield and purity (especially for the chromatography step), the albumin loss could be avoided by recovering the eluted portions which still contain albumin and repeating the chromatography. Also, changing the chromatography settings like flow rate, gradient slope, or sample concentration may contribute to better yields as these parameters can change the selectivity and resolution of the mixture in the chromatography (Issaq et al., 2003). Further studies may also address the presence of protein aggregates during the concentration process by tangential flow filtration as these aggregates can cause a pyrogenic reaction on the recipient (Kotb, 1995).

All evaluated techniques followed by chromatography were efficient to extract and purify canine albumin from cryopoor plasma. When considering canine plasma fractionation, it is important to balance the technique's yield with the final purity to achieve a viable product because the canine cryopoor plasma is not a readily available product. Therefore, our work proposes three different protocols that can be further improved for the purification of canine albumin.

3.5. Conclusion

All evaluated techniques were effective in extracting and purifying canine albumin from cryo-poor plasma. PEG precipitation followed by ion-exchange chromatography is a fast and simple protocol that can achieve high yields, especially when associated with tangential flow filtration. However, further studies are necessary to improve the yield and purity of the final product to reach similar results as seen in human plasma fractionation. Our study did not intend to evaluate the cost of a canine plasma fractionation production as it was a laboratory-scale study. This work is a first step towards overcoming the limitations of canine albumin clinical use with the development of an efficient canine plasma fractionation technique for the veterinary field, a field that is currently unexplored.

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3.6. References

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3.7. Figures and tables

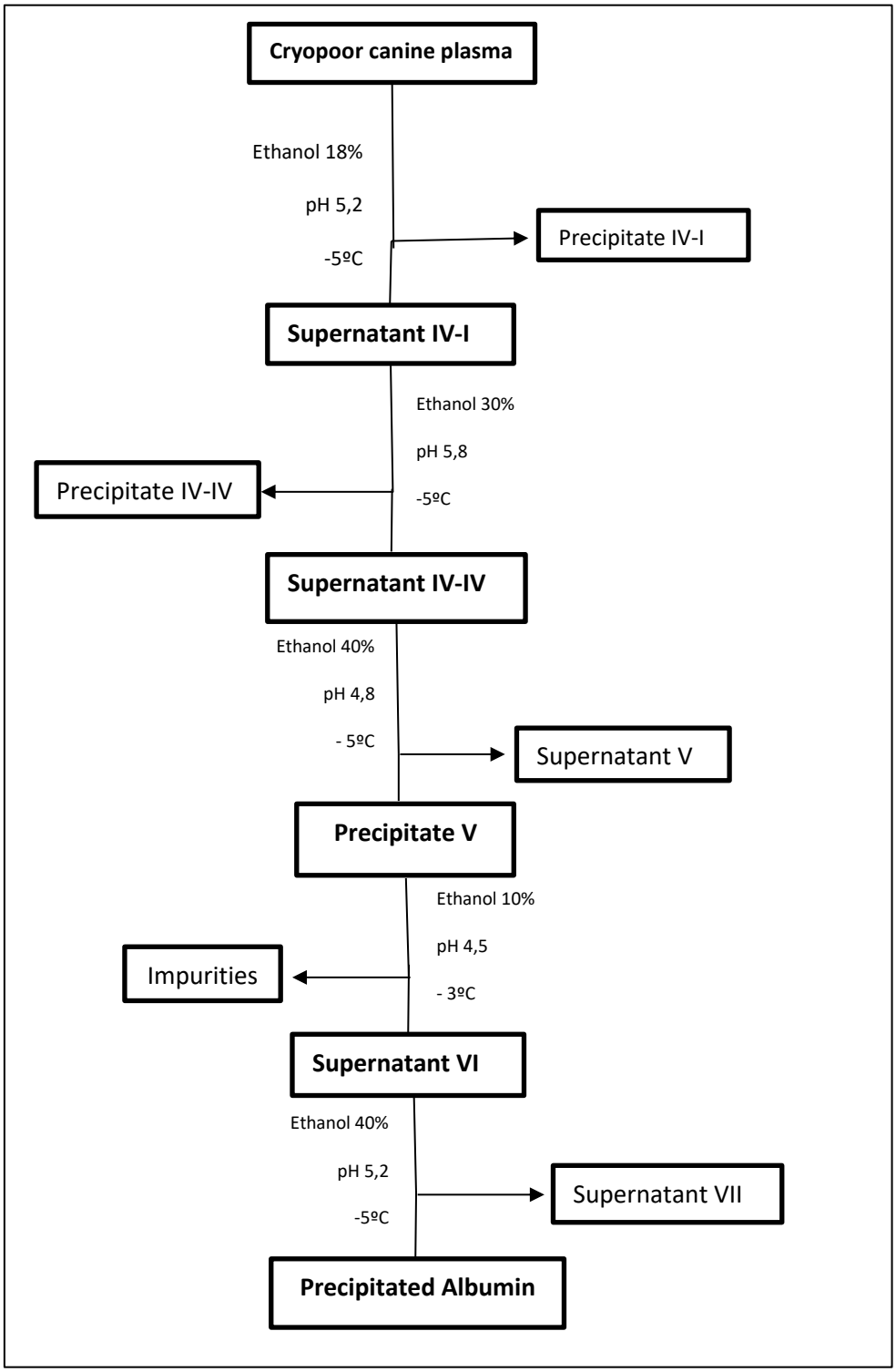

Figure 1: Modified Cohn's method 6 flowchart as applied in this study for the obtention of precipitated albumin, with the indications of ethanol concentration, pH, and temperature in each fractionation step.

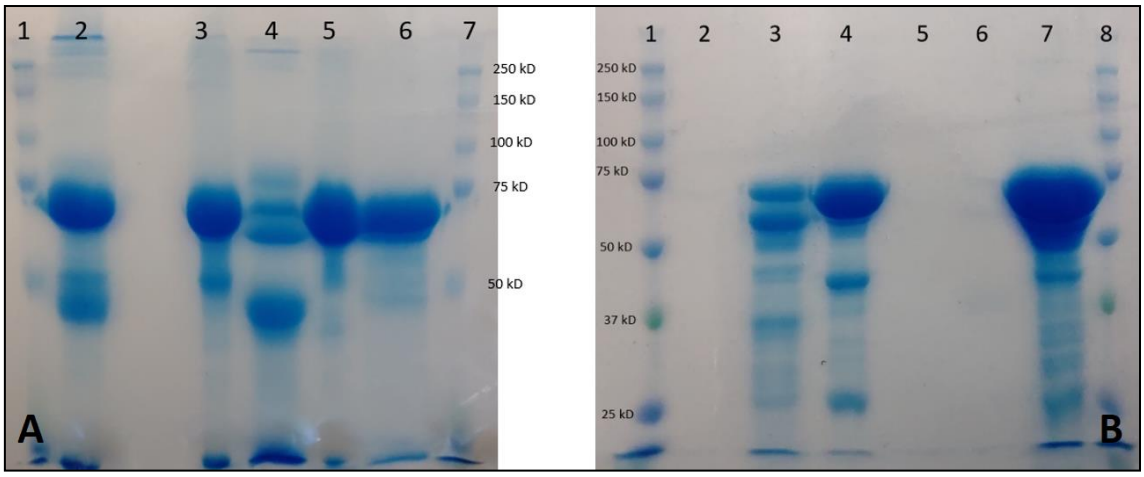

Figure 2: (A and B) Coomassie blue-stained 12.5% SDS-PAGE electrophoresis gel for the method of Cohn fractions. The columns are: (A1) Molecular weight marker; (A2) Untreated cryopoor plasma; (A3) Supernatant IV-I; (A4) Precipitate IV-I; (A5) Supernatant IV-IV; (A6) Precipitate IV-IV; (A7) Molecular weight marker. (B1) Molecular weight marker; (B2) Supernatant V; (B3) Precipitate V; (B4) Supernatant VI; (B5) Impurities; (B6) Supernatant VII; (B7) Precipitated albumin; (B8) Molecular weight marker.

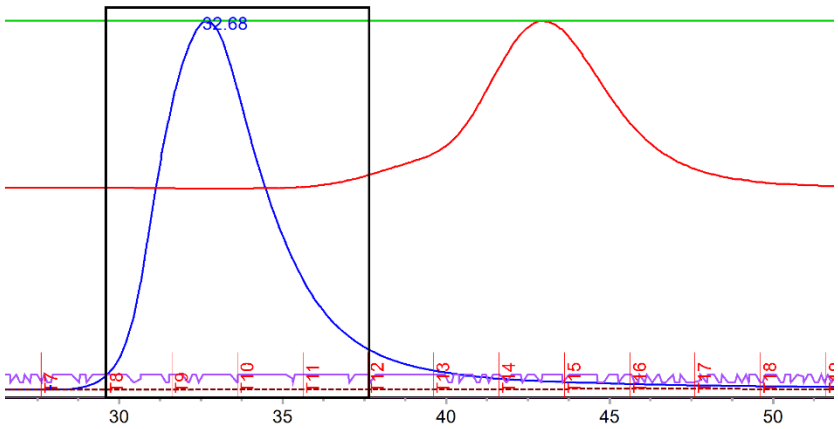

Figure 3: Size exclusion chromatography graphic for the sample obtained by the method of Cohn. The blue line indicates the UV absorption (mAU). The horizontal red line indicates the conductivity (mS/cm). The green line represents the system flow rate (mL/minute). The purple line indicates the system's pressure. The black rectangle indicates the collected eluted fraction.

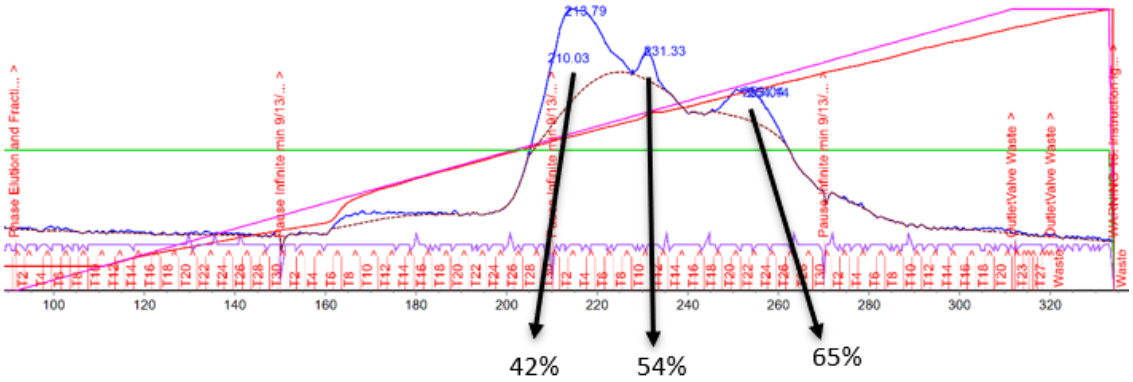

Figure 4: Buffer B gradient elution ion-exchange chromatography results using 0.5 mL of the sample obtained by size exclusion chromatography after fractionation by the method of Cohn. The blue line indicates the UV absorption (mAU) of the eluted flow, and the black arrows indicate the percentage of buffer B for each peak of absorption. The red line indicates the conductivity (mS/cm). The pink line indicates the percentage of buffer B (%) in the gradient. The green line indicates the system flow rate (mL/minute). The purple line indicates the system's pressure.

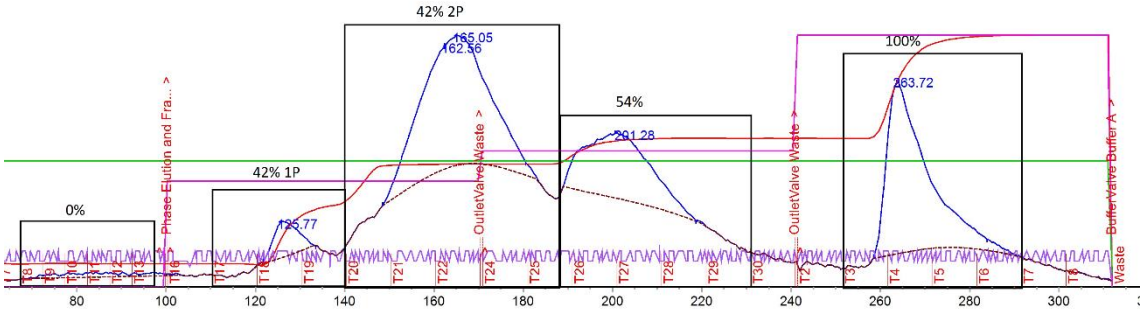

Figure 5: Stepwise ion-exchange chromatography applied to the samples obtained by the method of Cohn. The blue line represents the UV absorption (mAU). The red line represents the conductivity (mS/cm). The green line represents the system flow rate (mL/minute). The purple line represents the system's pressure (mPa). The black rectangles indicate the samples collected for further SDS-PAGE electrophoresis analyses with the corresponding percentage of buffer B.

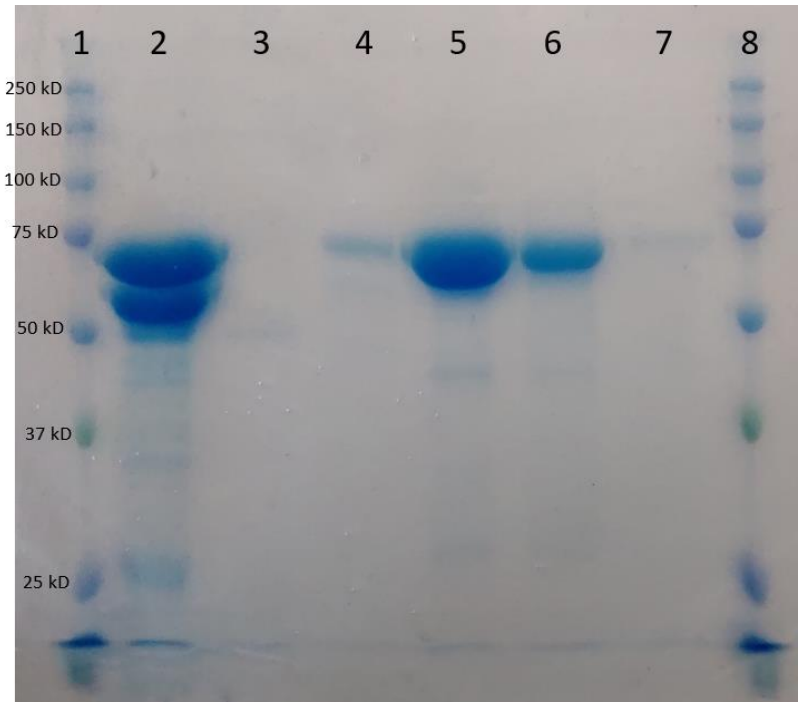

Figure 6: Coomassie blue stained 12.5% SDS-PAGE electrophoresis gel of fractions of the ion-exchange chromatography performed with sample precipitated with Cohn's method. (1) Molecular weight marker. (2) Initial sample before the chromatography. (3) Eluted fraction at 0% buffer B concentration. (4) First eluted fraction at 42% buffer B concentration. (5) Second eluted fraction at 42% buffer B concentration. (6) Eluted fraction at 54% buffer B concentration. (7) Eluted fraction at 100% buffer B concentration. (8) Molecular weight marker.

