FERNANDA LOPES DA SILVA

O USO DE SAIS DE FOSFATO NA INDÚSTRIA DE ALIMENTOS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos para obtenção do título de *Doctor Scientiae.*

Orientador: Antônio Fernandes de Carvalho

Coorientadores: Ítalo Tuler Perrone
Rodrigo Stephani

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Antônio Fernandes de Carvalho
Orientador

Eu dedico as pessoas importantes na minha vida, meus pais, meu irmão, meu namorado e meu amado afilhado.

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RESUMO

DA SILVA, Fernanda Lopes, D.Sc., Universidade Federal de Viçosa, março de 2023. **O uso de sais de fosfato na indústria de alimentos**. Orientador: Antônio Fernandes de Carvalho. Coorientadores: Ítalo Tuler Perone e Rodrigo Stephani.

Os fosfatos estão presentes em uma variedade de alimentos, isso porque na indústria de alimentos são usados como aditivos, onde servem para auxiliar o processamento, melhorar as propriedades organolépticas ou melhorar a segurança e o prazo de validade. Em produtos ricos em proteínas, como carnes, aves, frutos do mar e laticínios, onde suas principais funções são o controle do pH, agente quelante de íons metálicos, agente de retenção de água, agente tamponante, agente dispersante e/ou de suspenção e estabilizante. Sendo assim, são inúmeras as propriedades tecnológicas que agregam valor aos fosfatos, motivo pelos quais as indústrias devem se atentar a recomendação máxima de uso de acordo com a legislação. Haja vista a crescente atenção a quantidade adequada e segura para consumo de fosfato, recentes estudos vêm demonstrando que o consumo excessivo de fosfatos estão gerando doenças, e alguns chegam até a questionar se o fosfato não seria o novo sódio. Então a aplicação dos sais de fosfatos pela indústria deve ocorrer de forma consciente e utilizando o mínimo possível para ter o resultado desejado. Dessa forma, este estudo buscou realizar a aplicação de fosfatos em diferentes produtos alimentícios, como chá preto adoçado com stevia, queijo Minas Frescal e mistura láctea condensada, sempre buscando identificar as quantidades limítrofes necessárias para ter o resultado desejado. A aplicação em chá preto foi com o intuito de reduzir o sabor residual da stevia, e como demonstrado foi possível determinar os limiares hedônicos de aceitação e rejeição para uma mistura de sais de fosfato, sendo possível dessa forma, a indústria decidir qual a composição em que ela quer trabalhar utilizando esses fosfatos, e ainda tendo uma redução do sabor residual e uma aceitação do produto. Já quando aplicado a produção do queijo Minas Frescal em substituição parcial ou total do cloreto de cálcio, o MCP e um blend de fosfatos apresentaram composição e rendimento igual ao controle produzido somente com cloreto de cálcio. Demonstrando assim que os fosfatos de cálcio também são uma boa alternativa de substituição do cloreto de cálcio na fabricação do queijo Minas Frescal. Para a calda da mistura láctea condensada, etapa essa antes da concentração, os

fosfatos também foram aplicados, agora sendo uma mistura industrial de sais de fosfato. E o que se observou é que uma mistura de fosfatos de sódio e citrato de sódio promoveu uma melhor estabilidade térmica do produto, o que é muito interessante, visto que demonstra que problemas na concentração podem não acontecer, como a precipitação de proteínas devido ao calor. Já quando aplicamos uma mistura composta por ortofosfatos, polifosfatos e citrato de sódio o que se observa é um alto poder sequestrante de cálcio da mistura, isso devido a presença de polifosfatos que tem essa característica. Independente de qual produto os fosfatos foram aplicados, eles apresentaram boas características e aplicabilidade, permitindo a indústria gerar novas possibilidades de aplicação desses sais em diferentes produtos.

Palavras-chave: Fosfatos. Aplicação. Produtos lácteos.

ABSTRACT

DA SILVA, Fernanda Lopes, D.Sc., Universidade Federal de Viçosa, March, 2023. **The use of phosphates in food industry**. Adviser: Antônio Fernandes de Carvalho. Co-advisers: Ítalo Tuler Perone and Rodrigo Stephani.

Phosphates are present in variety of foods, because in the food industry they are used as additives, where they serve to aid processing, improve organoleptic properties or improve safety and shelf life. In protein-rich products, such as meat, poultry, seafood and dairy products, where their main functions are pH control, metal ion chelating agent, water retention agent, buffering agent, dispersing agent and/or suspension and stabilizer. Therefore, there are numerous technological properties that add value to phosphates, which is why industries must pay attention to the maximum recommendation for use in accordance with legislation. In view of the growing attention to the adequate and safe amount of phosphate to consume, recent studies have shown that the excessive consumption of phosphates is causing diseases, and some even question whether phosphate would not be the new sodium. So the application of phosphate salts by the industry must occur consciously and using as little as possible to get the desired result. Thus, this study sought to carry out the application of phosphates in different food products, such as black tea sweetened with stevia, Minas Frescal cheese and condensed milk mixture, always seeking to identify the borderline amounts necessary to have the desired result. The application in black tea was intended to reduce the residual taste of stevia, and as demonstrated, it was possible to determine the hedonic thresholds of acceptance and rejection for a mixture of phosphate salts, thus making it possible for the industry to decide which composition in that it wants to work using these phosphates, and still having a reduction in aftertaste and product acceptance. When applied to the production of Minas Frescal cheese in partial or total replacement of calcium chloride, MCP and a blend of phosphates showed composition and yield equal to the control produced only with calcium chloride. Thus, demonstrating that calcium chloride in the manufacture of Minas Frescal cheese. For the syrup of the condensed dairy mixture, this step before concentration, phosphates were also applied, now being an industrial mixture of phosphate salts. And what was observed is that a mixture of phosphate salts and sodium citrate promote better thermal stability of the product, which is very interesting, since it demonstrates

that concentration problems may not occur, such as the precipitation of protein due to heat. When we apply a mixture composed of orthophosphates, polyphosphates, and sodium citrate, what is observed is a high calcium sequestration power in the mixture, due to the presence of polyphosphates that have this characteristic. Regardless of which product the phosphates were applied to, they showed good characteristics and applicability, allowing the industry to generate new possibilities for applying these salts in different products.

Keywords: Phosphates. Aplication. Dairy products.

SUMÁRIO

1. INTRODUÇÃO GERAL

Os fosfatos são derivados do ácido fosfórico (H_3PO_4), podendo formar fosfatos inorgânicos quando estão ligados à íons carregados positivamente, como o sódio, ou formar fosfatos orgânicos, quando ligados com grupos orgânicos, como o fenil; e independente da forma como se encontram estão presentes na maioria dos organismos vivos (Dykes *et al.,* 2019).

Os fosfatos são classificados como ortofosfatos, pirofosfatos, trifosfatos ou polifosfatos, dependendo do número de grupos PO_4^{-3} presentes, sendo os ortofosfatos ou monofosfatos compostos por um grupo, os pirofosfatos ou difosfatos são compostos por 2 grupos, os trifosfatos ou tripolifosfatos apresentam 3 grupos, enquanto os polifosfatos apresentam mais de 3 grupos PO_4^{-3} (McBeath *et al.,* 2007; Dimitrelli *et al.,* 2005*)*. De acordo com o tamanho da cadeia os fosfatos apresentam características tecnológicas diferentes, onde os mono e difosfatos apresentam alto poder tamponante, sendo mais utilizados como agentes para correção de pH (Schär e Bosset, 2002; Guinee *et al.,* 2004; Mizuno e Lucey, 2005). Entretanto, os polifosfatos apresentam alta capacidade quelante de íons cálcio e outros íons, então são mais utilizados para aumentar a dispersão das moléculas de caseína ou quando é necessário complexar íons metálicos (Schär e Bosset, 2002; Guinee *et al.,* 2004; Mizuno e Lucey, 2005). Como apresentam essas características diferentes as indústrias utilizam misturas desses sais, de forma a terem tanto a capacidade tamponante quanto de sequestrante de cálcio, de acordo com a aplicação final desses sais e as características desejadas do produto (Schär e Bosset, 2002; Guinee *et al.,* 2004).

No entanto, os fosfatos sofrem hidrólise em solução, e a velocidade de hidrólise depende da concentração do fosfato, temperatura, pH e presença de H^+, que agem como catalisador (Lampila e Godber, 2001; McBeath *et al.,* 2007; Schen e Morgan, 1973). A Figura 1 ilustra o processo de hidrólise dos polifosfatos. Esse processo de hidrólise deve ser acompanhado, pois pode implicar em possível perda de função, visto que como comentado, pode acontecer a perda da capacidade quelante de íons, principalmente o Ca^{+2}, quando ocorre a hidrólise do polifosfato, para di- ou monofosfato, e dessa forma, pode resultar em produtos com características diferentes das desejadas (Molins, 1991). Os polifosfatos são muito estáveis em solução neutras mesmo em temperaturas superiores a 100 ºC (Halliwell *et al.,* 2001;

Torres-Dorante *et al.,* 2005), no entanto, quando ocorre a diminuição do pH, aumentando a concentração de H^+ em solução, a velocidade de hidrólise aumenta, principalmente em presença de Ca^{+2}, podendo originar ortofosfatos como produto final da reação (Jager e Heyns, 1998).

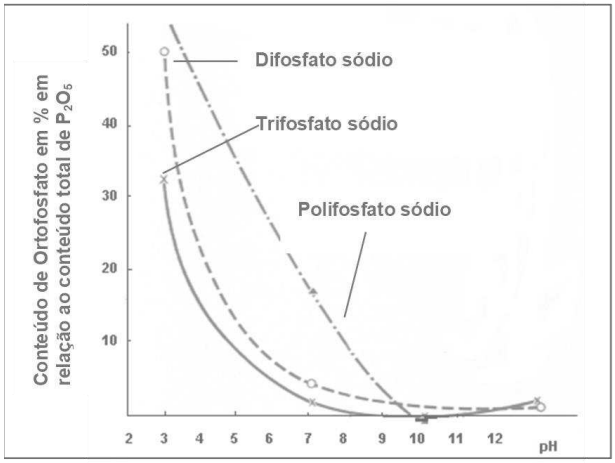

Figura 1 – Hidrólise dos fosfatos em função do pH (Fonte: Maurer-Rothmann e Scheurer, 2005).

Os fosfatos inorgânicos são usados como aditivos em uma variedade de produtos alimentícios, onde servem para auxiliar o processamento, melhorar as propriedades organolépticas ou melhorar a segurança e o prazo de validade. Eles estão presentes em uma variedade de alimentos, incluindo tipicamente produtos ricos em proteínas, como carnes, aves, frutos do mar e laticínios, suas principais funções podem ser: controle de pH, agente complexante de íons metálicos, agente de retenção de água, tampão, agente dispersante e/ou suspenção e estabilizante (Dykes *et al.,* 2019; Gonçalves e Ribeiro, 2008; Whiting, 2010; Xiong *et al.,* 2010). Na indústria de alimentos os fosfatos são utilizados em diferentes áreas, como em produtos cárneos, pães e massas, bebidas, laticínios etc.