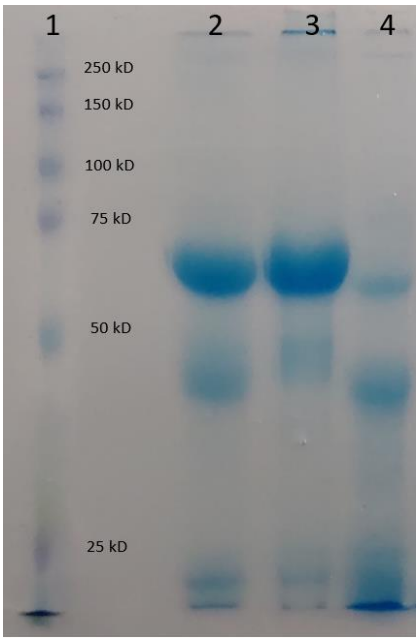

Figure 7: Coomassie-stained SDS-PAGE electrophoresis gel of the ammonium sulfate cryopoor plasma fractionation. (1) Molecular weight marker. (2) Cryopoor plasma before precipitation. (3) Supernatant after precipitation. (4) Precipitate.

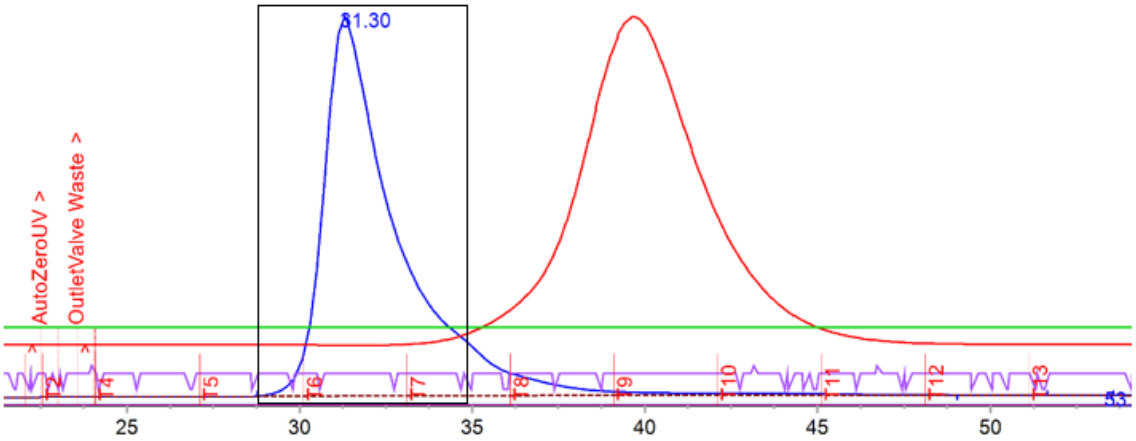

Figure 8: Size exclusion chromatography of the supernatant obtained after the ammonium sulfate fractionation. The blue line represents the UV absorption (mAU). The red line represents the conductivity (mS/cm). The green line represents the system flow. The purple line represents the system's pressure (mPa). The black rectangle represents the collected eluted fraction.

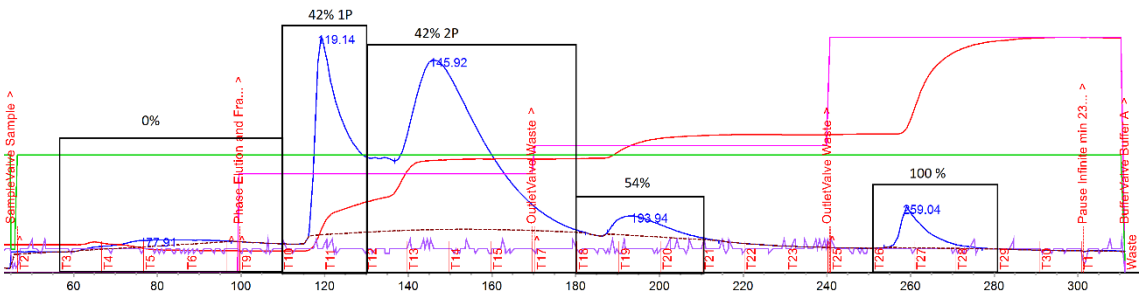

Figure 9: Stepwise ion-exchange chromatography applied to the samples obtained by the ammonium sulfate precipitation. The blue line represents the UV absorption (mAU). The red line represents the conductivity (mS/cm). The green line represents the system flow rate (mL/minute). The purple line represents the system's pressure (mPa). The black rectangles indicate the samples collected for further SDS-PAGE electrophoresis analyses with the corresponding percentage of buffer B.

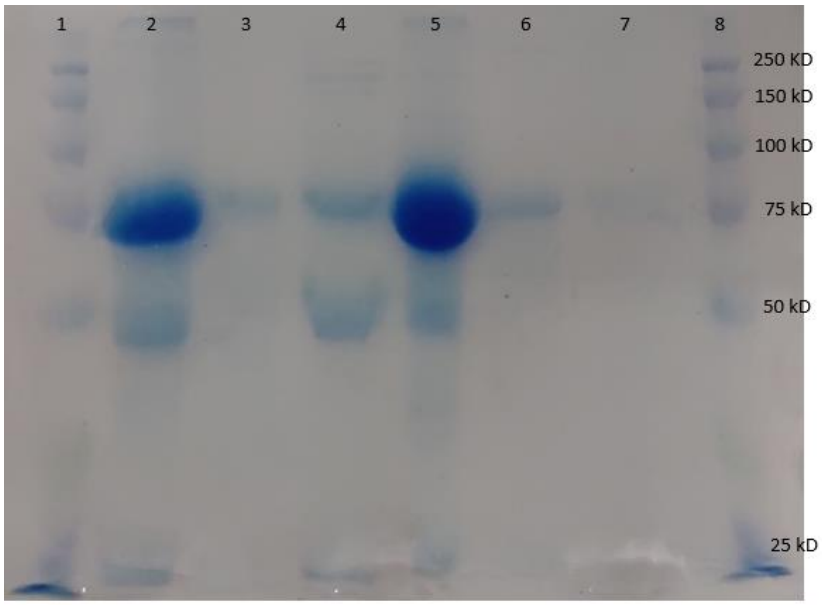

Figure 10: Coomassie-stained SDS-PAGE electrophoresis of the fractions collected by ion-exchange chromatography after ammonium sulfate fractionation. (1) Molecular weight marker. (2) Sample before ion-exchange chromatography. (3) Fraction eluted at 0% buffer B. (4) Fraction eluted at the first peak of 42% buffer B. (5) Fraction eluted at the second peak of 42% buffer B. (6) Fraction eluted at 54% of buffer B. (7) Fraction eluted at 100% buffer B. (8) Molecular weight marker.

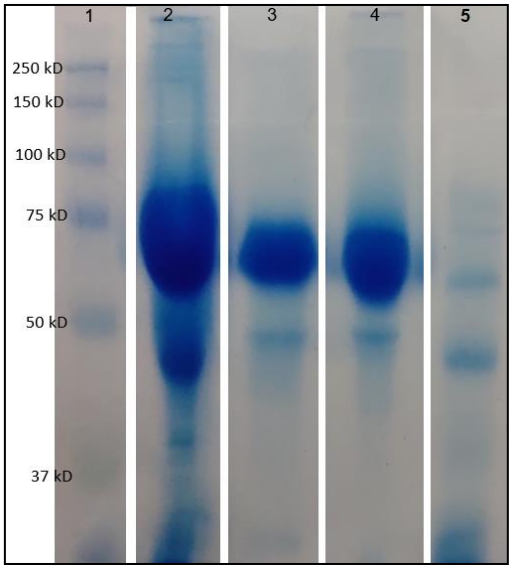

Figure 11: Coomassie-stained SDS-PAGE electrophoresis gel of the PEG cryopoor plasma fractionation. (1) Molecular weight marker. (2) Cryopoor plasma before precipitation. (3) Supernatant after precipitation. (4) Supernatant after ultrafiltration with 30 kDa filtering device. (5) Precipitate.

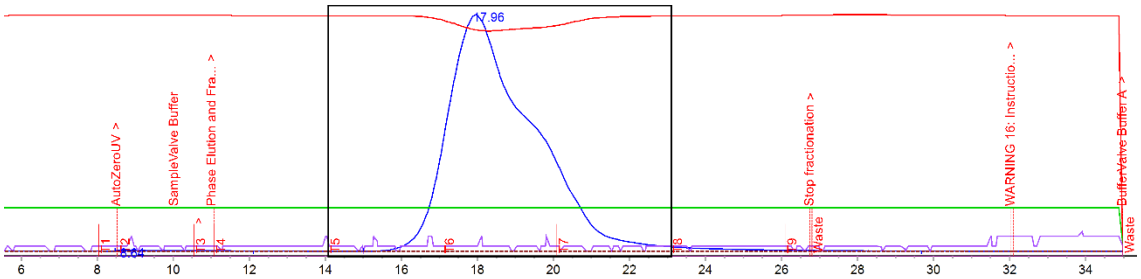

Figure 12: Size exclusion chromatography of the supernatant obtained after PEG fractionation with 30K filter. The blue line represents the UV absorption (mAU). The red line represents the conductivity (mS/cm). The green line represents the system flow. The purple line represents the system's pressure (mPa). The black rectangle represents the collected eluted fraction.

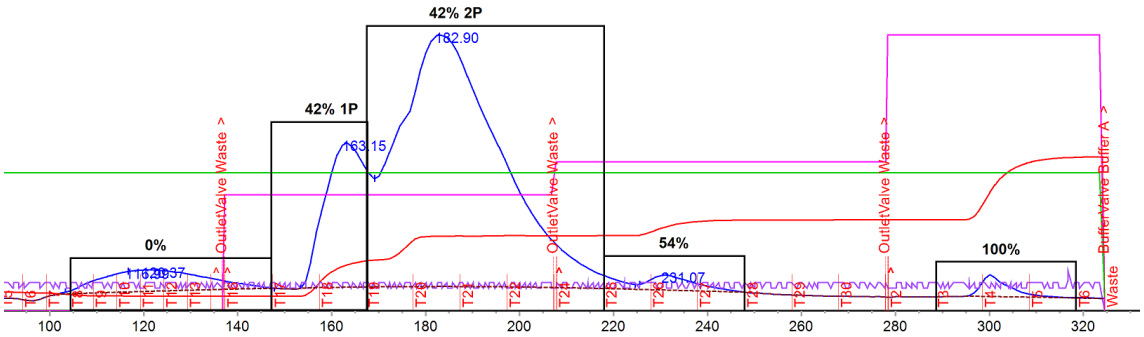

Figure 13: Stepwise ion-exchange chromatography applied to the samples obtained by PEG precipitation followed by 10 kDa (A) and 30 KDa (B) filters. The blue line represents the UV absorption (mAU). The red line represents the conductivity (mS/cm). The green line represents the system flow rate (mL/minute). The purple line represents the system's pressure (mPa). The black rectangles indicate the samples collected for further SDS-PAGE electrophoresis analyses with the corresponding percentage of buffer B.

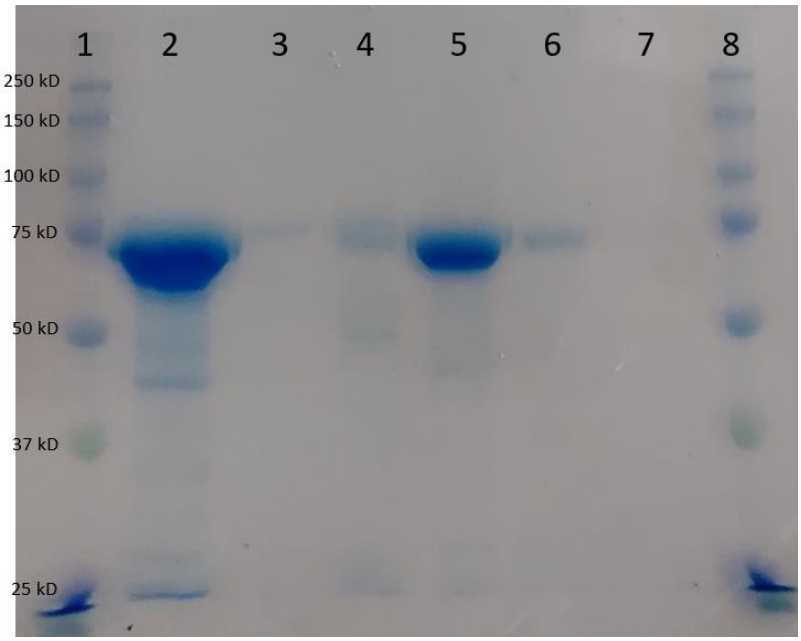

Figure 14: Coomassie-stained SDS-PAGE electrophoresis of the fractions collected by ion-exchange chromatography after PEG precipitation followed by a 30KDa filter. (1) Molecular weight marker. (2) Sample before ion-exchange chromatography. (3) Fraction eluted at 0% buffer B. (4) Fraction eluted at the first peak of 42% buffer B. (5) Fraction

eluted at the second peak of 42% buffer B. (6) Fraction eluted at 54% of buffer B. (7) Fraction eluted at 100% buffer B. (8) Molecular weight marker.

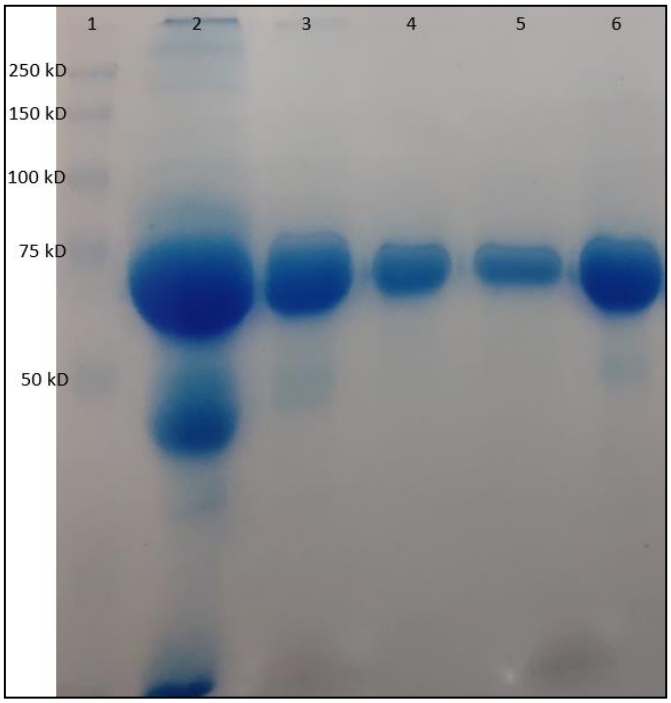

Figure 15: Coomassie-stained SDS-PAGE electrophoresis with all samples after the three fractionation protocols and subsequent ion-exchange chromatographies. (1) Molecular weight marker. (2) Plasma cryopoor. (3) Supernatant after ammonium sulfate precipitation. (4) Precipitate albumin after Cohn's method. (5) Supernatant after PEG precipitation. (6) Supernatant after PEG precipitation followed by 30 KDa filter.

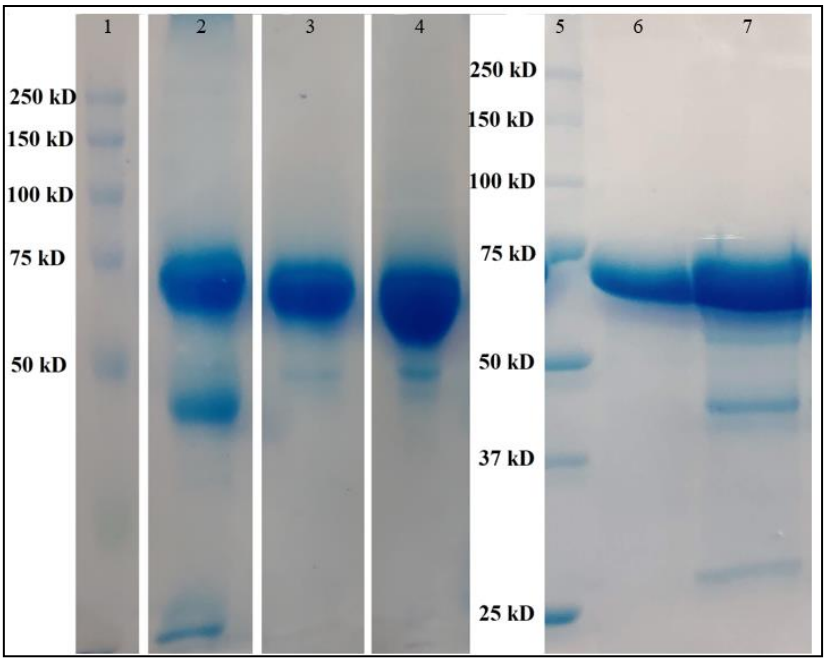

Figure 16: Coomassie-stained SDS-PAGE electrophoresis with supernatant collected during plasma fractionation with PEG followed by tangential flow filtration and ion-exchange chromatography. (1) Molecular weight marker. (2) Cryopoor plasma. (3) Supernatant after PEG precipitation. (4) Sample after tangential flow filtration. (5) Molecular weight marker. (6) Sample after ion-exchange chromatography. (7) Sample after concentration by tangential flow filtration.

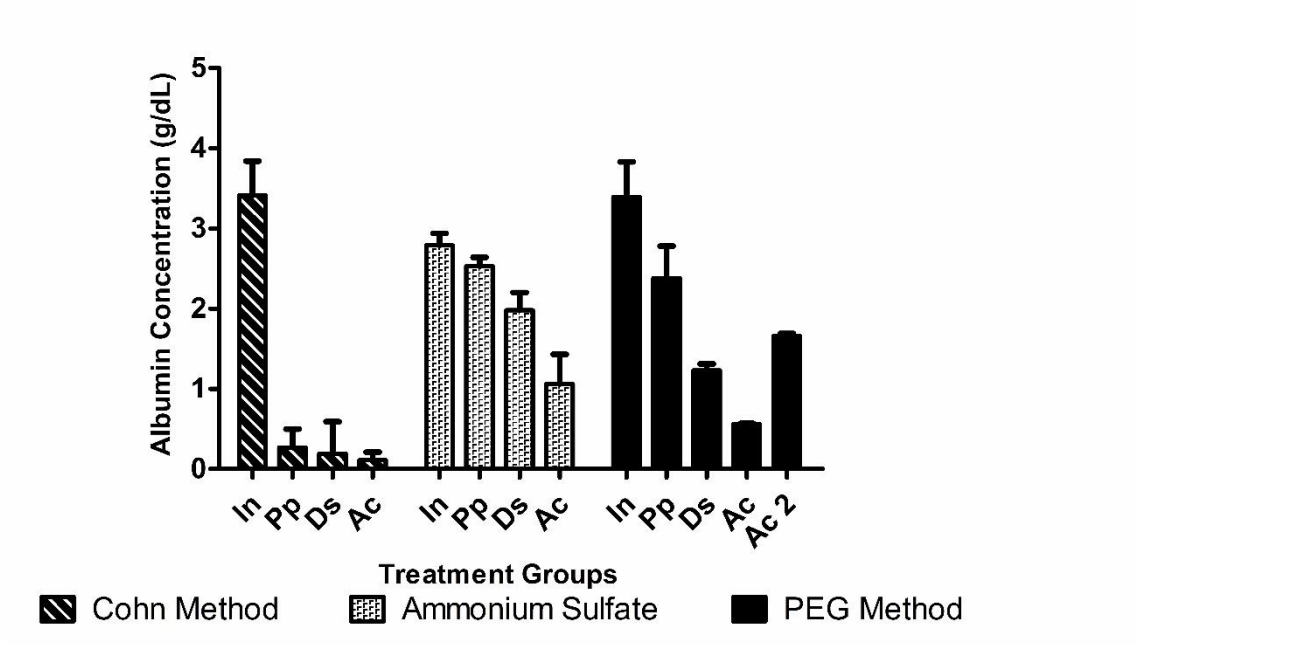

Figure 17: Comparison of albumin concentration at each fractionation process separated by the precipitation method. In = initial albumin concentration in cryopoor plasma. Pp = albumin concentration after precipitation method. Ds: albumin concentration after desalting. Ac = albumin concentration after ion-exchange chromatography. Ac2 = albumin concentration after ion-exchange chromatography and concentration by tangential filtration.

		Precipitation method		
		Cohn's method	Ammonium sulfate	PEG
	N	3	3	3
Cryopoor plasma	Albumin:Total protein ratio (%)	62.0 ± 2.4	55.2 ± 3.8	65.2 ± 2.37
	Final albumin purity (%)	65.5 ± 3.9	60.1 ± 5.6	80.6 ± 4.9
	Purity index	1.1 ± 0.1	1.1 ± 0.04	1.1 ± 0.19
	Albumin loss (%)	92.4 ± 5.4	9.1 ± 4.2	29.6 ± 10.6
	Protein loss (%)	92.9 ± 4.9	16.3 ± 7.3	43.2 ± 6.92

Table 1: Albumin and total protein ratio of cryopoor plasmas used for each precipitation technique and the final albumin purity, purity index, albumin loss, and protein loss reached after the precipitation technique. Data is shown by mean ± SD. The purity index was calculated by the ratio between final albumin purity and the initial albumin purity (Albumin/Total protein ratio). The percentage of the total protein loss and albumin loss was assessed by the difference between the final and initial protein or albumin mass. N: number of analyzes. PEG: polyethylene glycol

	Ultrafiltration		Desalting			Ion-exchange chromatography				Concentration by tangential filtration
	PEG + Filtering membrane device	PEG + Tangential filtration	Cohn's method	Ammonium sulfate	PEG + Filtering membrane device	Cohn's method	Ammonium sulfate	PEG + Filtering membrane device	PEG + Tangential filtration	PEG + Tangential filtration
N	2	2	3	3	3	3	3	2	2	2
Albumin purity (%)	87.9 ± 0.8	75.3 ± 6.2	82.6 ± 11.7	69.2 ± 6.6	83.2 ± 3.3	95.1 ± 2.8	79.2 ± 8.1	89.4 ± 4.2	76.0 ± 5.7	81.0 ± 14.2
Purity index	1.04 ± 0.01	0.9 ± 0.01	1.3 ± 0.2	1.3 ± 0.2	0.95 ± 0.05	1.2 ± 0,2	1.2 ± 0.14	1.1 ± 0.01	1.0 ± 0.01	1.0 ± 0.1
Albumin loss (%)	48.6 ± 5.0	9.1 ± 1.2	34.2 ± 8.2	21.8 ± 5.6	1.88 ± 1.1	42.5 ± 16.9	44.9 ± 24.5	54.2 ± 2.9	17.4 ± 18.4	7.8 ± 2.5
Protein loss (%)	51.2 ± 3.4	17.7 ± 20.9	47.2 ± 9.7	32.2 ± 0.9	3.1 ± 1.4	50.8 ± 11.8	47,2 ± 9,7	57.4 ± 2.4	18.1 ± 18.9	12.6 ± 11.2

Table 2: Albumin purity, purity index, albumin loss, and protein loss reached after each purification technique step. Data is shown by mean ± SD. The purity index was calculated by the ratio of final albumin purity percentage and initial albumin purity percentage. The percentage of the total protein loss and albumin loss was assessed by the difference between the final and initial protein or albumin mass. N: number of analyzes. PEG: polyethylene glycol

Total purity index	N	
Cohn's method	3	1.5 ± 0,1
Ammonium sulfate	3	1.4 ± 0.1
PEG + Ultrafiltration	2	1.4 ± 0.1
PEG + Tangential filtration	2	1.2 ± 0.2

Table 3: Total purity index at the end of each precipitation method combined with chromatography, with or without filtration by filtering membrane device or tangential filtration. The total purity index was calculated by the ratio of albumin purity at the end of the purification method and the initial albumin purity (Albumin/Total protein ratio) of the cryopoor plasma. Data is shown by mean ± SD. PEG: polyethylene glycol. N: number of analyzes

Capítulo 2

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4. Development of lyophilized canine albumin formulations: influence of buffer system and excipients on critical formulation temperatures and product performance

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ABSTRACT

In veterinary medicine, canine patients with hypoalbuminemia need a safe allogenic natural colloid solution for albumin supplementation. Although there is already lyophilized canine albumin commercially available produced by Animal Blood Resources, the high demand and prohibitive costs associated with importing prevent local stocking of the product which makes the development of a safe, effective, and stable albumin formulation extremely necessary. This research aimed to understand the influence of the buffer system and excipients used in the formulation of canine albumin on the product critical temperatures and performance. Three buffer systems were

evaluated (arginine 50 mM, HEPES 100 mM, and Tris 100 mM) with nine different combinations of excipients for each buffer, such as sucrose, trehalose, glycine, and mannitol. Within all evaluated formulations, canine albumin with arginine 50 mM buffer with 5% glycine (w/v) presented a superior performance regarding pharmaceutic elegance, residual moisture (below 2%), immediate reconstitution time, and maintenance of albumin's structural integrity. Furthermore, for this formulation, a high glass transition temperature (T_g') and collapse temperature (T_{col}) were observed, which allows a more aggressive and short freeze-drying cycle. Albumin robustness was observed as, even without lyoprotectants, the secondary structure was maintained immediately after lyophilization. The present study highlighted the importance of choosing the adequate buffer system and defined the main critical temperatures for an optimized lyophilization cycle for canine albumin aiming at the development of a high-performance product that meets the market demand.

KEYWORDS: Dog. Freeze-dying. Buffer system. DSC. FDM.

4.1. Introduction

Albumin is the main protein of the blood, corresponding to more than 50% of the total amount of protein in plasma, with a molecular mass of 69 kDa [1]. Critically ill patients generally are hypoalbuminemics, this condition being caused by low albumin production rates, accelerated protein loss, or a combination of both [2]. Albumin is a multifunctional protein, contributing to pressure regulation, vascular integrity, anticoagulation, acid-basic regulation, antioxidation, in addition to acting as a carrier of endogenous and exogenous substances [2,3]. Thus, it is not surprising that hypoalbuminemia is associated with a poor outcome, both in human and veterinary patients [4–6].

In veterinary medicine, the treatment for low serum albumin levels is focused mainly on the resolution of the underlying disease and proper nutrition [2]. However, for chronic or incurable diseases these interventions might not be sufficient, therefore, an albumin supplementation solution is necessary. The use of fresh frozen plasma for albumin replacement is no longer recommended because of the low albumin content in the plasma bag (25 to 30 g of albumin per liter) that requires high volumes for this purpose and can lead to a volume overload in the recipient and high risks of transfusion reactions [7–10].

There are reports of the use of human serum albumin (HAS) solutions in dogs, although this use is quite controversial [8,11,12]. The structural similarity of canine and human albumin is only 79,8% [13], and therefore, severe immediate and delayed adverse events can occur in healthy dogs even after the first transfusion procedure [14–16]. Also, death secondary to delayed-type III hypersensitivity reaction caused by HSA use in critically ill dogs was recently reported [17].