Esses fosfatos de qualidade alimentar ajudam a manter a estrutura e a hidratação dos produtos cárneos, aumentando a capacidade de retenção de água do músculo e de íons metálicos pró-oxidativos, isso porque eleva o pH da carne, aumentando assim a força iônica e consequentemente a capacidade de retenção de água (Lampila, 2013; Vasavada *et al.,* 2006). Dessa forma, são utilizados de forma a reduzir a perda devido ao cozimento, controlar o crescimento de patógenos, reduzir a perda por gotejamento e purga no armazenamento refrigerado e melhorar as propriedades de textura das carnes (Lindsay, 2008; Lampila, 2013). Na indústria de carnes e aves, os fosfatos mais utilizados são os polifosfatos ou pirofosfatos, que

atuam sequestrando os íons metálicos e dessa forma, fazendo a dissociação do complexo actomiosina (Fonseca *et al.,* 2011), além disso, um estudo demonstrou também que devido ao sequestro de ferro e cobre tem a capacidade de retardar o ranço em alguns produtos (Coultate, 2009).

Na indústria de pães e massas, o fosfato é utilizado como agente fermentador, melhorador da qualidade da farinha, aumentando a elasticidade do macarrão e reduzindo rachaduras na massa (Liu *et al.,* 2016; Tan *et al.,* 2009, Wu *et al.,* 2006). Como agente fermentador, é um dos ingredientes do fermento químico em pó, que neutraliza o bicarbonato de sódio ou potássio, e que controla a taxa de liberação de dióxido de carbono da fermentação química. Os fosfatos também têm a capacidade de fortalecer a rede de glúten durante a preparação da massa, promovendo o aumento da retenção de água e retardando a descoloração do macarrão fresco, isso porque promove a gelatinização do amido e reduz a perda por cozimento (Chen *et al.,* 2019; Fu, 2008; Tan *et al.,* 2009). Outros estudos vêm mostrando a aplicação de sais de fosfato em alimentos à base de trigo, mostrando que o fosfato monossódico, fosfato dissódico e fosfato dipotássio promoveram no macarrão uma aparência mais brilhante e amarela, aumentando a viscosidade de pico da farinha de trigo (Wang *et al.,* 2011); além disso, os fosfatos diminuíram a dureza e aumentaram ligeiramente a elasticidade, coesividade e resiliência de macarrão de trigo integral (Niu *et al.,* 2014).

Em bebidas, os fosfatos fazem parte da composição de refrigerantes, para controle da acidez da solução, oxidação de metais e preservação do produto (Biggs *et al.,* 2017). Além de ser usado também para aprimoramento do sabor e como modificador do pH de refrigerantes. A redução do pH melhora a estabilidade e vida útil do produto, pois atua na diminuição do crescimento microbiano. Além disso, os fosfatos podem ser usados em outras bebidas, como chás, shots e bebidas à base de frutas para estabilizar os componentes de frutas, a cor e a clareza da bebida (Dubey *et al.*, 2020).

Nos produtos lácteos, os fosfatos são usados como dispersante de proteínas em produtos lácteos secos, além de atuarem como sais de fusão para o processamento de queijo processado, como agentes de acificação, gelificação, suplementação de nutrientes, quelantes de cálcio e agente que auxiliam a emulsificação (Dykes *et al.,* 2019; Kapoor e Metzger, 2008). Uma das principais atuações dos fosfatos em laticínios, é a fabricação de queijos processados, como sais emulsificante/fundentes, como são chamados, atuando como sequestrante do cálcio

da rede cálcio-caseína-fosfato e doando o íon sódio, como pode-se ver na Figura 2 (Buňka *et al.,* 2014; Chen e Liu, 2012; Berger *et al.,* 1997). Como resultado, as principais forças moleculares que ligam os vários monômeros de caseína são parcialmente rompidas, esta ruptura leva à hidratação e dispersão da proteína (Guinee *et al.,* 2004; Chen e Liu, 2012). Os monômeros parcialmente dispersos da caseína, semelhantes a qualquer outra proteína, possuem porções hidrofílicas e hidrofóbicas que agora estão livres para interagir com a fase aquosa e com a fase gorda, respectivamente, levando a suas melhores propriedades de emulsificação (Mozuraityte *et al.,* 2019; Kapoor e Metzger, 2008). Durante a fabricação dos queijos processados, os sais emulsificantes desempenham um papel crucial, de forma a ter um produto homogêneo e com a consistência desejada (Guinee *et al.,* 2004; Kawasaki, 2008; Lee *et al.,* 2003). Em particular, o papel chave é o sequestro de Ca, levando ao desenvolvimento do paracaseinato de Na, que é mais solúvel, que por sua vez pode atuar como um emulsificante ativo, além de atuar também na emulsificação e estabilização da gordura presente na matriz dos queijos processados (Buňka *et al.,* 2014).

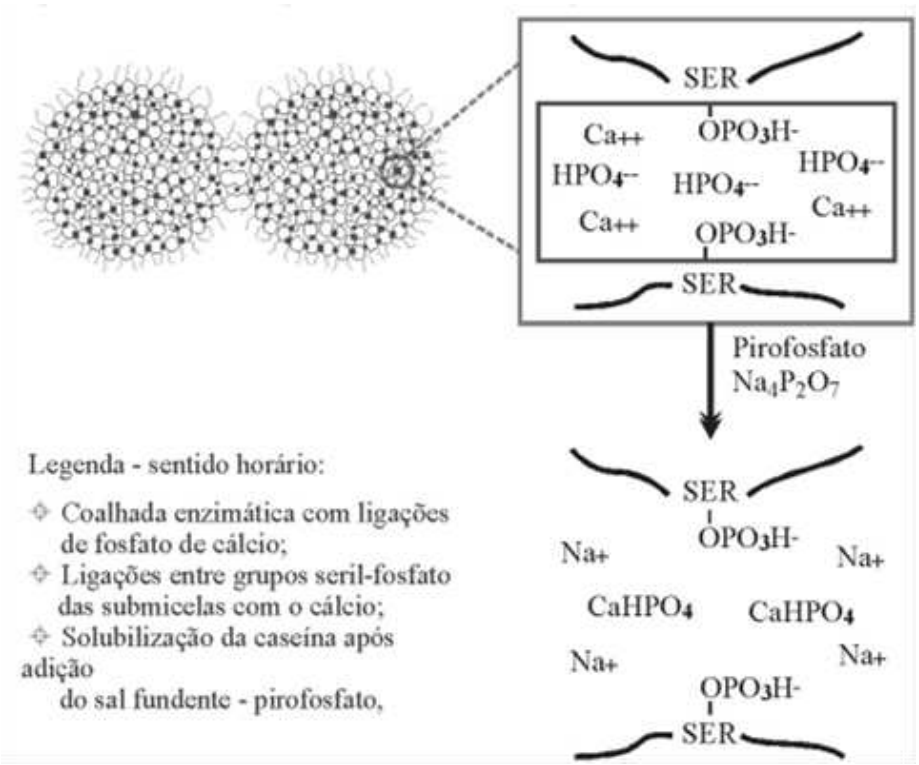

Figura 2 – Modo de ação dos sais fundentes (Fonte: Berger *et al.,* 1997).

Os fosfatos como sequestrantes de cálcio, tem um papel importante na indústria de laticínios, pois podem aumentar a estabilidade térmica do leite, bem como

modificar certos parâmetros do leite de forma a melhorar propriedades tecnológicas, como por exemplo, o sais sequestrantes de cálcio que são usados em queijos processados para melhorar suas características de emulsificação e derretimento (Kappor e Metzger, 2008), além disso, podem ser usados em sistemas para reduzir a incrustações geradas pelo tratamento térmico, como no caso dos tratamentos UHT (Prakash *et al.,* 2007; Scuedeller *et al.,* 2021). No entanto, nem todos os agentes sequestrantes de cálcio atuam da mesma forma, ou tem a mesma capacidade de se ligar ao cálcio, ou até mesmo a mesma capacidade de interagir com os íons cálcio e as proteínas da micela de caseína (De Kort *et al.,* 2009; Mizuno e Lucey, 2007). Um exemplo de sal de fosfato que é um ótimo quelante de cálcio, mas tem um forte efeito no sistema do leite, pois altera o equilíbrio da micela da caseína, isso porque, além de sequestrar os íons cálcio, também se liga a aminoácidos carregados positivamente da micela de caseína, e dependendo da concentração pode até promover a ruptura da micela é o hexametafosfato de sódio (SHMP) (Anema, 2015; De Kort *et al.,* 2011; Mizuno e Lucey, 2005). Inclusive, um estudo recente demonstrou o uso do SHMP em solução isolada de caseína micelar ((MCI), onde os autores identificaram que o SHMP afetou fortemente a estrutura micelar, no entanto, essas mudanças não prejudicaram a estabilidade das amostras quando comparado com as amostras sem adição do sal isso para amostras que sofreram tratamento térmico (121 ºC/8 min; 124 ºC/5 min; 140 ºC/5,8 s) e ficaram armazenadas por 60 dias à 20 ºC e 40 ºC (Garcia *et al.,* 2023). Dessa forma, é muito importante entender as características dos sais de fosfatos utilizados e para qual finalidade devem ser aplicados, para assim não ter problemas nas características do produto final, Lucey *et al.* (2011) traz uma lista de como selecionar os sais emulsificantes adequados para queijos processados considerando a idade do queijo, a composição do queijo, o retrabalho, o tipo de queijo processado, as características e composições padrões, condições de processamento, o custo do sal e características únicas requeridas para cada produto. Além disso, deve-se levar em conta a hidrólise dos sais, embora na prática é provável que a degradação hidrolítica seja baixa na maioria das aplicações em queijos processados (Maurer-Rothmann e Scheurer, 2005). Isso porque a taxa de hidrólise no queijo processado, provavelmente, depende da temperatura de processamento, pH, teor de água, concentração de Ca^{+2} e temperatura de armazenamento (Lucey *et al.,* 2011).

Outra função importante dos sais emulsificantes em queijos processados é em relação ao pH e a capacidade tampão, visto que o valor do pH é importante por

diversas razões: afetando a configuração da proteína, solubilidade e até que ponto os sais emulsificantes se ligam ao Ca^{+2} (Carić e Kaláb, 1993). Os sais emulsificantes usados na produção de queijo processado são geralmente de natureza básica, e seu uso resulta em um aumento do pH do queijo natural (~5,2) para pH ~5,6-6,0 (Lucey *et al.,* 2011). Um aumento do pH do queijo processado aumenta a carga líquida negativa das caseínas, aumentando a repulsão eletrostática na matriz de caseína, o que diminui as interações hidrofóbicas entre as moléculas de caseína individuais, resultando em uma rede de queijo processado mais aberta e solta com melhor capacidade de retenção de água e capacidade emulsificante durante a fabricação do produto (Marchesseau *et al.,* 1997; Horne, 1998; Fox *et al.,* 2000; Lucey *et al.,* 2003). Os ortofosfatos e pirofosfatos possuem alta capacidades de tamponamento nas faixas de pH 2-3, 4,5-9,0 e 10-12 (McCullough, 1973). A capacidade tamponante dos polifosfatos de sódio diminui com o aumento do comprimento da cadeia, e é efetivamente zero para os fosfatos de cadeia mais longa (n > 10) (Lucey *et al.,* 2011). Esta diminuição na capacidade de tamponamento com o comprimento da cadeia é devido à redução correspondente no número de funções de ácido fraco por molécula (McCullough, 1973).