Although a lyophilized canine albumin-based product is commercially available from Animal Blood Resources International (ABRI), a private veterinary blood bank, the high demand and prohibitive costs associated with importing prevent local stocking of the product. This makes the development of a safe, effective, and stable formulation extremely necessary. Currently, there are only two prospective, small-scale, clinical studies using the ABRI product. Craft and Powell [18] evaluated the use of CSA in 14 dogs with septic peritonitis. They observed an increase in serum albumin levels, colloid osmotic pressure, and Doppler blood pressure two hours after the infusion. Enders and colleagues [19] reported in an abstract the evaluation of the safety of repeated administration during 2 weeks of CSA in 6 healthy dogs. They concluded that repeated infusions seemed safe and that the CSA dose was effective in increasing serum albumin and colloid osmotic pressure.

Also termed freeze-drying, lyophilization is an industrial process of drying by freezing and sublimation of ice under vacuum and it is used to convert solutions of labile materials into solids of sufficient stability for distribution and storage [20]. During freezing, the first step of lyophilization, the liquid suspension is cooled, and ice is formed. Freezing is considered the most aggressive and stressful step of lyophilization and may cause protein denaturation and aggregation [21].

During lyophilization, a combination of suitable excipients is required to ensure adequate tonicity, reconstitution, and stability. Bulking agents are excipients that have a propensity to crystallize, and therefore, are used to achieve an elegant and stable cake, and can act as tonicity modulators [22]. Sugars or sugar alcohols such as lactose, sorbitol, and mannitol are typically used as bulking agents. Non-reducing compounds, such as mannitol, are preferred for protein-based formulations to avoid the Maillard reaction between protein and the excipient. Amino acids can also be used as bulking agents, such as histidine, glycine, and arginine. Lyoprotectants agents are excipients used for

stabilizing the active pharmaceutical ingredient (API) by remaining in an amorphous state. The most common agents are disaccharides like sucrose and trehalose [21,23].

Collapse and loosening of the porous structure can occur when the product is heated above the collapse temperature during the sublimation step. The onset of collapse or micro-collapse is characterized by the presence of small holes in the dried portion of the frozen matrix. [21,22]. Thus, a high collapse temperature is beneficial as the increase of 1ºC of the product temperature during lyophilization reduces primary drying time by 13% [24] An annealing step is often introduced into the freezing protocol to allow larger ice crystals to grow at the expense of small ones in a process denoted as "Ostwald ripening". Annealing is an isothermal step in which samples are maintained at a temperature above the glass transition temperature, followed by recooling. This thermal treatment has dramatic effects on the size of the ice crystals, and therefore on the primary drying time. It increases pores diameter in the freeze-dried matrix thereby facilitating water vapor to flow through the sample. The annealing procedure is also used to increase the crystallization of the bulking agents, such as mannitol and glycine [25].

Thus, for the development of a safe, effective, and stable albumin formulation with an elegant cake appearance, it is necessary studies about the ideal parameters and formulation. However, there is currently no systematic study about the freeze-drying of canine albumin. Therefore, this research aimed to understand the influence of the buffer system and excipients used in the formulation of canine albumin on the critical temperatures and the performance of the product. Parameters such as appearance, reconstitution time, residual moisture, and structural integrity of the canine albumin. For this purpose, we evaluated formulations with three buffer systems (arginine 50 mM, HEPES 100 mM, and Tris 100 mM) in pH 7.3, with nine different combinations of excipients for each buffer, such as sucrose, and trehalose, as lyoprotectant agents; and glycine, and mannitol, as bulking agents.

4.2. Materials and methods

All the chemicals used in this study were obtained from the following commercial vendors: L-arginine, 4-(2-hydroxyethyl)piperazine-1-ethane sulfonic acid (HEPES), Tris, Tris-base, polyethyleneglycol 6000 (PEG), 1,4-dithiothreitol (DTT), and 2-mercaptoethanol (MEtOH) (Sigma-Aldrich, MO, USA); mannitol (Cinética Produtos Químicos, PR, Brazil); D-(+)-trehalose dihydrate and sucrose (Fluka Biochemika, MO,

USA); glycine, and hydrochloric acid (Labsynth, SP, Brazil). All reagents used for SDS-PAGE electrophoresis were purchased from Sigma-Aldrich (MO, USA).

4.2.1. Ethics statement

The study was reviewed and approved by the Ethic Committee on Animal Use (CEUA/FMVZ; Institutional Animal Care and Use Committee (IACUC)) of the School of Veterinary Medicine and Animal Science (FMVZ) of the University of Sao Paulo (USP) (protocol 400013218).

4.2.2. Canine albumin solution

The canine albumin solution in 25 mM sodium phosphate buffer at pH 8.0 with a purity of >90% (by SDS-PAGE electrophoresis gel) was obtained from cryopoor plasma bags by precipitation with polyethyleneglycol 6000 (PEG) followed by ion-exchange chromatography. The cryopoor plasma bags were kindly provided by the Laboratory of Hemotherapy of the Department of Surgery of the School of Veterinary Medicine and Animal Sciences of the University of São Paulo.

4.2.3. Sample preparation and lyophilization conditions

During the formulation stage, three buffer systems were evaluated: arginine buffer 50 mM, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer 100 mM, and Tris 100 mM, all at pH 7.3. Buffer exchange was performed by dialysis (Membra-cell Dialysis Tub 12-16,000mw, 21mm, Viskase, Illinois, USA). The volume of buffer used corresponded to 10 times the volume of the membrane. Dialysis was maintained for a total period of 48 hours with three changes of buffer solution at regular intervals. For each buffer system tested, 9 different combinations of lyoprotectants and bulking agents were evaluated, such as sucrose, trehalose, glycine, and mannitol were evaluated (Table 1).

The lyophilization of canine albumin formulations was conducted using a pilot freeze-dryer Dura-Top MP Tray Dryer MNL-031-A (New York, USA). All formulations were filled into 2 mL type I (ISO 2R) glass tubing vials using a fill volume of 300 μL and partially stoppered with lyophilization stoppers. Calibrated thermocouples were placed in the bottom center in both edge and center vials to monitor the product temperature during the lyophilization cycle. The lyophilization cycles were carried out at an initial hold step at 5ºC for 30 min after which the temperature was reduced to −5ºC at a ramp rate of

1°C/min and held for 30 min. The temperature was then ramped to −40°C at 1°C/min and held for 8 h at a chamber pressure of between 100-150 mTorr. Secondary drying was conducted at a temperature of 25°C for 6 h at a ramp rate of 0.5°C/min.

At the end of the lyophilization, the vials were stoppered at 25°C under vacuum, then the freeze-dryer chamber was backfilled pressure until 750 Torr by injection of nitrogen gas. The vials were then crimped with aluminum crimps and stored at 5°C until further analysis. For each formulation, 7 vials were lyophilized.

It is important to emphasize that due to a temperature limitation of the freeze-dryer used in this study, only formulations with critical temperatures above -36°C were lyophilized, since the minimum temperature reached by the equipment shelves was -40°C.

4.2.4. Thermal analysis

Thermal analysis of solutions and freeze-dried samples was performed by a differential scanning calorimeter (DSC) (model Pyris 1; Perkin Elmer LLC, Norwalk, CT, USA) equipped with a cooling system (CryoFill, Perkim Elmer). It was calibrated using indium, mercury, and distilled water as standards. Approximately 10 µl of suspension were used in an aluminum pan and sealed. An empty pan was used as the reference. Cooling and heating rates of 1.0 and 20°C/min were applied throughout the analysis, respectively. The suspensions were cooled to -60°C, held for 1 min, and then heated to 25°C. Glass transition temperature of the liquid (T_g') corresponds to the midpoint of the base shift during the heating scan. The presence of a eutectic melting temperature (T_{eut}) was also assessed during the heating scan.

When the annealing procedure was applied, an isothermal step was added after the freezing step at 10 degrees above T_g' of the sample, which was held for 5 min. The second heating scan (to 25°C) was used to determine the glass transition temperature (T_g').

4.2.5. Collapse temperature measured by freeze-drying microscopy (FDM)

The collapse (T_{col}) and microcollapse (T_{mcol}) temperature were measured by a freeze-drying microscope (Linkam Scientific Instruments, Surrey, UK) equipped with a video camera, a computer to capture the collapse image (optical window), a liquid nitrogen cooling system, a vacuum pump, and a small freeze-drying chamber with a temperature controller. Direct observation of the freeze-dried sample was done using a polarized microscope (Nikon Elipse E600, Nikon, Japan). The samples were cooled at a

controlled rate (5°C/min) to -60°C, held for 1 min and then heated to 10°C (5°C/min). When the annealing procedure was applied, an isothermal step at 10 degrees above T_g' of the sample, which was held for 5 min, was added after the freezing step. The collapse temperature (T_c) corresponds to the lowest temperature of the overall loss of the initial frozen structure during freeze-drying, and the microcollapse temperature (T_{mcol}) corresponds to the onset of the collapse.

4.2.6. Cake appearance

The macroscopic appearance of the freeze-dried cakes was visually evaluated immediately after the lyophilization cycle. Irregularities were nominated according to the study of Patel and colleagues [26].

4.2.7. Residual moisture

The residual moisture of the lyophilized formulations was measured by Computrac® VAPOR PRO®(Arizona, USA), a green alternative to the Karl Fisher method. Approximately 30 mg of lyophilized powder was used to check if the water content was sufficiently low to ensure good stability during storage. Residual moisture (%) was calculated from the water content and the mass of the lyophilized powder, each formulation of canine albumin was performed in triplicate.

4.2.8. Reconstitution time

The samples of each formulation were reconstituted with 300 µL of water for injection (WFI) and gently agitated for 5 s. The reconstituted vials were visually observed against a black background and compared with a vial of WFI. The observation was performed at room temperature until the appearance of the reconstituted solution matched that of the control vial. No additional agitation and shaking was performed during the observation. The time required for complete dissolution was recorded as the reconstitution time, which was performed in triplicate for each formulation of canine albumin. Reconstitution time within 10 seconds was considered immediate.

4.2.9. SDS-PAGE

Pre-lyophilization and post-lyophilization albumin samples were subjected to an SDS-PAGE electrophoresis gel analysis using a 12.5% polyacrylamide gel to evaluate the fragmentation and/or aggregation. The samples were diluted with a sample buffer, containing both SDS and 2-mercaptoethanol, for a final concentration of 5 mg/ml of

protein, then 40 µL of this solution was boiled for 5 minutes to disrupt the disulfide bonds and coat the protein with SDS to provide a proper charge-to-mass relationship. Samples were loaded at equal concentrations of 5 µL/well and the molecular weight marker (LMW Calibration Kit for SDS Electrophoresis, GE Healthcare, SP, Brazil) was loaded at 10 µL/well. The gels run was performed with a running buffer (3 g/L Tris, 14,5 g/L glycine, and 1 g/L SDS) at a constant voltage of 130 V, for an average time of 90 minutes and subsequently stained using a standard Coomassie Brilliant Blue G-250 staining technique.

4.2.10. Raman spectroscopy

Raman spectra were acquired using a Raman microscope spectrometer model Alpha 300R (Witec Germany) equipped with a 20× objective (N.A. = 0.40) and a solid-state laser line at 532 nm (Nd:YAG, continuous wave) as the excitation source; the laser power was 2.5 mW. Structural analysis was conducted on liquid samples before lyophilization and on reconstituted samples after lyophilization with WFI. Sample preparation for Raman spectroscopy analyses was carried out by drying 2 µL of the liquid sample in an aluminum-coated blade. All spectra were obtained in the range of 250-1800 cm^{-1}, accumulating 200 scans with an integration time of 0.5 s each scan. The average spectrum was obtained with at least 10 spectra acquired per sample using the "Average Spectrum" tool of the Project FIVE 5.1 software from Witec,

4.3. Results and discussion

4.3.1. Preparation of formulations

The most common cause for pH change during freezing in lyophilization is the crystallization of the buffer compound, such as $Na_2HPO_4 \cdot 12\ H_2O$ crystallization in sodium phosphate buffer. Gómez, Pikal, and Rodríguez-Hornedo [27] reported that the freezing of 50 mM and 100 mM sodium phosphate buffer caused a pH decrease from 7.4 to 4.2. This pH shift can potentially lead to a considerable degree of perturbation on the secondary structure of the protein, raising the chances of physical and chemical degradation, deamination, and oxidation; therefore, affecting the storage stability of lyophilized proteins [21,28]. Therefore, the sodium-phosphate buffer solution exchange was necessary.

After the exchange of sodium phosphate buffer by a dialysis membrane, the final concentration of canine albumin solutions for the three evaluated buffers (arginine 50

mM, HEPES 100 mM, and tris 100mM) was approximately 57 mg/mL. Canine fresh frozen plasma bags have an average albumin concentration of 30 mg/mL [29]. Therefore, in our study, the protein solutions produced had an albumin concentration of approximately two-fold the concentration of a fresh frozen plasma bag. Moreover, this solution can be considered a high protein concentration solution, as the term is generally applied to preparations ranging between 50 and 150 mg/mL [30].

The use of fresh frozen plasma is not recommended for albumin reposition as there is a low albumin concentration per bag [31]. Therefore, a canine albumin solution with a high concentration is extremely useful to avoid a great infusion volume in clinical practice. Furthermore, a freeze-dried product provides great flexibility as it allows the achievement of solutions with different concentrations depending on the reconstitution volume used. In dogs, the use of 5% to 25% albumin solutions (HSA or CSA) is reported [32].

Additionally, for each buffer system, 9 different combinations with lyoprotectants (sucrose and trehalose) and bulking agents (glycine and mannitol) were evaluated (Table 1). All the solutions were adjusted to pH 7.3.

4.3.2. Critical temperatures by DSC and freeze-drying microscopy

Relevant critical temperatures for primary drying step optimization for the lyophilization process were determined by DSC (T_g' and T_{eut}) and FDM (T_{mcol} and T_{col}) (Table 2). The influence of an annealing step addition on critical temperatures of crystalline bulking agents was also evaluated.