Outras aplicações conhecidas dos fosfatos na indústria de lácteos que podemos citar são: como agente de viscosidade no leite achocolatado, onde é utilizado para manter os níveis de viscosidade mais altos para a dispersão do cacau em pó (Lampila, 2013); usado antes da secagem para fornecer hidratação e auxiliar na dispersão de proteínas (Dykes *et al.,* 2019); no leite evaporado para garantir que a gordura butírica não seja separada do meio aquoso (Lindsay, 2008); em produtos UHT para retardar o processo de gelificação (Anema, 2015).

Além disso, estudos mostram a utilização de fosfatos inorgânicos para o controle de microrganismos e de patógenos em alimentos, esses estudos surgiram após Tanaka (1982) e Tanaka *et al.* (1986) demonstrarem que o fosfato dissodico (DSP) prevenia o crescimento de *Clostridium botulinum* em pastas integrais processadas, embora estudos anteriores já buscavam entender o efeito desses sais sobre os microorganismos. Os ortofosfatos tem baixa atividade microbiana, devido a sua baixa capacidade quelante de metal, no entanto, os pirofosfatos e polifosfatos tem mostrado atividade microbiana em bactérias gram positivas, que são mais susceptíveis que as bactérias gram negativas (Buňkova e Buňka, 2017; Davidson *et al.,* 2002). Alguns exemplos de estudos que demonstraram a efetividade dos sais de

fosfatos no controle microbiano são: uso do fosfato trissódico (TSP) para o controle de *Salmonella* e *Campylobacter* em aves, pois devido ao seu alto pH (10-12), age sobre as membranas celulares (Koolman *et al.,* 2014; Alonso-Hernando *et al.,* 2013; Sarjit e Dykes, 2015; del Rio *et al.,* 2007); polifosfatos de cadeia longa em níveis ≤1% inibiu o crescimento de *Clostridium tyrobutyricum* em queijo processado (Löessner *et al.,* 1997); polifosfato de cadeia longa apresentou efeito bactericida ou bacteriostático maior que polifosfatos de cadeia curta em queijos processados contra *Bacillus cereus* INV 10; *Bacillus subtilis* ATCC 19659, *Bacillus thuringiensis* CFBP 3476, *Clostridium perfringens* ATCC 13124, *Escherichia faecalis* FAIR-E 179, *Listeria monocytogenes* Scott A e *Staphylococcus aureus* ATCC 6538 (Fusieger *et al.,* 2023). O mecanismo de inibição de bactérias por polifosfatos, provavelmente, está relacionado à sua capacidade para quelar íons metálicos de sítios de ligação nas paredes celulares de microorganismos, enquanto também há algumas indicações de que os polifosfatos podem interferir na função do RNA e as atividades metabólicas de células bacterianas (Lee *et al.*, 1994; Ellinger, 1972).

Os fosfatos como todos os outros aditivos alimentares só podem ser utilizados se cumprirem uma função tecnológica, e para o caso dos fosfatos há uma lista de mais de 100 diferentes formas químicas de fosfato que podem ser utilizadas em alimentos para diversos fins que é fornecida pelo Comitê Conjunto de Especialistas em Aditivos Alimentares da FAO/OMS (JECFA). Os regulamentos das organizações como FAO/OMS determinam o nível residual máximo desses aditivos que podem ser encontrados em um produto alimentício destinado ao consumo humano, além disso, o Codex no seu padrão 192 (Codex Stan 192-1995) lista todos os aditivos alimentares e suas diferentes formas químicas que podem ser usados na alimentação humana, de forma a promover o comércio internacional. E há diversas legislações especificas no Brasil que determinam se os fosfatos podem ser utilizados em determinado produto e se sim, qual a quantidade máxima que deve ser utilizada, que é expressa em P_2O_5 (pentóxido de fósforo).

Do ponto de vista da nutrição humana, o fosforo apresenta propriedades importantes, e uma proporção ideal de Ca e P absorvido é 1:1 (Schäffer *et al.,* 1999, Kůrová *et al.,* 2022). No entanto, os produtos alimentícios processados tendem a ter um teor de fosfatos mais alto do que o naturalmente presente nos alimentos, como o caso dos queijos processados em que essa proporção acima mencionada é geralmente reduzida para 1:1,5-3,0 devido à presença dos sais emulsificantes à base

de fosfato (Schäffer *et al.,* 1999, Kůrová *et al.,* 2022). Outro exemplo são as carnes processadas e produtos de aves que têm o dobro da quantidade de fosfato em comparação com produtos naturais. Os níveis de fosfatos permitidos para uso em alimentos devem ser “geralmente considerados seguros” pela Food and Drug Administration (FDA). Os fosfatos apresentam um requisito dietético essencial para os seres humanos. Fosfatos orgânicos de carne, grãos, laticínios e nozes e fosfatos inorgânicos (Pi) de aditivos alimentares são prontamente absorvidos no intestino delgado, processado no fígado, armazenados nas células e ossos e reabsorvidos nos rins (Dykes *et al.,* 2019). O fosfato tem muitos papéis fisiológicos, bioquímicos e de sinalização celular no corpo, e dessa forma, é necessário encontrar um equilíbrio adequado entre o uso de fosfatos como aditivos e possíveis efeitos nocivos associados ao consumo excessivo. Como uma biomolécula, a geometria molecular tetraédrica do fosfato permite que ele forme ligações com outras quatro moléculas e crie compostos moleculares complexos, como ácidos nucléicos, proteínas, ATP e fosfolipídios (Azevedo e Saiardi, 2017). A importância do fosfato no corpo humano é evidente pelo fato de que 80%-90% do fosfato plasmático filtrado é ativamente reabsorvido nos túbulos renais em um indivíduo saudável (Prasad e Bhadauria, 2013). No entanto, a quantidade de aditivos de fosfato na dieta do norte-americano médio mais que dobrou desde 1990, estimando-se que metade da população dos EUA esteja consumindo em excesso a IDA recomendada de fosfatos (Ritz *et al.,* 2012; Uribarri e Calvo, 2013).

Essa quantidade excessiva de fosfato consumida vem chamando atenção, pois tem provocado doenças. Em estudo realizo por Abrams e Atkinson (2003), os autores evidenciaram que o excesso de fósforo pode causar em crianças hipocalcemia e fraturas, principalmente quando esse não está associado a um aumento de cálcio na dieta, uma prática comum, mas não comprovada é a recomendação de ingestão de 1,5-2,0:1 na proporção de cálcio para fósforo em base molar. Os mesmos autores identificaram um consumo maior que o recomendado para crianças na África e no México. A hiperfosfatemia é uma concentração sérica de fosfato anormalmente elevada (> 1,46 mmol/L), geralmente associada a resultados negativos para a saúde, se experimentada por um período prolongado de tempo (Shaman e Kowalski, 2016). Essa condição é comumente associada com função renal prejudicada, mas também pode ser o resultado de aumento da ingestão de fosfato na dieta, síndrome de lise tumoral e hiperparatireoidismo (Nguyen e Wang, 2012). Há

evidências crescentes de que o aumento dos níveis de fosfato aumenta o risco de doenças cardiovasculares, como a aterosclerose (Lau *et al.,* 2010; Nguyen e Wang, 2012); aumento o risco de doenças cardiovasculares devido a calcificação vascular (Adeney *et al.,* 2009; Lau *et al.,* 2010).

Assim, alguns estudos têm associado o fosfato ao novo sódio. Visto que a ingestão de sódio presente no queijo processado costuma ser maior (325-798 mg/50 g) do que no queijo natural (95-697 mg/50 g) devido à adição de NaCl e sais emulsionantes (Agarwal *et al.,* 2011; Johnson *et al.,* 2009). Portanto, a indústria como um todo deve buscar formas de reduzir o uso de fosfato, como já tem buscado reduzir o sódio, e substituir as formulações para conter baixo teor de sódio e fosfato sem alterar a qualidade do produto final e o perfil sensorial.

Algumas opções para redução de sais emulsificantes podem ser realizado de acordo com três abordagens conforme descrito: primeira opção seria realizar a substituição parcial por outras substâncias, segunda opção seria substituir completamento por outros aditivos ou misturas alimentares, e por fim, uma outra opção seria remover parcialmente os íons de cálcio da matéria prima por métodos físicos ou físico-químicos (Červíková *et al.,* 2010; Červíková *et al.,* 2017). E se essa substituição dos sais emulsificantes por hidrocoloides, como ágar, carragena ou gelatina, fosse bem-sucedida pode permitir (1) uma diminuição do nível de P e um aumento da razão Ca:P; (2) uma diminuição da concentração de Na; (3) utilização de aditivos alimentares biodegradáveis de fontes alternativas em vez de P; (4) formação de produtos com potencial benefícios para os consumidores (Kratochvílová *et al.,* 2022).

Considerando as informações expostas anteriormente, o objetivo do presente trabalho foi analisar o uso de fosfatos em diferentes produtos, buscando identificar qual seria o menor percentual a ser adicional de forma a obter os resultados desejados com a utilização de fosfatos, sem que comprometa também a qualidade do produto final e o sensorial. Com isso, foi realizado estudos com a utilização de fosfatos para a redução do sabor residual da estevia em chá preto, o uso de fosfato em substituição do cloreto de cálcio no queijo Minas Frescal e a utilização de fosfatos na calda de uma mistura láctea condensada produzida com gordura vegetal, conforme descrito nos diferentes artigos:

- **Artigo 1:** Influence of phosphates in reduction of the aftertaste of steviol glycoside (derived from *Stevia rebaudiana* Bertoni) in black tea drinks
- **Artigo 2:** Effects of calcium chloride substitution on the physicochemical properties of Minas Frescal Cheese
- **Artigo 3:** Influence of the use of phosphates salts and different proportions of casein and whey protein on the syrup characteristics of a blend of sweetened condensed skimmed milk and vegetable fat

** Os artigos apresentados a seguir estão no formato solicitado pelas diferentes revistas em que os trabalhos foram (artigo 1) ou serão (artigo 2 e 3) publicados.

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2. ARTIGO 1

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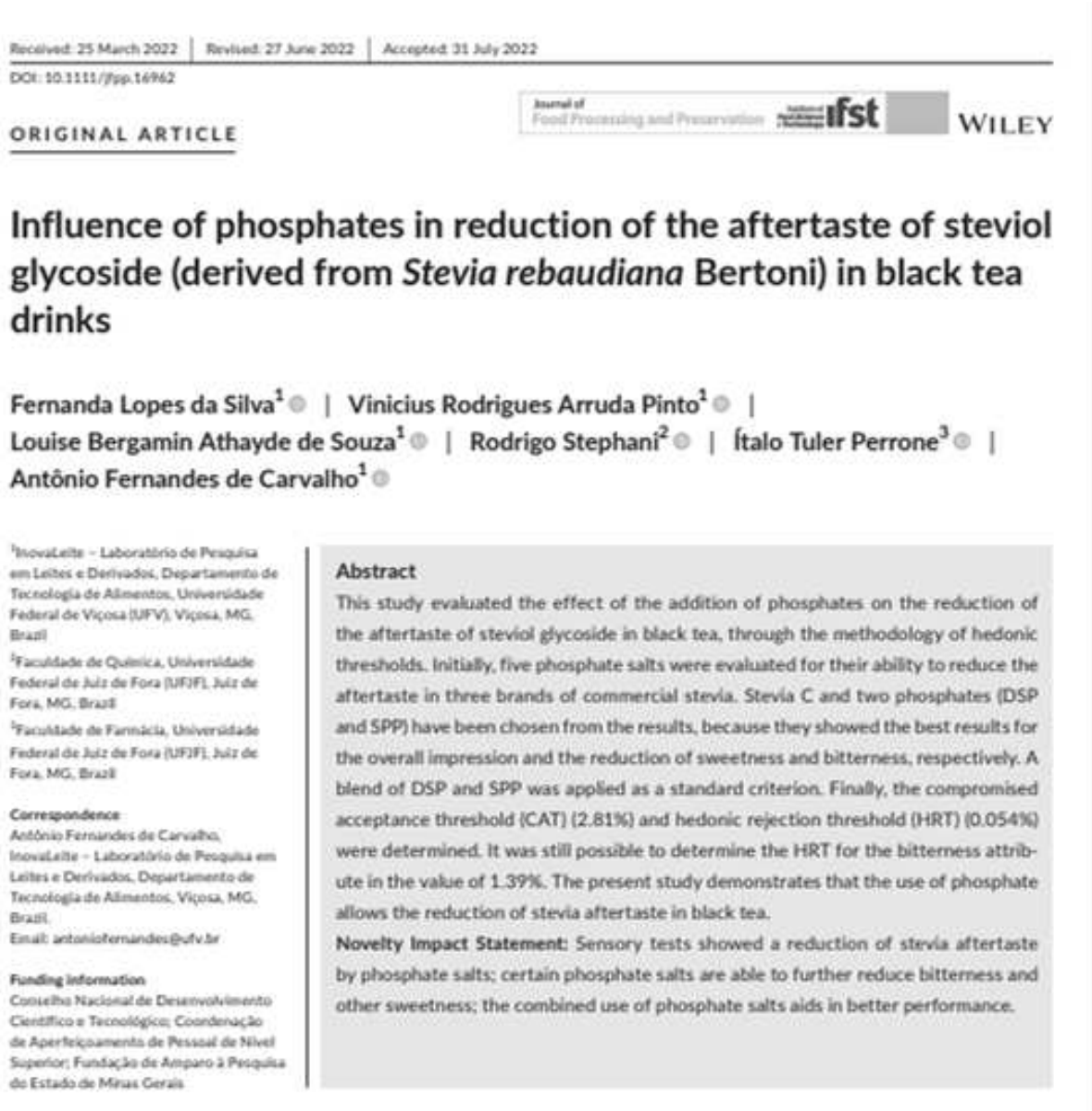

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Influence of phosphates in reduction of the aftertaste of steviol glycoside (derived from *Stevia rebaudiana* Bertoni) in black tea drinks

Fernanda Lopes da Silva[1] | Vinicius Rodrigues Arruda Pinto[1] | Louise Bergamin Athayde de Souza[1] | Rodrigo Stephani[2] | Ítalo Tuler Perrone[3] | Antônio Fernandes de Carvalho[1]

[1]InovaLeite – Laboratório de Pesquisa em Leites e Derivados, Departamento de Tecnologia de Alimentos, Universidade Federal de Viçosa (UFV), Viçosa, MG, Brazil
[2]Faculdade de Química, Universidade Federal de Juiz de Fora (UFJF), Juiz de Fora, MG, Brazil
[3]Faculdade de Farmácia, Universidade Federal de Juiz de Fora (UFJF), Juiz de Fora, MG, Brazil

Correspondence
Antônio Fernandes de Carvalho, InovaLeite – Laboratório de Pesquisa em Leites e Derivados, Departamento de Tecnologia de Alimentos, Viçosa, MG, Brazil.
Email: antoniofernandes@ufv.br

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Abstract

This study evaluated the effect of the addition of phosphates on the reduction of the aftertaste of steviol glycoside in black tea, through the methodology of hedonic thresholds. Initially, five phosphate salts were evaluated for their ability to reduce the aftertaste in three brands of commercial stevia. Stevia C and two phosphates (DSP and SPP) have been chosen from the results, because they showed the best results for the overall impression and the reduction of sweetness and bitterness, respectively. A blend of DSP and SPP was applied as a standard criterion. Finally, the compromised acceptance threshold (CAT) (2.81%) and hedonic rejection threshold (HRT) (0.054%) were determined. It was still possible to determine the HRT for the bitterness attribute in the value of 1.39%. The present study demonstrates that the use of phosphate allows the reduction of stevia aftertaste in black tea.

Novelty Impact Statement: Sensory tests showed a reduction of stevia aftertaste by phosphate salts; certain phosphate salts are able to further reduce bitterness and other sweetness; the combined use of phosphate salts aids in better performance.

Influence of phosphate in reduction of the aftertaste of steviol glycoside (derived from *Stevia rebaudiana* Bertoni) in black tea

Fernanda Lopes da Silva[1], Vinicius Rodrigues Arruda Pinto[1], Louise Bergamin Athayde de Souza[1], Rodrigo Stepani[2], Ítalo Tuler Perrone[3], Antônio Fernandes de Carvalho[1*]

[1] InovaLeite – Laboratório de Pesquisa em Leites e Derivados, Departamento de Tecnologia de Alimentos, Universidade Federal de Viçosa (UFV), 36570 900, Viçosa, MG, Brazil.
[2] Faculdade de Química, Universidade Federal de Juiz de Fora (UFJF), 36036 900, Juiz de Fora, MG, Brazil.
[3] Faculdade de Farmácia, Universidade Federal de Juiz de Fora (UFJF), 36036 900, Juiz de Fora, MG, Brazil.

* corresponding author: A. F. Carvalho (antoniofernandes@ufv.br; +55 (31) 3612-6807)

Abstract

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Keywords: Phosphate salts; hedonic thresholds; aftertaste; sweetener; black tea.

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1. Introduction

There is a growing interest in the food industry to replace sucrose in foods, as they are associated with several diseases such as obesity, type 2 diabetes, hypertension, cardiovascular disease, and other comorbidities (Wang, Coxson, Shen, Goldman, & Bibbins-Domingo, 2012; WHO, 2015), and relatedly, there is a desire by consumers to purchase foods deemed to be healthier (Mintel, 2021).

An alternative to reducing sugar in foods is to replace them with artificial sweeteners, such as aspartame and saccharin, and natural sweeteners, such as stevia. The food industry may, however, have some difficulty in transitioning to artificial and other sweeteners, as sugars have certain crucial characteristics such as texture, structure, flavor, and preservation of food (Ahmad, Khan, Blundell, Azzopardi, & Mahommodally, 2020). In addition to the reduction being difficult without influencing sensory perception (Hutchings, Low, & Keast, 2018), for example, the artificial sweeteners commonly used add a characteristic residual mineral flavor to the product (Luo, Arcot, Gill, Louie, & Rangan, 2019; Guggisberg, Piccinali, & Schreiner, 2011; Puri, Sharma, & Tiwari, 2011).

Of the more than 200 known stevia species, *S. rebaudiana* Bertoni it's the only one with a sweet taste (Shivanna, Naika, Khanum, & Kaul, 2013). The sweet taste of stevia species is attributed to some steviol glycosides (SGs); however, these are also responsible for a bitter aftertaste (Lemus-Mondaca, Veja-Gálvez, Zura-Bravo, & Ah-hen, 2012; Goyal, Samsher, & Goyal, 2010).

Industrial processes as biotransformation, transglycosylation, chemical interaction with other compounds (e.g., proteins), pre-treatment of stevia leaves with ethanol before extracting, using new varieties, encapsulation and use of taste modifiers and/or flavor enhancer can perform the extraction and purification of SGs, in order to reduce bitter aftertaste (Ahmad et al., 2020).

Studies showed that it is not possible to perform the substitution by stevia beyond 50% of sucrose, without compromising the characteristics of the product, including sensory acceptance (Gao, Brennan, Mason, & Brennan, 2017; Lisak, Jelicic, Tratnik, & Bozanic, 2011; Giri, Rao, & Ramesh, 2014; Andersen, Mielby, Viemose, Bredie, & Hyldig, 2017). However, soft drinks and more than 300 teas containing stevia have been introduced to the market, and ready-to-drink iced teas containing stevia have

increased by 86% in the last five years (Ahmad et al., 2020; Hatman, 2017; PureCircle, 2017). Phosphates are already part of the formulation of some ready-to-drink teas, wherein manufactures use in order to control acidity, chelating agent, or even to control tea turbidity (Dubey, Janve, Ray, & Singhal, 2020). Therefore, phosphates can also be a good alternative to be used in these drinks to reduce stevia aftertaste (Purkayastha et al., 2015).

However, it is important to know how to dose the required amount of phosphates, as well as which phosphates can be used in order to reduce this aftertaste of stevia, and a methodology that can be used is the hedonic limit methodology (HTM). This methodology allows us to determine the thresholds of compromised acceptance (CAT) and compromised rejection (HRT), using for this a control and some stimuli, and when applied to quality control in the food industries, allows for cost reduction, and makes more precise the modifications in the formulations (Souza et al., 2021; Lima Filho et al., 2017). Some studies have already demonstrated the use of the HTM to assess the reduction of sucrose in strawberry yogurt (Souza et al., 2021) and in grape nectar (Lima Filho, Minim, Silva, Della Lucia, & Minim, 2015), sodium reduction in hamburger (Lima Filho, Della Lucia, Minim, Gamba, Lima, & Minim, 2019), among other. However, no study was found using hedonic thresholds to evaluate as a stimulus the use of phosphates to reduce aftertaste in stevia.

Therefore, the aim of this study is to evaluate the effect of addition phosphates on the aftertaste of SGs in iced black tea, using sensory analysis to determine CAT and HRT values for a phosphate blend.

2. Material and methods

The present study was approved by the Ethics Committee on Human Research of the Federal University of Viçosa (UFV), Brazil, under number 23845319.2.0000.5153. The analyses were performed at the Sensory Analysis Laboratories of the UFV, in individual booths with white light.