Based on Table 2 results, it is noticeable the considerable influence of the buffer system used on the canine albumin formulation regarding the critical temperatures. Additionally, in some formulations, T_{col} was greater than T_g' up to 12 degrees (F4T), which is consistent with high protein concentration solutions. Colandene and colleagues [33] reported that in monoclonal antibody formulations without a crystalline bulking agent, as the protein concentration increased, T_{col} became progressively higher than T_g', leading to a difference of up to 15 degrees.

For the formulation with pure albumin in Tris 100 mM buffer (F9T), there was an 0.7-degree difference between T_g' (-29.2ºC) and T_{col} (-28.5ºC) contrasting with the same formulations with arginine 50 mM (F9A) and HEPES 100 mM buffer (F9H), which have a difference between T_g' (-25.5ºC and -33.9ºC, respectively) and T_{col} (-16.5ºC and -25.0ºC,

respectively) of approximately 9 degrees, consistent with the robustness of high protein formulations. Albumins have a T_g' around -13 to -11ºC [21,34], which was close to T_{col} of F9A. However, for F9T and F9H, T_{col} was lower, in agreement with the lower T_g's for the same formulations. Possibly, the low T_g's are associated with the respective buffering agents T_g's, which are around -50ºC for Tris, and -65ºC for HEPES [34,35], as both had a higher concentration (100 mM) than arginine (50 mM) on CSA formulations.

Interestingly, formulations in Tris 100 mM buffer containing 5% (w/v) of mannitol (F7T) or glycine (F8T) presented T_{col} (-41.1ºC and -35.6ºC, respectively) around 3 to 13 degrees lower than its respective T_g' (-38.5ºC and -22.2ºC, respectively), contrasting with same albumin formulations with arginine 50 mM or HEPES 100 mM buffer, which presented, as expected for a high protein concentration formulation, T_{col} around 7 to 10 degrees (-24.8ºC for F7A and -9.2ºC for F8A) and 4 to 9 degrees (-31.5ºC for F7H and -22.5ºC for F8H) higher than its respective T_g' (-35.1ºC for F7A, -16.2ºC for F8A, -35.0ºC for F7H, and -31.5ºC for F8H). Probably, for F7T and F8T, a certain degree of mannitol or glycine crystallization inhibition occurred. However, mannitol had a higher degree of crystallization inhibition, as glycine tends to almost completely crystalize at low freezing rates [36]. The low T_{col} for F7T and F8T agrees with the low T_g's of amorphous glycine and mannitol, which are around -35ºC for mannitol and -37ºC for glycine [37].

Similarly, for formulations with glycine and trehalose 4:1 (w/v) or glycine and sucrose 4:1 (w/v) in HEPES buffer (F2H and F4H, respectively), T_{col} (-35.3ºC and -36.8ºC, respectively) were lower from their respective T_g's (-32.8ºC and -33.2ºC, respectively), although this difference was small (around 2 to 4 degrees). This suggests a partial inhibition of glycine crystallization. Glycine crystallization inhibition caused by the proportion of sucrose in the formulation is well known and reported in literature [38]. The low T_g's of F2A (-42.9ºC), F4A (-38.4ºC), F2T (-33.4ºC), and F4T (-44ºC) can be due to the low T_g' of amorphous glycine [37].

For formulations with only sucrose as lyoprotectant (F6), a similar trending towards T_{col} was observed. For Tris 100 mM and HEPES 100 mM buffer, T_g's (-31.4ºC and -30.6ºC, respectively) were approximately 5 to 6 degrees below the respective T_{col} (-25.9ºC and -25.2ºC, respectively), being close to sucrose T_g' (-32ºC) [38]. For arginine 50 mM buffer, interestingly, T_g' and T_{col} were -26.3ºC and -20.7ºC, respectively. This suggests a general crystallinity increase in the formulation with arginine.

For formulations with mannitol and glycine 1:1 (w/v) (F5), T_{col} was higher than respective T_g's, with a difference of 4, 10 and 6 degrees for F5A (-35.2ºC and -39.2ºC), F5T (-31.6ºC and -42.3ºC) and F5H (-36.1ºC and -41.9ºC), respectively. The low T_g' around -40ºC suggests a crystallinity reduction possibly by crystallization inhibition of glycine and mannitol, to some degree, caused by each other, depending on the buffer system [36].

Similarly, for formulations containing mannitol and trehalose 4:1 (F1), T_{col} was higher than T_g', regardless of the buffer system. The differences were 7, 10, and 6 degrees for F1A (-25.2ºC and -31.7ºC), F1T (-30.6ºC and -40.8ºC), and F1H (-30.6ºC and -36.4ºC), respectively, which is consistent to high protein concentration formulations behavior. The low T_g' from F1T and F1H could be due to the low T_g' from Tris and HEPES. Another possibility is the inhibition of mannitol crystallization, which presents a low T_g' in the amorphous state, by the CSA concentration. Jena and colleagues [39] reported that on lyophilization of mannitol, trehalose, and BSA formulations with annealing treatment, trehalose and BSA proportion considerably impact crystallization of mannitol and trehalose itself. An increase in BSA concentration caused a decrease in the formulation crystallinity as a whole.

For formulations with mannitol and sucrose 4:1 (F3), T_{col} was higher than the respective Tg' regardless of the buffer system. F3A (-29.3ºC and -32.6ºC, respectively) had a difference of 3 degrees, and F3T (-30.6ºC and -37.3ºC, respectively) and F3H (-27ºC and -34.5ºC, respectively) had a difference of approximately 7 degrees, which is consistent with a high protein concentration formulation, especially for F3T and F3H. The low T_g' of F3T (-37.3ºC) can be related to the presence of amorphous mannitol, caused by crystallization inhibition by sucrose [40]; or be related to the low T_g' of Tris buffer.

Besides having a low T_g', the retention of amorphous mannitol on the lyophile increases the chances of mannitol crystallization during product storage, leading to a release of unfrozen water associated with amorphous mannitol, compromising the stability of the API and excipients. Moreover, if an excipient is retained as a hydrate in the lyophilized formulation, dehydration may occur during storage and the consequent release of water of crystallization compromising the stability of the formulation as a whole [41,42].

After the addition of an annealing step (except for pure albumin and 5% sucrose formulations), similar differences were observed regarding critical temperatures depending on the buffer system and excipients used. Interestingly, for formulations with trehalose and mannitol 1:4 (w/v) (F1) regarding the buffer system, a considerable increase on T_g' and T_{col} was not observed after annealing treatment. This was unexpected as annealing promotes an additional mannitol crystallization and, therefore, increases the critical temperatures [25]. Similar behavior was observed for formulations with sucrose and mannitol 1:4 (w/v) (F3) regardless of the buffer system. Thus, it is suggested that the amorphous compound (CSA, trehalose, or sucrose) could influence the physical state of the crystalline compound (mannitol) even after annealing [39,40].

In arginine 50 mM buffer, formulations with trehalose and glycine 1:4 (w/v) (F2A), and sucrose and glycine 1:4 (w/v) (F4A), had an expressive difference of 20 to 23 degrees between T_g' before and after the annealing step, also there was a considerably higher T_{col} on samples that after the thermal treatment. After annealing, T_{col} had an increase around 19 and 23 degrees for F2A (-34.2ºC to -15.4ºC) and F4A (-34.5ºC to -11.6ºC), respectively. Similarly, for the same formulations in Tris 100 mM buffer (F2T and F4T), T_{col} increased around 12 degrees for both formulations (-30.4ºC to -18ºC for F2T, and -32.5ºC to -20.5ºC for F4T); and for T_g', an increase around 14 degrees for F4T (-44.0ºC to -29.1ºC), and an increase of around 4 degrees for F2T (-33.4ºC to -29.6ºC). In HEPES 100 mM buffer, for F2H and F4H, there was an increase of approximately 15 degrees for T_{col} (-35.3ºC to -20.1ºC, and -36.8ºC to -21.3ºC, respectively). Possibly, the annealing step promoted additional crystallization of glycine, and therefore, increased the critical temperatures of these formulations [25].

For formulations with glycine and mannitol 1:1 (w/v) (F5), an interesting behavior was observed. In general, a difference of 3 to 6 degrees in T_{col} was observed after annealing. For Tris 100 mM buffer (F5T), surprisingly, an approximately 3-degree reduction in T_{col} was observed after annealing (-31.6ºC to -34.3ºC), while with arginine 50 mM and HEPES 100 mM, there was an increase in T_{col} (-35.2ºC to -30.0ºC, and -36.1ºC to -30.5ºC, respectively). This suggests the influence of the buffer system upon the crystallinity of the formulations as Tris could be inhibiting the crystallization of mannitol and glycine even after annealing. Moreover, mannitol can influence the crystallinity degree of glycine; such that amorphous glycine is retained in the final dry matrix [36], thus maintaining T_g' and T_{col} around -30ºC.

For formulations with only 5% (w/v) mannitol or glycine (F7 and F8, respectively), a distinct variation on critical temperatures after annealing was observed depending on the buffer system. For the formulation in arginine 50 mM buffer with mannitol (F7A), an increase in T_g' around 14 degrees was observed (-35.1ºC to -21.4ºC), contrasting to F7T and F7H, which presented a minor increase in T_{col} of only 4 degrees approximately (-41.4ºC to -37.3ºC, and -31.5ºC to -27.2ºC, respectively), without a considerable difference in respective T_g'. Formulations with glycine (F8) also differ depending on the buffer system. For F8A, no considerable difference in critical temperatures was observed after annealing. However, for F8T, there was an increase of approximately 18 degrees on T_{col} after annealing (-35.6ºC to -18.0ºC). For F8H, a decrease of around 4 degrees in T_{col} was observed (-22.5ºC to -26.4ºC). Again, this suggests the influence of the buffer system upon the crystallinity of the formulations.

Several reports have shown that the API (e.g., protein), the bulking agent (mannitol and/or glycine), the lyoprotectant (sucrose or trehalose), and the buffer of a formulation, can inhibit the crystallization of the compounds themselves depending on the concentration and ratio [22,36,39,40], and therefore, influencing the critical temperatures.

However, the use of an X-ray diffraction analysis is essential for the confirmation of the hypothesis about the crystalline state of the excipients in the evaluated formulations. Moreover, although the inclusion of an annealing step did not present a measurable benefit on T_g' and T_{col} for some formulations, this thermal treatment can increase the size of the ice crystals via Ostwald ripening, increasing sublimation rates and, therefore, decreasing primary drying time due to the formation of pores with a larger diameter and less resistance to vapor flow [25]. Although, for the evaluation of the addition of a post-freezing treatment, the use of scanning electron microscopy (SEM) and determination of porosity in freeze-dried solids is necessary. Therefore, the lack of these techniques in our study is a limitation that will be addressed in further studies.

From all the formulations evaluated, arginine buffer with glycine 5% (w/v) had the highest critical temperatures without annealing (T_g' of -16.2ºC and T_{col} of -9.2ºC).

DSC profiles (heating ramp) of formulations in arginine 50 mM buffer with sucrose 1% (w/v) and mannitol 4% (w/v) (F3A) and sucrose 1% (w/v) and glycine 4% (w/v) (F4A) before and after annealing step (second heating ramp) are presented in Figure

1 and 2. Figure 3 shows determination of T_{mcol} and T_{col} for the formulation in arginine buffer with trehalose 1% (w/v) and mannitol 4% (w/v) (F1A) by FDM.

4.3.3. Cake appearance, residual moisture, and reconstitution time

After lyophilization, cakes were visually evaluated and classified according to their defects (Table 3). On visual inspection, uniform shrinkage of the cake and cracks were observed in all lyophilized formulations, with some formulations (Figures 4E, 4F, and 4G) presenting a total loss of contact with the wall and bottom of the vial. This can be due to a great unfrozen water content remaining on formulation before desorption during secondary drying [43]. Nonetheless, according to Patel and colleagues [26], in general, lyophilized cake shrinkage and cracking do not impact product quality attributes and are acceptable appearance characteristics.

Similarly, Garidel and Presser [30] reported an association between increasing protein concentration and cracking. Lyophilized cakes with a high concentration of a monoclonal antibody had larger cracks and cake fragments compared with formulations with less concentrated formulations. For some formulations (Table 3), macro-collapse, in which there is an important shrinkage and loss of structural integrity of the cake, was observed. Additionally, some vials presented dried material between vial and stopper and minor splashing on the wall of the vial. These irregularities could be a result of the manual filling of the vials and are considered a critical defect for the risk of a failure to complete sealing the vial and therefore compromising the sterility assurance of the product and deliverable dose [26].

Although the evaluation of a freeze-dried product appearance is subjective, as it only provides information on the macroscopic structure, the appearance of a lyophilized cake often goes paired with activity and stability. A lyophilized cake with macroscopic loss of structure often contains a higher level of residual moisture and has a lower specific surface area, which leads to an increased reconstitution time, and potential degradative reactions along with the storage [44]. However, it has been reported that for some protein formulations, a small degree of collapse did not compromise the activity of the product, and did not affect reconstitution time [45].

Several authors have already evaluated the effect of residual moisture on the physical and chemical stability of lyophilized protein formulations [46–48]. In most cases, the high residual moisture content in lyophilized protein formulations can accelerate

several degradation pathways, since water acts as a plasticizer, decreasing the glass transition temperature of the lyophilized product (T_g) and thus increasing the degree of molecular mobility [47].

With exception of F1A and F7A, residual moisture from lyophilized samples was below 2%. Small variations in moisture content were predicted depending on the formulation composition and by the variability on the vial position by the freeze-dryer shelf. There is point-to-point temperature variation on the shelf added to the effect of a greater heat transfer by radiation to the vials positioned on the sides and close to the freeze-dryer chamber door [49].

Surprisingly and despite such differences, the reconstitution time for all formulations without exception was immediate, even with the high albumin concentration in the evaluated formulations. Some degree of collapse and larger cracks may have improved the wettability of the freeze-dried cakes, thus reducing reconstitution time [45]. However, additional studies that correlate the reconstitution time with cake porosity, such as SEM, specific surface area (BET), and mercury intrusion porosimetry (MIP), are necessary to validate this hypothesis.