The work was performed using three steps, namely:

Step 1: Determination of the best phosphate salts to reduce the aftertaste of three brands of commercial stevia and the best brand of national commercial stevia by the sensory acceptance test using the ideal scale.

Step 2: Selection of the best blend of phosphate salts to reduce the aftertaste of SGs by sensory preference test.

Step 3: Determination of the hedonic threshold from the preferred blend of phosphate salts in the black iced tea sweeten by stevia.

In step 1, phosphate salts were evaluated for their ability to reduce the aftertaste of stevia in iced black tea. The ability of phosphates to reduce the aftertaste was evaluated by sensory acceptance using ideal scale for evaluating the reduction of sweetness, bitterness, and global impression assessment. The phosphates that presented values that showed a reduction in the bitter and sweet aftertaste were chosen as the best phosphates and used in the following steps. And the stevia that presented the closest characteristics to the ideal in the global impression was chosen as the best stevia to proceed with the analyses. The best phosphates from step 1 were used in step 2 in the form of blends, and a preference ordering test was used to determine the most preferred blend. In the final stage, the most preferred blend was used as control and other four concentrations of the blends were used as stimuli to determine the CAT and HRT values for black tea sweetened with the stevia. In each of these stages the tasters were not the same, but only tasters who were tea drinkers were always used.

2.1. Material

The following ingredients were used to produce tea: tea leaves trademark, stevia from different national brands, and phosphates provided by ICL Food Specialties (São José dos Campos, SP, Brazil). The stevia was named as stevia A, B and C, where stevia A and C were formed SGs, and stevia B is Rebaudioside A. The phosphates used Disodium Phosphate Anhydrous (DSP – Na_2HPO_4 – 141.96 g mol^{-1}), Monosodium Phosphate Anhydrous (MSP – NaH_2PO_4 – 119.98 g mol^{-1}), Sodium Hexametaphosphate (SHP – $(NaPO_3)_6$ – 465.40 g mol^{-1}), Sodium Polyphosphate (SPP – $(NaPO_3)_n$ – 662.70 g mol^{-1}) and control, without phosphate.

Pure water from the Department of Food Science and Technology was used for all of the experiments. Coffee glasses of 50 mL codified with three-digit numbers were used for sensory analysis and plastic bottles of 1 L were used for tea storage.

2.2. Methods

2.2.1. Preparation of tea infusion

The black tea was steeped in distilled water with a leaf/water ratio of 1.75 g 100 mL^{-1} at 80 ºC for 5 min, the concentration of tea samples was defined according to Nishiyama et al. (2010). The extract was filtered out through a 600-mesh screen and then stevia was added in a concentration of 0.06 g 100 mL^{-1}. The stevia concentration used in the tea followed as determined in Brazilian legislation, considering the recommended concentration for the total substitution of sugars (Brasil, 2008).

With the tea ready and sweetened, portioning was carried out in equal parts, and the phosphates were added at a concentration of 0.14 g 100 mL^{-1} diluted in the same amount of water according to the volume of portioned tea. This concentration used in the tea followed as determined in legislation, considering the concentration of P_2O_5 in the final product (Brasil, 2007).

After preparation, the tea samples were bottled and closed and kept refrigerated (6 ± 1 ºC) until the next day, when sensory analyzes were performed. And during the analysis the samples were served cold (8 ± 1 ºC).

2.2.2. Sensory acceptance test

The sensory acceptance test was used to evaluate among the three commercial stevia to determine which one had the best sensory profile, as well as among the evaluated phosphates which had the best capacity to reduce stevia aftertaste. For this, a just right scale was used to assess the sweetness and residual bitterness, and overall impression, one end of the scale being a much more intense flavor than the ideal (7), and the other end being a weaker flavor than the ideal (1), where the middle of the scale represented the flavor of ideal intensity (4), according to Minim (2013).

The samples, about 20 mL, were delivered to consumers at random, being delivered one sample at a time along with the evaluation form. The samples presentation order was balanced according to MacFie, Bratchell, Greenhoff, & Vallis (1989). Each session consisted of one stevia per day, totaling three sessions. One hundred per session female and male tea consumers, of ages between 18 and 60 years were used.

After the evaluation of all test samples, consumers were asked to complete a demographic questionnaire. Questions included age, gender, frequency of and reasons for tea consumption, consumption of sweetened tea or not, if sweetened the use is sucrose or sweetener and knowledge of stevia.

2.2.3. Sensory preference test

The sensory preference test was used to determine the preference of the consumer for the blend of phosphates used. In the previous section, DSP and SPP phosphates were chosen. Thus, a geometric progression was used to formulate four blends using the phosphates DSP and SPP, the concentrations being used as follows: Blend 1 - 80 g 100 mL^{-1} DSP and 20 g 100 mL^{-1} SPP; Blend 2 - 60 g 100 mL^{-1} DSP and 40 g 100 mL^{-1} SPP; Blend 3 - 40 g 100 mL^{-1} DSP and 60 g 100 mL^{-1} SPP; Blend 4 - 20 g 100 mL^{-1} DSP and 80 g 100 mL^{-1} SPP, maintaining the final concentration of 0.14 g 100 mL^{-1} of phosphate.

For this, an ordering scale was used, going from more preferred at one and less preferred at the other. The four samples, about 20 mL each, were delivered to consumers at the same time and randomly, together with the evaluation form, and consumption was standardized from left to right (Minim, 2013). This test was conducted by 70 consumers (Newell, & Macfarlane, 1987).

2.2.4. Determination of the hedonic thresholds

The sample that preferred in the previous step was used as a control (40 g 100 mL^{-1} DSP and 60 g 100 mL^{-1} SPP), since we did not have a commercial sample to be used. Based on this control sample, a geometric progression (Prescott, Norris, Kunst, & Kim, 2005) was performed again to determine the concentrations of phosphates DSP and SPP that would be used in this step as stimuli, and the concentrations used were stimuli 1 - 20 g 100 mL^{-1} DSP and 80 g 100 mL^{-1} SPP; stimuli 2 - 10 g 100 mL^{-1} DSP and 90 g 100 mL^{-1} SPP; stimuli 3 - 5 g 100 mL^{-1} DSP and 95 g 100 mL^{-1} SPP; and stimuli 4 - 2.5 g 100 mL^{-1} DSP and 97.5 g 100 mL^{-1} SPP.

The hedonic thresholds, CAT and HRT were determined according to procedures proposed by Lima Filho et al. (2015).

102 consumers participated in four acceptance test sessions. In each session two samples were served (control and one of the stimulus samples). Between sessions, tea pairs were provided in descending order of DSP concentration and ascending order of SPP, and the stimulus sample position within each pair was randomized.

Approximately 20 mL of each black tea sample was served at about 8 ºC. Consumers rated how much they approved or disapproved the tea's using a 9-point hedonic scale. After rinsing the mouth with water, they received a new pair of samples.

The CAT and HRT values of black tea were determined following the description made by Lima Filho et al. (2015). The CAT was determined through a t-test between the

paired samples comparing the values of the hedonic scale for the control sample and for the stimulus sample. The t values obtained in each session (y-axis) were plotted against the DSP concentration in the blend (x-axis). The CAT was calculated from the mathematical regression, where the DSP concentration corresponded to the point at which the calculated t value became equal to the tabulated t value ($p = 0.05$). The HRT value was obtained through the hedonic scale values for the stimulus samples (y-axis) versus the DSP concentration (x-axis), and the HRT value was calculated through the mathematical regression obtained where the y value was equal to 5, value that is indifferent in the hedonic scale. In both cases, a regression model was made, where the regression model which had the highest R^2 value, and which best fitted the data was chosen.

2.2.6. Statistical analyses

For statistical analyses, the results were compared considering the treatments by Tukey test ($P < 0.05$) to identify significant differences at 95% of confidence level, using SISVAR® software system version 5.6 (Ferreira, 2011). And to assess hedonic thresholds, Excel ® software system (version 2020) was used, testing among all types of regression models the one that presented the best results of r^2.

3. Results and discussion

3.1. Step 1 - Determination of the best phosphate salt

Table 1 shows the results obtained on the ideal scale for the values of sweetness, bitterness, and overall impression for the three types of commercial stevia and the four phosphates tested.

The criterion for choosing stevia was the one that presented the results closest to the ideal (4) for the overall impression of the tea without the addition of phosphates, thus being the evaluation only of the stevia used. Thus, stevia C was the stevia with a profile closest to the ideal (4.30), compared to the others which presented values of 4.38 (stevia A) and 4.62 (stevia B). The information in the product's technical file indicates that stevia C has at least 95% of SGs, and since at least 75% is Rebaudiosideo A, that is considered among the superior components of stevia in terms of sweetness and flavor quality (Lemus-Mondaca et al., 2012).

At this stage, it was possible to identify which phosphates showed the best values for reducing bitterness and sweetness for black iced tea sweetened with stevia. The criterion for choosing the phosphates was the one with the greatest ability to reduce the sweetness and bitterness of the tea, that is, those with values below four, as shown in Table 1.

Therefore, when evaluating the values for sweetness, we see that phosphates SPP and SHP were the ones that showed the best characteristics for reducing the sweetness of black tea, as they presented values lower than four, while DSP and MSP phosphates presented higher values, for all brands of stevia evaluated. However, when evaluating stevia C, which was chosen to continue the studies, phosphate SPP had an overall impression value closer to the ideal (4.45) compared to SHP (4.74), which is why it was chosen to continue our studies.

As for the bitterness values, the phosphate salts that presented the ability to reduce the bitterness of black tea with stevia were DSP, followed by MSP, SHP, and SPP. In the same way as the previous one, DSP phosphate was chosen, since, evaluating the overall impression of stevia C, it was the one that presented values closer to the ideal (4.17).

These results showed that phosphate salts are modifying the sensory perception of black tea sweetened with stevia, as in a study by Behrens et al. (2017), who showed that the mixture of saccharin with cyclamate reduces the bitter taste, because saccharin blocks the bitter taste receptors activated by cyclamate and cyclamate inhibits the receptors activated by saccharin. This combination of cyclamate and saccharin leads to a reduction in bitterness and an increase in sweetness (Behrens et al., 2017), as we can see in Table 1, where the phosphate salts in which there is a reduction in bitterness, we have an increase in sweetness, this same behavior was observed for Liang et al., 2022 (a). One observation made by Liang et al., 2022 (b) is that molecules larger than 1 kDa have greater bitterness, while molecules smaller than 1 kDa have greater sweetness, umami, and saltiness. But further studies are needed relating the sensory aspect to the response in the cell.

Another evaluation was carried out with Stevia C, separating consumers who have the habit of drinking sweetened tea and unsweetened tea, this separation was possible due to the questionnaire applied during the evaluation, the results being shown in Table 2.

Based on Table 2, it is possible to see the difference in evaluation that exists between these consumers, mainly related to sweetness and bitterness. Where tasters who do not sweeten the tea can no longer differ between samples, thus not showing a significant difference to the sweetness and bitterness attribute. Furthermore, sweetness values, in general, are closer to ideal, and bitterness values are less than ideal when we compare the groups of consumers who sweeten the tea and those who do not. This difference in assessment may be related to taste acuity. This may indicate a difference in the detection thresholds of bitter and sweet attributes for this group of people, as well as one study, showed this difference in a group of elderly and young people (Mojet, Heidema & Christ-Hazelhof, 2004), however, more studies should be carried out to identify whether there is in fact a change in this detection threshold for these groups of people.

3.2. Step 2 - Determination of a standard concentration

The previously chosen phosphates, DSP and SPP, were used in this stage in the form of a blend, in order to improve the performance in reducing the stevia aftertaste, since the DSP presented a greater aftertaste reduction characteristic for bitterness and SPP for the sweetness. The preference test allowed us to determine which concentration of the blend differed from the others, thus making in the standard concentration between the previously chosen mixtures of DSP and SPP phosphates, as shown in Table 3.

Blend 3 (40% DSP and 60% SPP) differed significantly from the others, being the most preferred and it was chosen as the control for the next step.

This step was necessary to determine a control to be used in determining hedonic thresholds, as there was no commercial standard that could be used.

3.3. Step 3 - Hedonic thresholds

Figure 1 shows the t values and the average hedonic for determining the CAT and HRT for black tea.

In Figure 1, the CAT obtained was equal to 2.81%, which means that the industry can safely reduce the amount of DSP in the blend from 40% to 2.81%, without compromising the sensory acceptability of black tea. Although the amount of DSP can be reduced to almost zero within the blend, the HRT estimate showed that a very low value of DSP in the blend, less than 0.054% can lead to sensory rejection of the tea.

These results can have an important impact for the industry, as they provide more precise formulation of phosphate blends allowing the total replacement of sugar by stevia in tea without compromising sensory acceptance or resulting in product rejection.

Stevia C has a composition at least 75% of Rebaudioside A, a molecule with a sweeter and more bitter characteristic than other SGs (Mayank, 2015). Studies have already shown that a chemical change in the molecule can increase its sweetness and reduce its bitterness, as has already been shown for aspartame (Temussi, 2012) and neohesperidin (Shin, Kim, Shin, & Kim, 1995; Naim, Rogatka, Yamamoto, & Zehavi, 1982), as these chemical modifications reduce the activation of the bitter receptor and increase the sweetness, and vice versa. This reinforces the hypothesis that the studied phosphate salts led to some chemical modification of SGs, which led to a modification of its bitter and sweet perception by consumers.

In a second instance, it was tested the sweetness, color, and bitterness attribute to determine which attributes of the tea could ostensibly lead to the product's sensory rejection. The regression models to identify the HRT obtained are shown in Figure 2.

The model for the color did not generate any value for HRT, although it did generate a mathematical model, as the values assigned to this attribute were all above the indifference value (5), that is, not having values in the rejection region of the sample.

While for the attribute of sweetness it was not possible to generate any mathematical model through the software, but the HRT for the bitterness attribute found was 1.39% of DSP. This value shows us that the reduction of DSP in the blend can occur from 40% to 1.39% without a sensory rejection of the product, indicating once again that it is not possible to completely remove the DSP from the blend. Determining the rejection threshold for bitterness and not for sweetness further reinforces that the detection limit of bitterness is much higher than sweetness since the genes that identify bitter taste are many, making it the 2nd in a sensory scale, just behind the smell, in this way bitter compounds are generally detected at much lower levels of concentration than sweet ones (Di Pizio et al., 2018; Meyerhof, 2005; Purves et al., 2001).

4. Conclusion

Based on these assessments, it is possible to show that phosphates present some modification of SGs, which affect the perception of stevia aftertaste, requiring further

studies to identify the type of interaction that may be occurring between these compounds.

In addition, it was possible to identify the CAT and HRT values for the mixture of DSP and SPP phosphates that can aid the industry in tea formulations with the total replacement of sugar by stevia, in such a way that doesn't reduce the sensory acceptance of the product. And without changing the product's ingredient list, as phosphates are already used in industrialized iced tea formulations.

This study was used to identify whether phosphate salts have the ability to help reduce stevia aftertaste, but further studies are needed in order to better understand the sensory relationship with consumer perception, as the processes in the detection of sweet and bitter in the cell are very complex.

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Conflict of Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Author's contributions

AFC was responsible for the conceptualization, funding acquisition and supervision of the activities. FLS, VRAP, RS, ITP and AFC designed this paper. FLS, VRAP and LBAS conducted the laboratory analysis and validated the results. FLS and AFC were responsible for the data analysis. FLS wrote the original draft of the manuscript. FLS, VRAP, RS, ITP and AFC reviewed and edited the manuscript.

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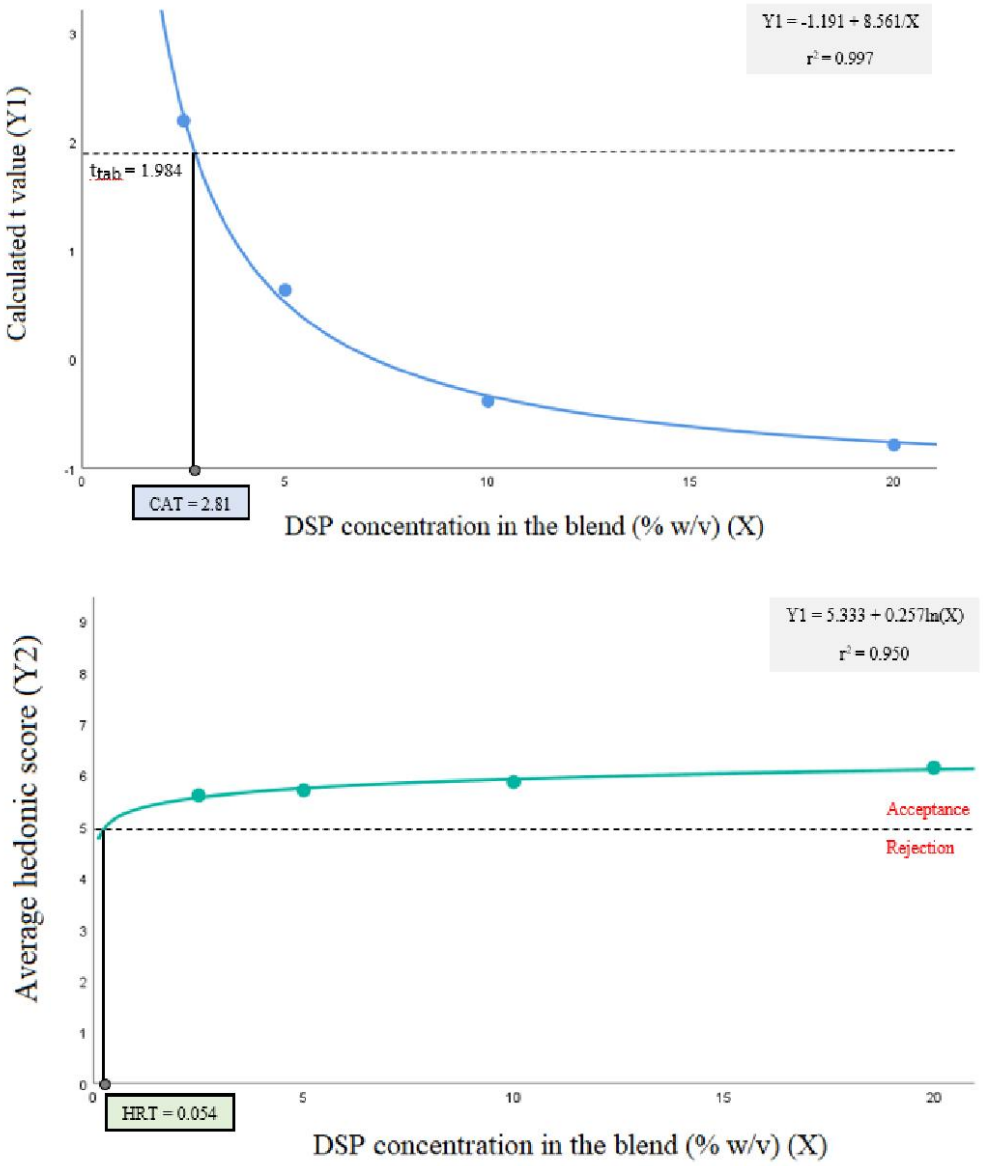

Figure 1. Calculated t values and average hedonic scores in function of the DSP concentration in the blend (n=102 and p = 0.05) (ttab = 1.984).

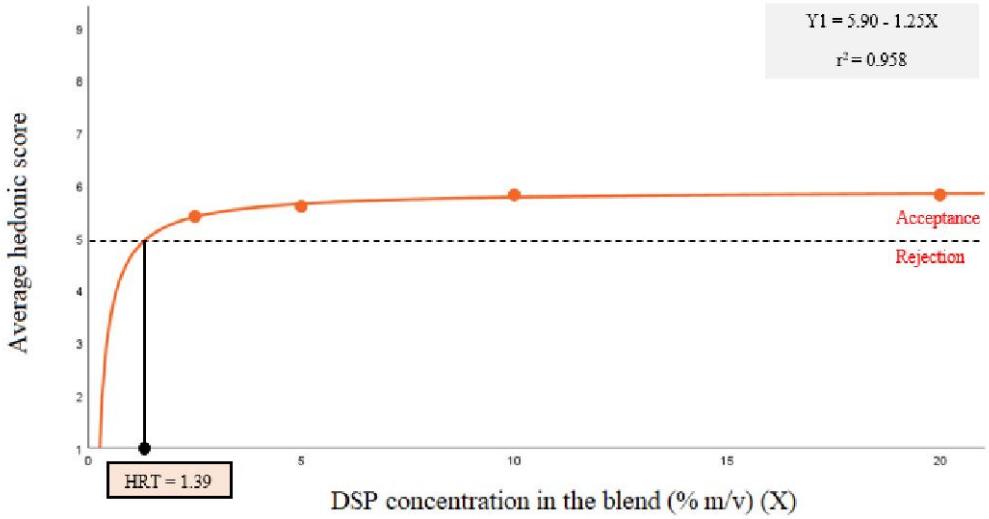

Figure 2. Calculated average hedonic scores in function of the DSP concentration in the blend, for bitterness attribute (n=102 and p = 0.05). The black dashed line represents the hedonic score 5, referring to the hedonic term "indifferent".