It is important to emphasize that the reconstitution time can be related to the freezing step during lyophilization. Beech and colleagues [50] reported longer reconstitution times for BSA 50 mg/ml formulations quench cooled with liquid nitrogen, despite having the largest surface area. The authors concluded that the surface area was not a predominant factor in determining the reconstitution time of the lyophilized material, but the presence of closed pores and the pore size of the dry matrix. Furthermore, the opening and connectivity of the pores were considered important to reduce the resistance to vapor flow during the drying steps. It was further highlighted that a more open network of pores could also allow better penetration of the diluent into the dry freeze-dried product.

4.3.4. SDS-PAGE

Fragmentation and/or aggregation of the lyophilized samples were assessed by SDS-PAGE electrophoresis gel in reducing conditions. SDS-PAGE gel showed that, in all formulations, canine albumin remained intact (Figure 5). The presence of other bands with lower molecular weight can be related to the albumin preparation process itself as the purity was not 100%. The same band pattern was observed for all the formulations evaluated before and after lyophilization, indicating no considerable changes in the

protein integrity caused by the lyophilization and after resuspension of canine albumin, regardless of the buffer system and excipients used in each formulation of this study.

4.3.5. Structural analyzes by Raman spectroscopy

Raman spectroscopy is used for analyzing samples in a non-invasive way, thus not damaging the sample to be analyzed. It is used in lyophilization studies to analyze secondary structural changes [51–53]. Therefore, this technique was used to compare the integrity of CSA before and after the lyophilization process. For this purpose, formulations containing only CSA in the three buffers solutions (F9A, F9T, and F9H) were analyzed before and after lyophilization.

Figure 6 shows the Raman spectra obtained from the formulations F9A, F9T, and F9H before lyophilization. To the best of the authors knowledge, there are no studies about the characterization of the Raman spectra of CSA. However, it is noticeable that regardless of the buffer used in lyophilization, the spectral pattern of CSA is very similar to other albumins, such as bovine and human albumin. Both bovine and human albumin have the assignment of its bands been well described [54,55]. Therefore, the assignment of CSA Raman peaks can be made using the current literature. The tentative assignment of the peaks obtained in the Raman spectrum of the samples is presented in Table 6. It is noticeable in the sample with HEPES 100 mM buffer a peak at 1037 cm^{-1} that could be from the buffer itself [56].

The fingerprint region of the protein is located at 800-1800 cm^{-1} frequency. The compounds of the secondary structure are typically associated with the amide I region (1600-1700 cm^{-1}), the amide III region (1230-1340 cm^{-1}), and C-C stretching bands (890-1060 cm^{-1}) of the protein backbone [57]. Amide I band is due to the in-plane C=O stretching vibration, weakly coupled with C-N stretching and in plane N-H bending [58,59]. Amide III band correlates mainly to the in-phase combination of N-H in-plane bending and C-N stretching vibrations [60,61].

However, the integrity analysis of the secondary structure of CSA focuses mainly on the α-helix content because approximately 66% of the amino acids in CSA are folded into this structure [62]. The main α-helix regions are located primarily at 1650 cm^{-1} (in amide I region), at 1320 cm^{-1} (in amide III region), and at 938 cm^{-1} (attributed to α-helical skeletal by C-C-N stretching) [51,52,54]. Therefore, these 3 regions will be compared before and after lyophilization for secondary structural changes in CSA.

From Figure 7 that compares the samples pre- and post-lyophilization, it is noticeable that the spectrum pattern was maintained in all samples (amide I, amide III, and α-helical skeletal) without frequency shift or general broadening of the bands, hence, the secondary structure was maintained upon rehydration of the lyophilized albumin or, at least, there were not irreversible disturbances in the secondary structure of the CSA. This result corroborates with Hédoux and colleagues [51] report, that used real-time micro-Raman spectroscopy during BSA lyophilization. They found structural changes on BSA only during primary drying and those were reversible upon rehydration. Albumins are robust proteins in general as albumin itself is used as a cryo- and lyoprotectant on freeze-drying formulations and may act as a stabilizer for other proteins [21,28,63], which justifies the few structural changes on the samples after lyophilization even without lyo- or cryoprotectants.

4.4. Conclusions

An influence of the buffer system on the critical temperatures (T_g', T_{col}) evaluated by DSC and FDM was evidenced. Moreover, the influence of the interactions between excipients related to the crystalline state of bulking agents in the formulation characterizations was observed as well. Within all evaluated formulations, canine albumin with arginine 50 mM buffer with 5% glycine (w/v) presented the highest glass transition temperature (T_g') and collapse temperature (T_{col}), which allows a more aggressive and, consequently, faster freeze-drying cycle. Furthermore, this formulation presented pharmaceutic elegance, residual moisture below 2%, immediate reconstitution time, and maintenance of albumin's structural integrity. Albumin robustness was also observed as the secondary structure was maintained immediately after lyophilization even without lyoprotectants.

Although additional characterizations, like sample morphology and porosity, superficial area, excipients crystallinity, and protein aggregation, need to be done, the present study highlighted the importance of choosing the adequate buffer system used in CSA formulations. Moreover, the main critical temperatures necessary for the an optimized lyophilization cycle were defined as well, thus enabling the development of a product with high-performance that meets the market demand for a safe allogenic albumin solution for supplementation in hypoalbuminemic dogs.

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4.5. References

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4.6. Figures and tables

Table 1

Overview of formulation, composition, and sample assignment in the present study.

Sample	Composition					
	CSA (mg/mL)	Suc (% w/v)	Tre (% w/v)	Man (% w/v)	Gly (% w/v)	Buffer
F1A	57	-	1	4	-	pH 7.3, 50 mM Arg
F2A	57	-	1	-	4	pH 7.3, 50 mM Arg
F3A	57	1	-	4	-	pH 7.3, 50 mM Arg
F4A	57	1	-	-	4	pH 7.3, 50 mM Arg
F5A	57	-	-	2.5	2.5	pH 7.3, 50 mM Arg
F6A	57	5	-	-	-	pH 7.3, 50 mM Arg
F7A	57	-	-	5	-	pH 7.3, 50 mM Arg
F8A	57	-	-	-	5	pH 7.3, 50 mM Arg
F9A	57	-	-	-	-	pH 7.3, 50 mM Arg
F1T	57	-	1	4	-	pH 7.3, 100 mM Tris
F2T	57	-	1	-	4	pH 7.3, 100 mM Tris
F3T	57	1	-	4	-	pH 7.3, 100 mM Tris
F4T	57	1	-	-	4	pH 7.3, 100 mM Tris
F5T	57	-	-	2.5	2.5	pH 7.3, 100 mM Tris
F6T	57	5	-	-	-	pH 7.3, 100 mM Tris
F7T	57	-	-	5	-	pH 7.3, 100 mM Tris
F8T	57	-	-	-	5	pH 7.3, 100 mM Tris
F9T	57	-	-	-	-	pH 7.3, 100 mM Tris
F1H	57	-	1	4	-	pH 7.3, 100 mM HEPES
F2H	57	-	1	-	4	pH 7.3, 100 mM HEPES
F3H	57	1	-	4	-	pH 7.3, 100 mM HEPES
F4H	57	1	-	-	4	pH 7.3, 100 mM HEPES
F5H	57	-	-	2.5	2.5	pH 7.3, 100 mM HEPES
F6H	57	5	-	-	-	pH 7.3, 100 mM HEPES
F7H	57	-	-	5	-	pH 7.3, 100 mM HEPES
F8H	57	-	-	-	5	pH 7.3, 100 mM HEPES
F9H	57	-	-	-	-	pH 7.3, 100 mM HEPES

CSA, canine serum albumin; Suc, sucrose; Tre, Trehalose; Man, Mannitol; Gly, Glycine; Arg, Arginine.

Table 2
Summary of thermal properties of all canine serum albumin formulations.

Sample	Convencional thermal cycle				Thermal cycle with annealing			
	T_g' (ºC)	T_{eut} (ºC)	T_{mcol} (ºC)	T_{col} (ºC)	T_g' (ºC)	T_{eut} (ºC)	T_{mcol} (ºC)	T_{col} (ºC)
F1A	-31.7	ND	-26.9	-25.2	-31.8	-0.20	-26.0	-25.9
F2A	-42.9	0.53	-37.2	-34.2	-19.9	-0.11	-20.4	-15.4
F3A	-32.6	ND	-31.3	-29.3	-33.1	0.53	-32.3	-31.1
F4A	-38.4	-0.32	-36.6	-34.5	-18.0	-0.30	-12.5	-11.6
F5A	-39.2	ND	-36.6	-35.2	-39.8	ND	-31.0	-30
F6A	-26.3	*	-22.8	-20.7	*	*	*	*
F7A	-35.1	ND	-26.4	-24.8	-21.4	ND	-28.6	-26.6
F8A	-16.2	-0.29	-10.1	-9.2	-17.7	-1.90	-11,0	-10.6
F9A	-25.5	*	-21.9	-16.5	*	*	*	*
F1T	-40.8	2.75	-33.2	-30.6	-36.3	ND	-32.3	-31.8
F2T	-33.4	-3.25	-33.3	-30.4	-29.6	0.10	-18.6	-18
F3T	-37.3	ND	-31.7	-30.6	-36.8	8.01	-33.4	-32.7
F4T	-44.0	-0.70	-35.5	-32.5	-29.1	-0.08	-21.5	-20.5
F5T	-42.3	ND	-33.3	-31.6	-43.1	ND	-35.4	-34.3
F6T	-31.4	*	-27.9	-25.9	*	*	*	*
F7T	-38.5	7.56	-45.7	-41.4	-37.4	1.79	-39.6	-37.3
F8T	-22.2	-0.29	-36.7	-35.6	-20.9	0.04	-18.7	-18.0
F9T	-29.2	*	-32.7	-28.5	*	*	*	*
F1H	-36.4	ND	-32.8	-30.6	-36.2	0.47	-32.4	-31.2
F2H	-32.8	-0.07	-37.5	-35.3	-30.6	-0.17	-21.9	-20.1
F3H	-34.5	ND	-28.8	-27.0	-35.1	0.14	-30.8	-29.0
F4H	-33.2	-0.49	-39.6	-36.8	-29.6	0.28	-22.6	-21.3
F5H	-41.9	ND	-37.8	-36.1	-41.2	ND	-32.4	-30.5
F6H	-30.6	*	-27.3	-25.2	*	*	*	*
F7H	-35.0	ND	-35.5	-31.5	-36.2	0.49	-29.1	-27.2
F8H	-31.5	0.63	-24.5	-22.5	-30.9	-0.21	-28.7	-26.4
F9H	-33.9	*	-27.9	-25,0	*	*	*	*

T_g', glass transition temperature; T_{eut}, eutectic melting temperature; T_{mcol}, microcollapse temperature T_{col}, collapse temperature; ND, not determined; *for the amorphous formulations (canine albumin without excipients and canine albumin with sucrose) the post-freezing annealing step was not performed.