3. ARTIGO 2

Effects of calcium chloride substitution on the physicochemical properties of Minas Frescal Cheese

Summary

The aim in this research paper was to investigate the effect of using calcium monophosphate (MCP) and MCP mixed with commercial phosphates salts, in total or partial replacement of calcium chloride ($CaCl_2$) in the manufacture of Minas Frescal cheese. Initially, model cheeses were made to perform the rheological analysis during the coagulation process. Of these, the five best treatments were chosen to carry out the production of Minas Frescal cheese, used only $CaCl_2$ and MCP, and partial replacements of MCP + polyphosphate, MCP + potassium monophosphate (MKP) and MCP. The cheeses showed no significant difference in physicochemical composition, yield and syneresis, however, the cheese with partial replacement of $CaCl_2$ by MCP + polyphosphate and MCP + MKP showed the highest hardness values, like the control. This demonstrates that it is possible to replace calcium chloride without significant changes in the physicochemical characteristics and yield of Minas Frescal cheese, and it is still possible to modulate the hardness of the cheese produced according to the type of calcium/phosphate source used. This allows the industry to replace the source of calcium in the manufacture of Minas Frescal cheese according to the desired hardness.

Keywords: calcium substitute; phosphate; quality; texture; yield.

Introduction

Minas Frescal is a fresh and soft white cheese (Codex, 1978), typically manufactured in Brazil, obtained by enzymatic coagulation of pasteurized milk with rennet or supplemented or not of specific lactic acid bacteria (Brasil, 1996; Brasil, 2004). During its manufacture, heat treatment leads to a reduction of soluble calcium (Ca^{2+}), leading to a change in the salt balance of the milk (Wang & Ma, 2020).

Therefore, it has long been a consensus on the need to add calcium to improve manufacturing yield of the cheese, thus replacing the calcium lost during the heat treatment, with an addition of up to 10 mM Ca^{2+} having an effect to increasing the strength of the gel, above that, the opposite effect may occur (Lucey & Fox, 1993; Solorza & Bell, 1998; Santos *et al*. 2013).

This effect occurs because calcium is involved in the clotting process of milk. A clear understanding of the role of calcium within this coagulation process is hampered by the complexity of forms in which calcium is present in milk. However, it is known that the effect of addition of Ca^{2+} is related to reducing the surface potential of the para-casein micelles (Ong *et al.* 2013). Ca^{2+} ions bind to the casein micelles via electrostatic cross linking of the phosphate moiety of the colloidal calcium phosphate, thereby neutralizing their charge and resulting in increased aggregation of the rennet micelles (Dalgleish, 1983; Ong *et al.* 2013). In cheese industry, the agent used to replace this lost calcium is $CaCl_2$.

Some studies on the influence of adding $CaCl_2$ for cheese production show an increase in the hardness of cheddar cheese (Ong et al. 2013) and cured Minas cheese (Santos et al., 2013), which leads to the formation of more homogeneous gels (Tarapata *el al.* 2020), improves the hardness of the milk coagulum and increases the degree of syneresis and to yield in the cheese (Wolfschoon-Pombo, 1997); there is no change in the composition of the cheese (Santos *et al.* 2013). However, there are no studies that demonstrate the use of other sources of calcium to replace $CaCl_2$ during milk clotting (or enzymatical coagulation of milk).

An alternative source of calcium may be calcium monophosphate (MCP), since in the casein micelle, the non-protein components expressed in ions are mostly calcium (37.5%) and phosphate (50%). In addition, calcium phosphate is the main inorganic constituent of the micelle (Sleigh *et al.* 1983; Bak *et al.* 2001; Kolar *et al.* 2002). In a

study carried out by Guo *et al.* (2003), the presence of calcium phosphate in a standard β-casein solution led to total protein precipitation/separation, while the presence of Ca^{2+} alone led to only minor protein separation. The authors concluded that the separation is caused by the indiscriminate co-precipitation of proteins by organic compounds compared to the selective precipitation of β-casein by calcium ions. Thus, the objective of this work was to evaluate the partial or total replacement of $CaCl_2$ by MCP and the mixture of this MCP with other types of phosphate salts, like monopotassium phosphate (MKP) and polyphosphate, in the production of Minas Frescal cheese (type of fresh cheese).

Material and methods

As experimental design, this study was conducted in two steps: (I) previous evaluation of the best concentrations of total or partial replacement of $CaCl_2$ by MCP and/or Blend 1 and Blend 2, through rheological analysis of cheese models (Supplementary material); and (II) determination of physical chemical characteristics, texture and yield of Minas Frescal cheeses produced with the best treatments determined in step I.

Material

Chemical reagents of analytical grade were used for the physicochemical analyses. The following ingredients were employed for Minas Frescal cheese manufacture: raw cow's milk (Laticínio Funarbe, Viçosa, MG, Brazil); lactic acid 85% (v/v) P.A. (Dinâmica Química Contemporânea, Indaiatuba, SP, Brazil); calcium chloride 40% (w/v) (Dinâmica Química Contemporânea); rennet Maxiren XDS BF (bovine chymosin; DSM Food Specialties, Delft, the Netherlands); commercial sodium chloride (Cisne, Cabo Frio, RJ, Brazil); phosphates salts: monocalcium phosphate (MCP); a mixture of MCP with polyphosphate, named Blend 1; and a mixture of MCP with monopotassium phosphate (MKP), named Blend 2. Blend 1 and 2 contained around 14.5-15.5% calcium content.

Methods

Based on the results of step I (Supplementary material), five treatments were carried out with 50 L of raw milk and the addition of different calcium sources were considered for step II: (T2) control with addition of 0.24 gL^{-1} $CaCl_2$; (T4) 0.5 gL^{-1} MCP; (T9) 0.25 gL^{-1} Blend 1 + 0.12 gL^{-1} $CaCl_2$; (T10) 0.25 gL^{-1} Blend 2 + 0.12 gL^{-1} $CaCl_2$; and (T11)

0.25 gL^{-1} MCP + 0.12 gL^{-1} $CaCl_2$. The Minas Frescal cheese manufacture (Figure S1) was performed with the pasteurization of the milk at 65 ± 1 ºC for 30 minutes and cooled to 38 ± 1 ºC. Calcium sources were added according to the treatments and mixed for 2 minutes. In the next step, 0.16 mLL^{-1} of lactic acid (10% v/v) and 0.05 mLL^{-1} diluted of rennet (used according to the manufacturer's recommendations) were added and mixed for 2 minutes; coagulation was conducted at 38 ± 2 °C for 40 minutes, after which the curd was cut into 1 cm cubes edge, kept at rest for 2 minutes, mixed slowly for 20 minutes and the whey partially drained (26 L; of which 500 mL were collected for further analysis). Then, sodium chloride (NaCl) was added (2.1% w/v considering the 24 L remaining in the tank), mixed for 2 minutes, and the rest of the whey was removed, and the curd was added to the molds. The remaining whey was drained, and 550- to 600-g cheeses were molded (2 h); the cheeses were kept in the molds until the following day at 5 ± 2 ºC in a ripening chamber with 85 to 86% relative humidity, and then they were packed in plastic bags and stored at 5 ± 2 ºC for 15 days. Three independent repetitions of each treatment were performed. Cheeses samples were subjected to physicochemical analyses, yield, and texture profile analyses (TPA) at day 1 of storage, and syneresis analysis at day 1, 3, 5, 7, 9, 11, 13 and 15 of storage.

Physicochemical analyses

Whey and cheese samples were subjected to physicochemical analysis. Moisture, fat, protein, ash, and pH were determined for pasteurized milk and whey samples; moisture, fat in dry matter, protein, ash, pH, water activity (Aw), and syneresis were determined for cheese samples. The moisture content was determined gravimetrically by drying 5 g of samples at 105 ± 2 ºC until a constant mass was obtained (ISO 5534:2004). Fat content was measured by Gerber-van Gulik method (ISO 3432:2008). Protein was calculated by determination of total nitrogen by the Kjeldahl method, using a conversion factor of 6.38 (ISO 8968-1:2014). The total content of ash was determined gravimetrically by the incineration method at 550 ºC (IDF 27:1964). The pH of cheese samples was measured by blending 20 g of cheese with 20 mL of distilled water, whereas the pH of pasteurized milk and whey was directly determined on the pHmeter (Hanna Instruments Ltd., Leighton Buzzard, UK). An AquaLab (3TE; Decagon Devices Inc., Pullman, WA, USA) was used to measure water activity; the sample cup was filled

to half its depth, placed in the sample chamber and the Aw measured using the standard procedure for the instrument.

Yield and syneresis of Minas Frescal cheese

The mass of Minas Frescal cheese (kg) was determined by weighing the samples after packaging. The yield (kgL^{-1}) was calculated based on the amount of cheese samples produced with the 50 L of milk (Fritzen-Freire *et al*. 2010). The syneresis was calculated by Equation 1, where mw (g) is the mass of the whey released from three cheeses in its package during the storage and mc (g) is the mass of each cheese in the package (Sant'Ana *et al.* 2013).

$$\text{Syneresis (\%)} = (mw/mc) \times 100 \qquad (1)$$

Texture profile analysis (TPA)

TPA was carried out using the universal testing machine (Instron – Series 3367, Canton, MA, USA, 2005). Compression measurements were performed with a cylinder 53 mm in diameter, exerting a force of up to 250 N, with a compression distance of 60% of the initial cube height, for a 2 mm sample. The test speed was 0.8 mm/s with two penetration cycles (Pons & Fiszman, 1996). The force exerted on the sample was automatically recorded and the hardness parameter (N) was automatically evaluated from the force (N) × time (s) curves generated during the test by the Blue Hill 2.0 software (Instron, USA, 2005). Three samples of each treatment were prepared, and at least 5 measurements were performed in each sample.

Statistical analyses

For statistical analyses, the results were compared considering the treatments by Tukey test ($p<0.05$) to identify significant differences at 95% of confidence level, using SISVAR® software system version 5.6 (Ferreira, 2011).

Results and Discussion

The average composition of the milks used to produce Minas Frescal cheese were 87.700 ± 0.30 g 100 g^{-1} of moisture content, 3.60 ± 0.1 g 100 g^{-1} of fat content, 3.03 ± 0.31 g 100 g^{-1} of protein content, 1.123 ± 0.50 g 100 g^{-1} of ash content and 6.68 ± 0.0 of pH. All values are in accordance with the Brazilian milk quality regulation (Brasil, 2018).

Regarding to the whey obtained during Minas Frescal cheese manufacture, Table 1 shows the composition. No differences were observed between the different treatments for the physicochemical parameters of whey ($p>0.05$), the parameters of pH and total solids are in accordance with the Brazilian whey quality regulation (Brasil, 2020). In addition, the results are in accordance with results reported by Silva *et al.* (2013), who evaluated the effect of whey on the production of pasty dulce de leche. This study was also conducted with the production of Minas Frescal cheese to obtain the whey and the manufacture of the cheese was similar. It is important to emphasize this, since to compare the physical and chemical values of the whey, it is fundamental to observe its origin (from which type of cheese it was obtained). The amount of whey expelled in the cheese vat was not significantly different ($p>0.05$) (data not shown), suggesting that any subtle differences in the protein gel network do not have a large impact.