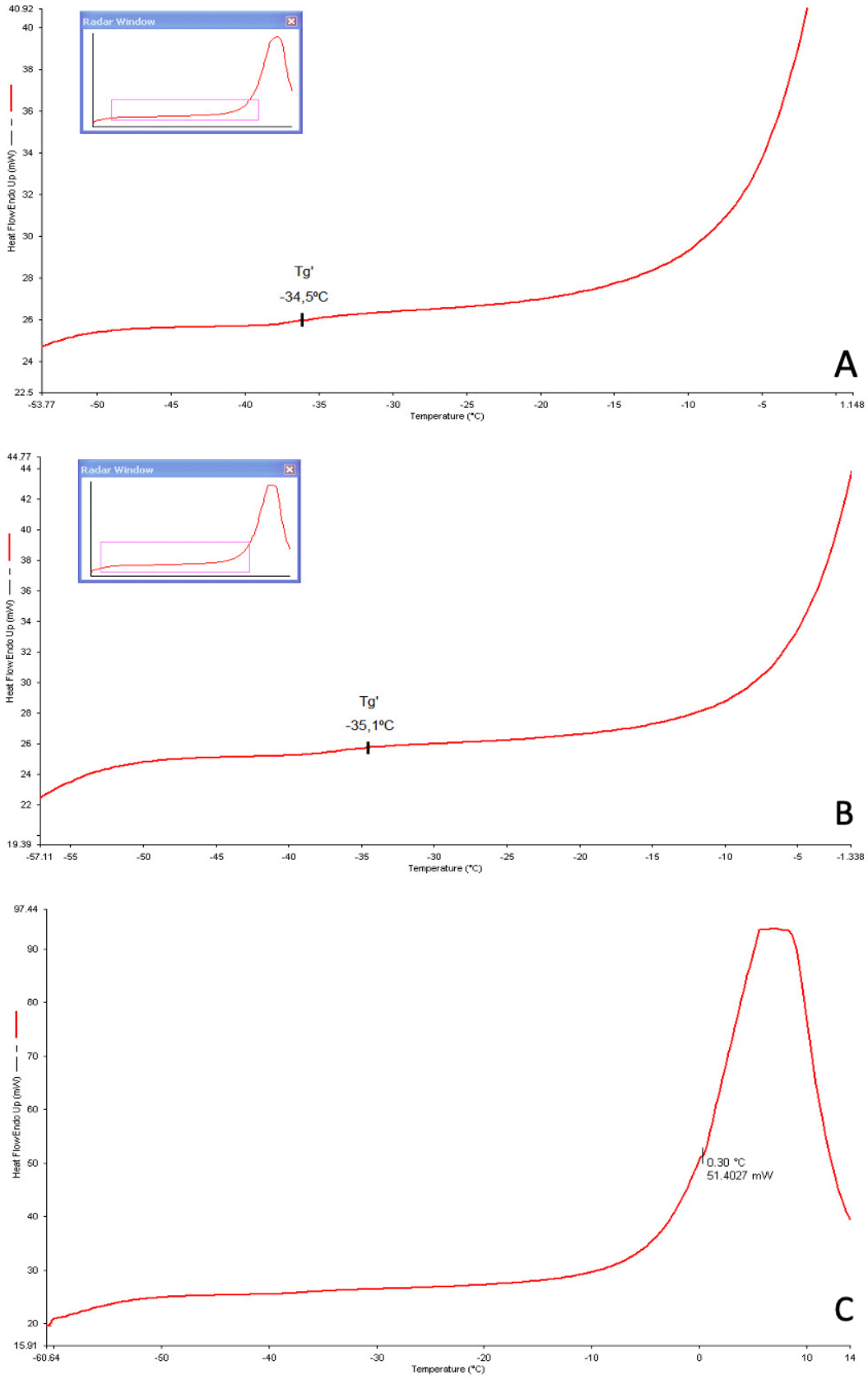
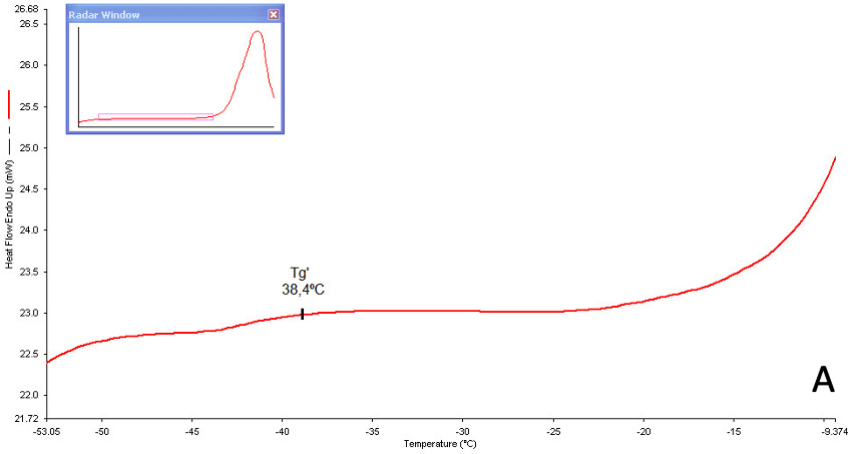
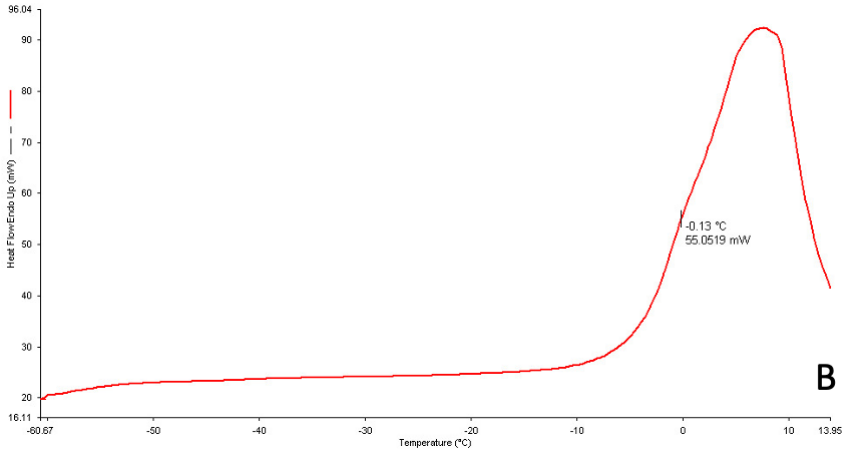
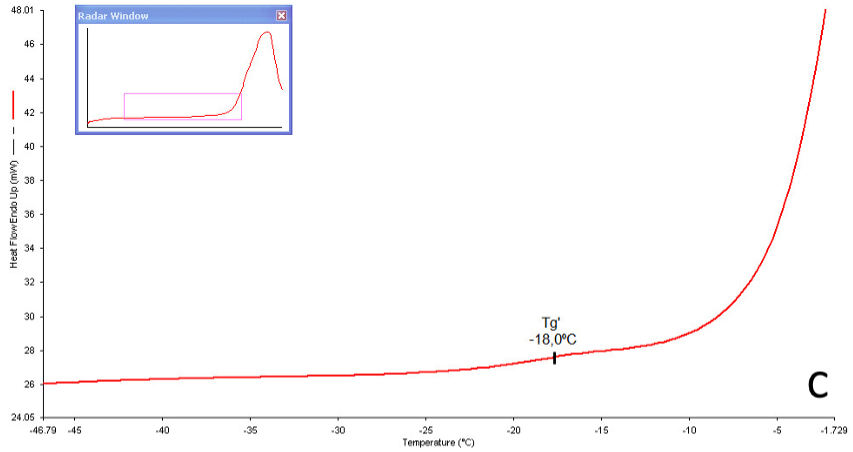


Figure 1: DSC heating profile of frozen canine albumin in 100 mM HEPES buffer with sucrose and mannitol 1:4 (% w/v). A: Curve without annealing showing T_g'; B: Curve

with annealing showing T_g'. C: Curve with annealing showing T_{eut}. The suspensions were cooled from room temperature to -60°C at 1°C/min, held for 1 min, and heated to 25°C at 20°C/min. When the annealing procedure was applied, the suspensions were cooled from room temperature to -60°C at 1°C/min, heated to the annealing temperature, annealed for 5 min, cooled to -50°C, and reheated to 25°C at 20°C/min. The second heating scan is shown here.


Radar Window
Tg'
38,4ºC
A
Heat Flow Endo Up (mW)
Temperature (°C)


-0.13 °C
55.0519 mW
B
Heat Flow Endo Up (mW)
Temperature (°C)


Radar Window
Tg'
-18,0ºC
C
Heat Flow Endo Up (mW)
Temperature (°C)

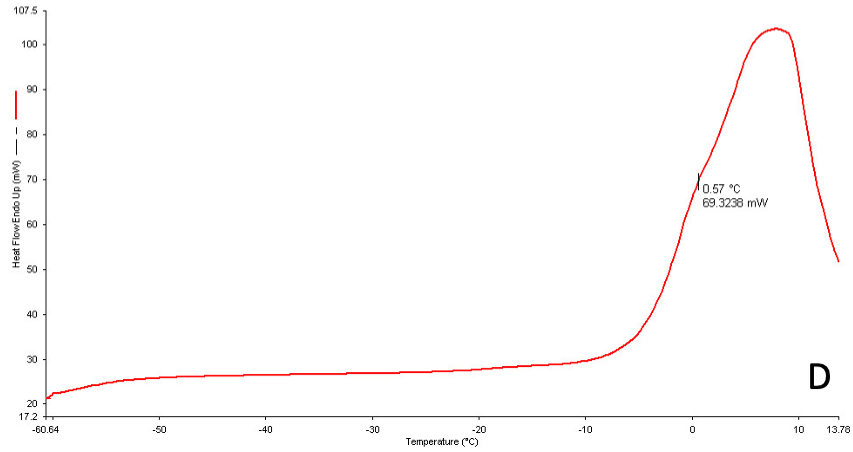

0.57 °C
69.3238 mW
D
Heat Flow Endo Up (mW)
Temperature (°C)

Figure 2: DSC heating profile of frozen canine albumin in 50 mM arginine buffer with sucrose and glycine 1:4 (% w/v). A: Curve without annealing showing T_g'; B: Curve without annealing showing T_{eut}; C: Curve with annealing showing T_g'; D: Curve with annealing showing T_{eut}. The suspensions were cooled from room temperature to -60°C at 1°C/min, held for 1 min, and heated to 25°C at 20°C/min. When the annealing procedure was applied, the suspensions were cooled from room temperature to -60°C at 1°C/min, heated to the annealing temperature, annealed for 5 min, cooled to -50°C, and reheated to 25°C at 20°C/min. The second heating scan is shown here.

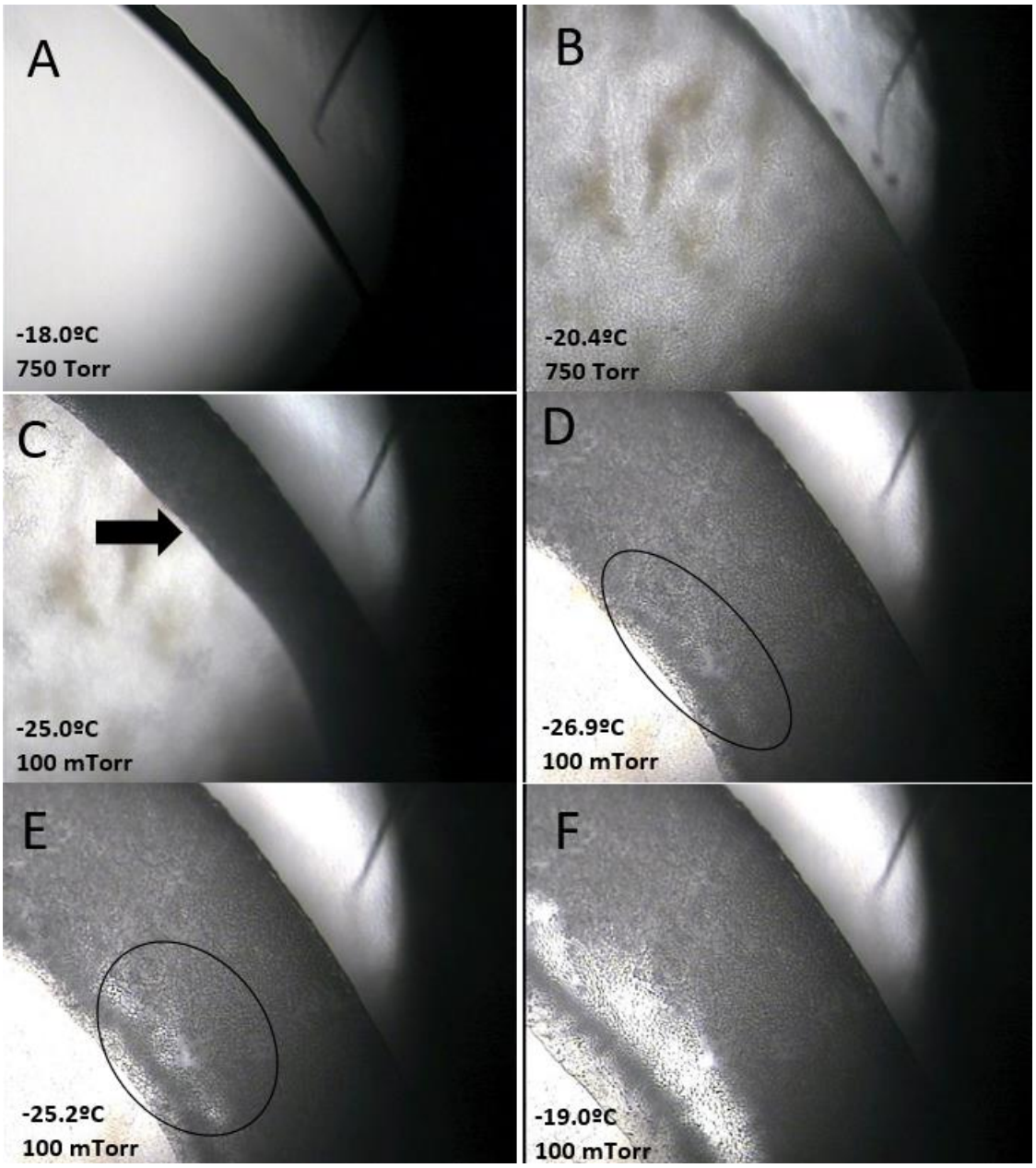


Figure 3: Determination of collapse temperature of canine albumin in 50 mM arginine buffer with trehalose and mannitol 1:4 (% w/v). A: Liquid sample; B: Frozen sample; C: Sublimation front (arrow); D: Microcollapse (circle); E: Onset of overall collapse (circle); and F: Overall collapse.

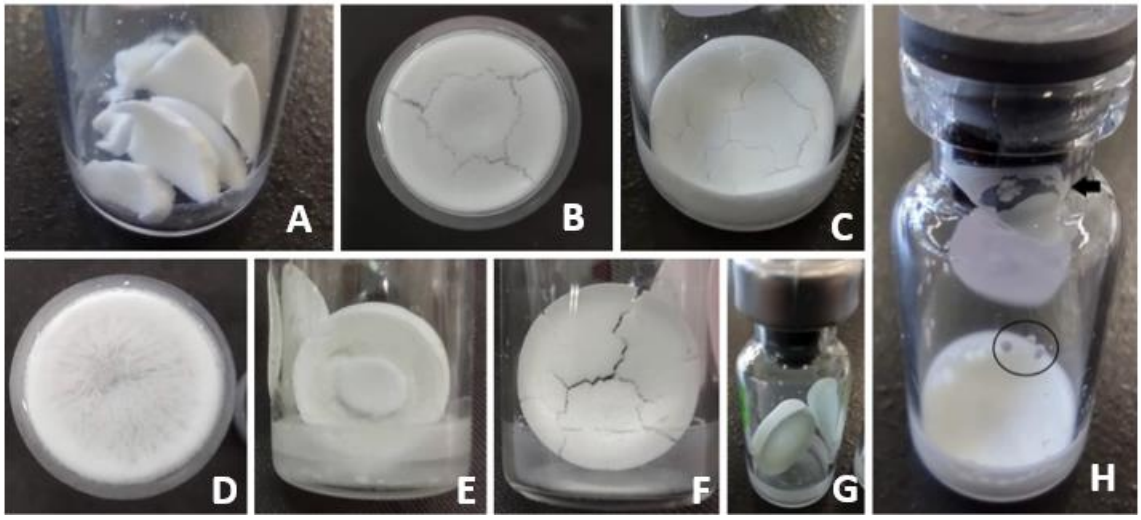


Figure 4: A: Broken cake; B: Bottom of cracked cake; C: Cracked cake; D: Bottom of collapsed cake; E: Collapsed cake with shrinkage; F: Cracked cake with shrinkage; G: Cake shrinkage; H: Dried product between vial and stopper (arrow) and minor splashing on the wall of the vial (circle). Only formulations with critical temperatures above -36°C were lyophilized since the minimum temperature reached by the freeze-dryer shelves was -40°C.

Table 3: Results of physical properties of initial lyophilized cakes

Sample	Appearance	RM (%)	RT
F1A	Cracked cake (2)	2.3±0.22	Immediate
F3A	Minor splashing on the wall of the vial (1), cake shrinkage (1)	1.3±0.1	Immediate
F6A	Collapsed cake (7), cracked cake (1), cake shrinkage (3)	1.1±0.1	Immediate
F7A	Dried product between vial and stopper (1), cake shrinkage (1)	2.1±0.7	Immediate
F8A	Cracked cake (7), minor splashing on the wall of the vial (1), dried product between vial and stopper (1)	1.0±0.3	Immediate
F9A	Cake shrinkage (1), broken cake (4), cracked cake (2)	1.7±0.3	Immediate
F2T	Collapsed cake (7), cracked cake (4), dried product between vial and stopper (1), minor splashing on the wall of the vial (1)	1.0±0.2	Immediate
F6T	Collapsed cake (7), cracked cake (1)	1.6±0.2	Immediate
F8T	Cracked cake (7)	0.9±0.3	Immediate
F9T	Cake shrinkage (5)	1.2±0.2	Immediate
F2H	Cake shrinkage (3)	0.9±0.2	Immediate
F3H	Collapsed cake (7), cake shrinkage (3)	1.0±0.1	Immediate
F6H	Collapsed cake (7), cake shrinkage (3)	1.2±0.2	Immediate
F7H	Broken cake (1), Cake shrinkage (1)	0.8±0.0	Immediate
F8H	Minor splashing on the wall of the vial (4)	0.7±0.02	Immediate
F9H	Cake shrinkage (5)	1.0±0.01	Immediate

RM, Residual moisture; RT, Reconstitution time; (N), number of vials presenting the physical appearance. RM is shown by mean±SD

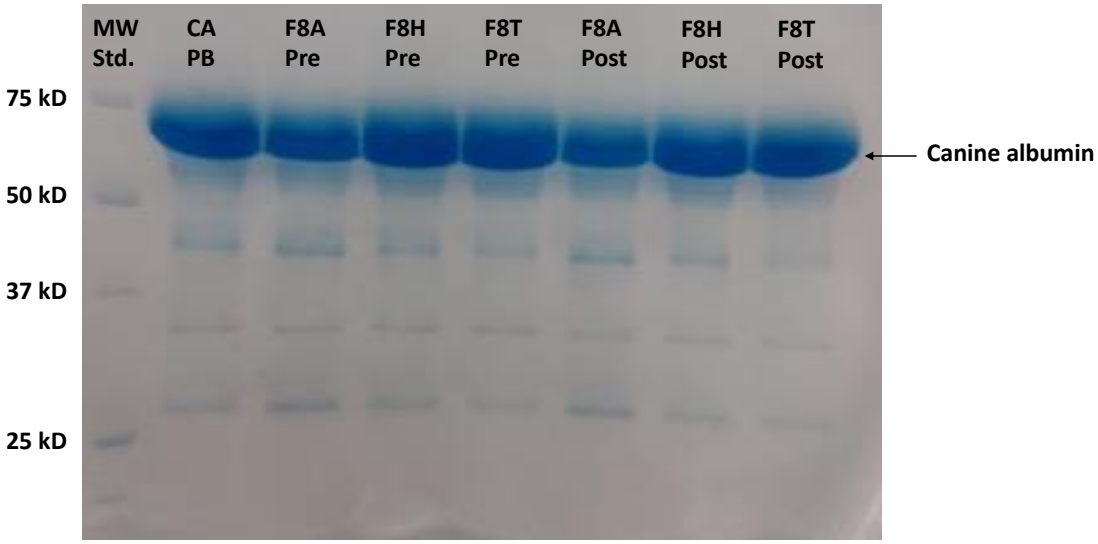


Figure 5: Reducing SDS-PAGE showing intact canine albumin (69 kDa). The same banding pattern and intensity could be seen in all the freeze-dried formulations, indicating that there are no considerable changes seen upon freeze-drying. Lane assignments: The

first lane represents MW standard. Lanes assigned as 1 represent canine albumin in phosphate buffer before buffer exchange. Lanes assigned as 2 and 5 represent F8A formulation pre- and post-lyophilization respectively. Lanes assigned as 3 and 6 represent F8H formulation pre- and post-lyophilization respectively. Lanes assigned as 4 and 7 represent F8T formulation pre- and post-lyophilization respectively.

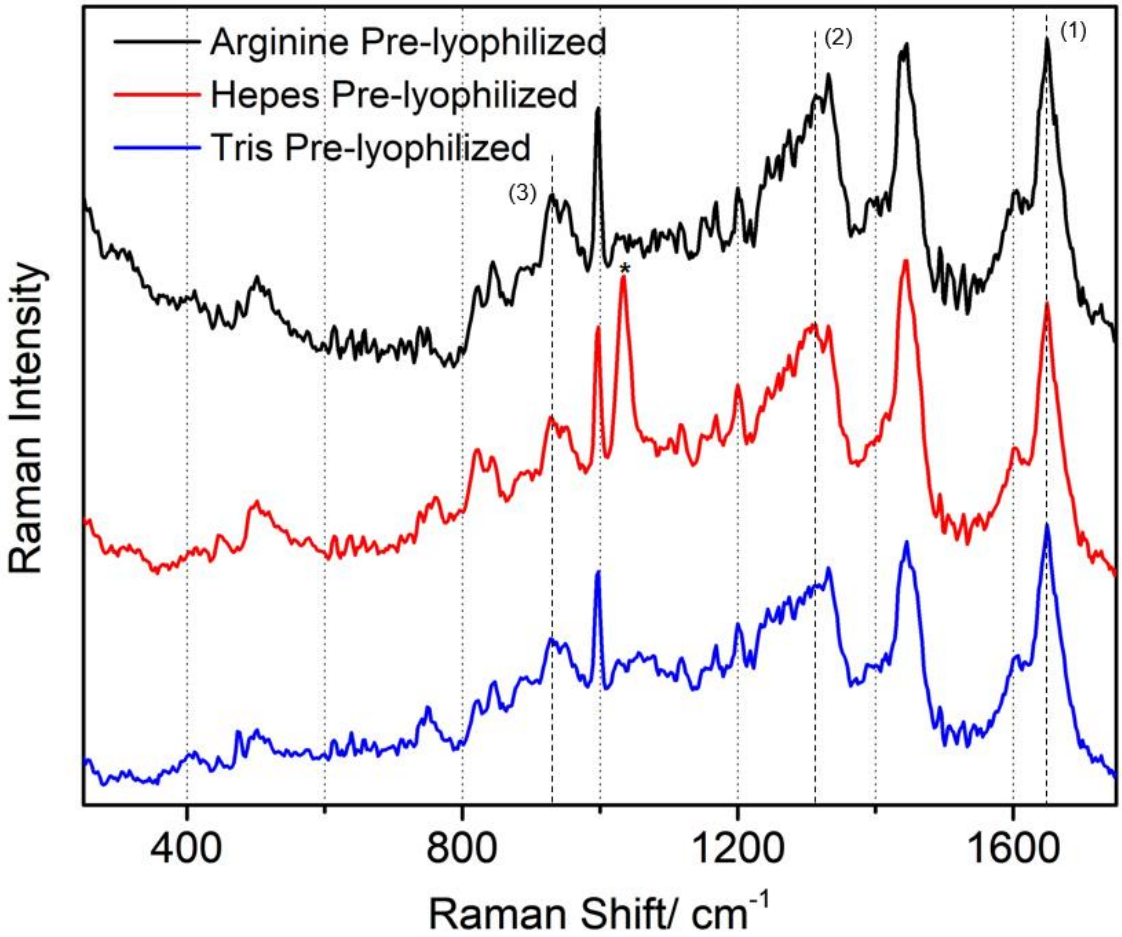


Figure 6: Raman spectra of CSA samples without cryo- and lyoprotectants before lyophilization in arginine buffer (black line), HEPES buffer (red line), and Tris buffer (blue line). The number above the dashed lines indicates the fingerprint regions assigned to α-helix content. (1) 1650 cm-1 in amide I region. (2) 1320 cm-1 in amide III band. (3) α-helical skeletal (938 cm^{-1}). * Peak from HEPES buffer at 1037 cm^{-1}.

Table 6: Tentative assignment of the main Raman peaks of CSA in each buffer system.

Band position (cm^{-1})			
Arginine buffer	HEPES buffer	Tris buffer	Tentative Assignment
502 (w)[a]	505 (w)	502 (w)	ν^d (SS)
-	755 (w)	748 (w)	Tyr, ν (CS)
824 (w)	820 (w)	824 (w)	Tyr
847 (m)[b]	843 (m)	847 (m)	Tyr
888 (w)	888 (w)	888 (w)	Trp
938 (m)	938 (m)	938 (m)	α-helical skeleton, ν (CCN),$_{sym}$
995 (m)	995 (m)	995 (m)	ring breath
-	1037 (s)	-	ν (SO_3)
1320 (s)[c]	1320 (s)	1320 (s)	δ^e (CH), Amide III
1446 (s)	1446 (s)	1446 (s)	δ (CH_2/CH_3)
1601	1601	1601	Trp, Tyr, Phe
1650 (s)	1650 (s)	1650 (s)	Amide I

[a]Weak (w), [b]medium (m), [c]strong (s), [d]stretching (ν), [e]bending (δ).

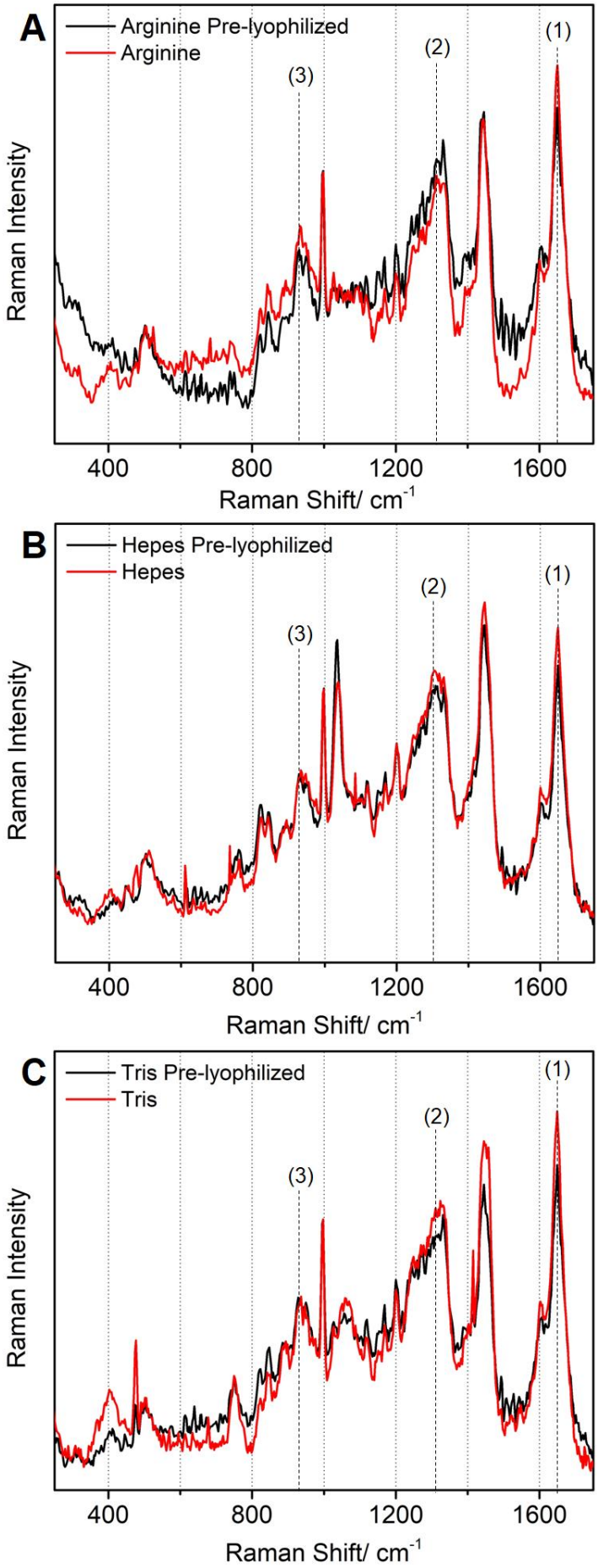

A
Arginine Pre-lyophilized
Arginine
(1)
(2)
(3)
Raman Intensity
400
800
1200
1600
Raman Shift/ cm-1
B
Hepes Pre-lyophilized
Hepes
(1)
(2)
(3)
Raman Intensity
400
800
1200
1600
Raman Shift/ cm-1
C
Tris Pre-lyophilized
Tris
(1)
(2)
(3)
Raman Intensity
400
800
1200
1600
Raman Shift/ cm-1

Figure 7: Comparison of Raman spectra of CSA pre- and after lyophilization with different buffers. A: CSA in arginine 50 mM buffer. Blackline: pre-lyophilization spectrum. Redline: post-lyophilization spectrum. B: CSA in HEPES 100 mM buffer. Blackline: pre-lyophilization spectrum. Redline: post-lyophilization spectrum. C: CSA in Tris 100 mM buffer. Blackline: pre-lyophilization spectrum. Redline: post-lyophilization spectrum. Numbers above dashed lines indicate the fingerprint regions assigned to α-helix content: (1) 1650 cm^{-1} in amide I region. (2) 1320 cm^{-1} in amide III band. (3) 938 cm^{-1} (α-helical skeletal).

5. Considerações finais

Com esse trabalho, foi possível avaliar a aplicação de técnicas de fracionamento para a produção de derivados do plasma canino, já que, apesar dessas técnicas já serem amplamente utilizadas para o fracionamento do plasma humano, este ainda é um campo não muito explorado na medicina veterinária. Os protocolos avaliados foram efetivos em purificar albumina, com a técnica com PEG combinada com filtração tangencial obtendo o maior rendimento. Além disso, foi possível realizar um estudo inédito acerca da liofilização da albumina canina que evidenciou a importância da escolha adequada do sistema tampão utilizado, bem como a identificação das principais temperaturas críticas necessárias para o desenvolvimento de um ciclo de liofilização otimizado. Também foi possível avaliar a performance do produto liofilizado em relação a elegância da pastilha, tempo de reconstituição, umidade residual e integridade estrutural. Mesmo sendo necessários mais estudos para a melhora do processo de fracionamento do plasma e acerca da estabilidade a longo prazo da albumina canina liofilizada, o estudo foi um primeiro passo para um produto derivado do plasma canino que supre uma demanda no mercado veterinário. Como perspectiva futura, pretende-se produzir uma albumina liofilizada estéril para testes clínicos na espécie canina.

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