Table 1 also shows the evolution of physicochemical parameters and Minas Frescal cheese hardness. No differences were observed between the different treatments for the physicochemical parameters ($p>0.05$), demonstrating the feasibility of partial or total replacement of $CaCl_2$. In addition, all cheeses produced have moisture content values within the legislation (Brasil, 1996; Brasil, 2004). However, the values for fat content are above, being classified as full fat (fat dry matter 45 to 59.9 g 100 g^{-1}). Studies have shown that this content is quite variable, only 40% of commercial samples meet this standard, and the values found in this study are within the values reported by Magenis *et al.* (2014) with values for fat in dry matter between 23.12 to 52.47 g 100 g^{-1}. Values for protein and ash content are in line with results reported by Magenis *et al.* (2014) and Oliveira *et al.* (2014). A greater standard variation is due to the quality of the initial milk as well as the type of heat treatment used in this milk, which leads to changes in the physical and chemical values of the cheese (Cichoscki *et al.* 2002; Guo *et al.* 2004; Martín-González *et al.* 2007).

The use of different phosphates sources did not affect statistically ($p>0.05$) the results for cheese yield (Table 1), as the cheese yield basically depends on the fat and protein, that is, it varies according to the composition of the milk (Guo *et al.* 2004), and the results for yield agree with other studies in the literature (Sant'Ana *et al.* 2013; Fritzen-Freire *et al*. 2010). Likewise, the partial or total replacement of $CaCl_2$ by MCP or by its mixture with polyphosphates and MKP did not significantly affect the physicochemical

composition and the pH of the cheeses according to the proportions evaluated in this study ($p>0.05$).

However, the texture profile of the cheeses determined in term of hardness was statistically affected ($p<0.05$) (Table 1), with the higher values in T2 with addition of 0.24 gL^{-1} $CaCl_2$ and T10 with 0.25 gL^{-1} of Blend 2 + 0.12 gL^{-1} of $CaCl_2$. And it is observed that the treatments that used the total or partial replacement of $CaCl_2$ by MCP (T4 and 11, respectively) presented the lowest values, while cheeses containing the mixture of MCP with polyphosphate or MKP (T9 and T10, respectively) demonstrated similar results the control sample (T2). Thus, what is observed is that the addition of MCP only in total or partial replacement of $CaCl_2$ did not improve cheese hardness; on the contrary, it reduced it. This behavior was different from what was expected, since, as demonstrated by Guo *et al.* (2003), the presence of monophosphate and calcium in a β-casein solution led to greater precipitation of casein and these precipitates are more resistant than precipitates formed in the presence of calcium alone. What is observed is that this precipitate robustness mechanism may be different in the presence of other proteins present in milk.

Other results for the texture profile of cheeses produced with partial or total replacement of $CaCl_2$ were obtained (Table S2). For the gumminess profile, the T11 treatment, with the partial replacement by MCP, presented lower values than the control (T2). On the other hand, T9 and T10 treatments in which the $CaCl_2$ was partially replaced by the blends, presented statistically similar values ($p<0.05$) to the control. The same behavior was verified for the chewiness profile. The gumminess and chewiness of the cheeses also followed a similar trend to the hardness (Table 1). No significant trend ($p>0.05$) was observed in cohesiveness and springiness of the samples. Similar results were found by Ong *et al.* (2013) when verifying the effect to different concentrations of $CaCl_2$ on the production of Cheedar cheese. The results obtained for the texture profiles of the cheeses show that the replacement only by MCP of $CaCl_2$, either partially or totally, does not produce cheeses with texture similar to the control, requiring the addition of another source of phosphate for the cheeses to resemble the cheese control. This may also demonstrate the effect of phosphate on the texture of the final product, and not just calcium as observed in other studies (Ong *et al.* 2013; Ong *et al.* 2015).

The values obtained for syneresis over the 15 days were also not significantly different ($p>0.05$), according to Table 2. However, there was an increase in syneresis with storage time. Sant'Ana *et al.* (2013) also monitored the syneresis of Minas frescal cheeses produced with milk from different sources, and the authors observed an increase in syneresis over the 21-day storage period, accompanied by a decrease in pH during the same period. The authors pointed out that the increase in syneresis was due to the increase in hydrogen ions and the acidification of the medium, which led to a reduction in repulsive forces of the casein micelles and, consequently, greater aggregation and expulsion of whey from the cheese mass. Another observation is that the T11 that presented the lowest hardness value was the treatment that also presented the highest levels of syneresis from day 1 (Table 2).

Therefore, the replacement of $CaCl_2$ by MCP or MCP mixed with polyphosphate or MKP did not affect the general properties of Minas Frescal cheese, indicating that is a sufficient amount of Ca is still present in the milk. Thus, as noted by Ong *et al.* (2013) for the production of Cheddar cheese in which various concentrations of $CaCl_2$ addition did not change characteristics such as final cheese composition, yield and there were small variations in cheese hardness as the $CaCl_2$ concentration increased, from of 50 mgL^{-1}, there was also an increase in hardness.

Table 3 summarizes the results obtained in this study, which facilitates the industry's understanding of the possibilities of replacing $CaCl_2$ in Minas Frescal cheese according to the characteristics evaluated. This table was built by comparing the control treatment (T2) with the other treatments, showing which characteristics are the same or different. When different, if they are better (>) or worse (<) than the control, according to the results obtained in this study, and in order to summarize these observations. The modulation of cheese hardness is possible according to the industrial objective, without changing the physicochemical characteristics, yield and syneresis of the cheese.

Conclusion

This study shows that it is possible for the industry to manufacture Minas Frescal cheese using other sources of calcium, such as MCP, without changing its physical chemical characteristics and yield. It is also possible to modulate the hardness of the cheese according to the proportion of MCP or the blends used in this study, without any change in syneresis during storage. Cheeses produced with Blend 2 and $CaCl_2$

(0.25 gL^{-1} of Blend 2 + 0.12 gL^{-1} of $CaCl_2$) showed higher values for hardness, however, further studies are needed to identify which characteristics this cheese presents differently from other, such as soluble calcium content.

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Conflict of Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Authors' contributions

AFC was responsible for the conceptualization, funding acquisition and supervision of the activities. FLS, AF, NFNS, RS and AFC designed this paper. FLS, AF, MTCM, ICO and NFNS conducted the laboratory analysis and validated the results. FLS and AFC were responsible for the data analysis. FLS wrote the original draft of the manuscript. FLS, AF, ITP, NFNS, RS and AFC reviewed and edited the manuscript.

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Table legends

Table 1: Evaluation of the composition and characterization of the whey and Minas Frescal cheese produced by the partial or total substitution of $CaCl_2$.

Table 2: Evaluation of the syneresis of the Minas Frescal cheese produced by the partial or total substitution of $CaCl_2$.

Table 3: Modulation of Minas Frescal cheese samples produced by the partial or total substitution of $CaCl_2$ by MCP and its blends and compared with the control treatment (T2).

Table 1:

	Treatment				
	T2	T4	T9	T10	T11
Whey					
Moisture content (g 100 g^{-1})	93.313 ± 0.25[a]	93.537 ± 0.31[a]	93.297 ± 0.172[a]	93.350 ± 0.35[a]	93.470 ± 0.14[a]
Fat content (g 100 mL^{-1})	0.4 ± 0.0[a]	0.3 ± 0.1[a]	0.4 ± 0.1[a]	0.3 ± 0.1[a]	0.3 ± 0.1[a]
Protein content (g 100 mL^{-1})	0.85 ± 0.06[a]	0.80 ± 0.05[a]	0.83 ± 0.02[a]	0.83 ± 0.02[a]	0.80 ± 0.05[a]
Ash content (g 100 g^{-1})	0.577 ± 0.05[a]	0.620 ± 0.10[a]	0.670 ± 0.10[a]	0.610 ± 0.09[a]	0.567 ± 0.10[a]
pH	6.55 ± 0.2[a]	6.50 ± 0.1[a]	6.52 ± 0.1[a]	6.48 ± 0.2[a]	6.68 ± 0.0[a]
Minas Frescal cheese					
Moisture content (g 100 g^{-1})	59.367 ± 1.78[a]	59.300 ± 1.79[a]	59.690 ± 1.79[a]	59.503 ± 2.87[a]	59.860 ± 2.37[a]
Fat in dry matter (g 100 g^{-1})	48.5 ± 4.2[a]	47.2 ± 2.4[a]	49.0 ± 0.7[a]	51.3 ± 1.3[a]	51.7 ± 1.7[a]
Protein content (g 100 g^{-1})	14.67 ± 0.7[a]	13.85 ± 1.2[a]	13.94 ± 1.2[a]	14.49 ± 1.4[a]	15.25 ± 0.4[a]
Ash content (g 100 g^{-1})	3.390 ± 0.87[a]	3.457 ± 0.85[a]	3.380 ± 0.31[a]	2.880 ± 0.26[a]	2.893 ± 0.14[a]
pH	6.82 ± 0.1[a]	6.71 ± 0.2[a]	6.82 ± 0.1[a]	6.81 ± 0.2[a]	6.70 ± 0.1[a]
Aw	0.989 ± 0.01[a]	0.991 ± 0.01[a]	0.985 ± 0.01[a]	0.991 ± 0.01[a]	0.990 ± 0.01[a]
Mass of Minas cheese (kg)	8.613 ± 0.04[a]	8.640 ± 0.11[a]	8.390 ± 0.33[a]	8.527 ± 0.36[a]	8.330 ± 0.41[a]
Yield (Lkg^{-1})	5.80 ± 0.0[a]	5.79 ± 0.1[a]	5.96 ± 0.2[a]	5.87 ± 0.2[a]	6.01 ± 0.3[a]
Hardness (N)	7,6443 ± 0,358[c]	5,9026 ± 0,329[a,b]	6,5457 ± 0,078[b,c]	7,2983 ± 0,589[b,c]	4,6549 ± 0,189[a]

[a-b] Within a line, different superscript lowercase letters denote significant differences ($P<0.05$) among the samples by Tukey's test. Treatments: (T2) control with addition of 0.24 gL^{-1} $CaCl_2$; (T4) 0.5 gL^{-1} MCP; (T9) 0.25 gL^{-1} Blend 1 + 0.12 gL^{-1} $CaCl_2$; (T10) 0.25 gL^{-1} Blend 2 + 0.12 gL^{-1} $CaCl_2$; and (T11) 0.25 gL^{-1} MCP + 0.12 gL^{-1} $CaCl_2$. Blend 1 is a mixture of MCP with polyphosphate and Blend 2 is a mixture of MCP with MKP.

Table 2:

Treatment	Syneresis (%)							
	Day 1	Day 3	Day 5	Day 7	Day 9	Day 11	Day 13	Day 15
T2	0.206 ± 0.218[a]	1.012 ± 0.806[a]	1.894 ± 1.763[a]	2.560 ± 1.570[a]	3.234 ± 1.748[a]	4.164 ± 1.446[a]	4.550 ± 1.574[a]	5.273 ± 0.925[a]
T4	0.433 ± 0.162[a]	1.179 ± 0.548[a]	1.870 ± 1.090[a]	2.760 ± 0.699[a]	3.249 ± 0.899[a]	3.793 ± 0.907[a]	4.114 ± 1.282[a]	4.498 ± 1.069[a]
T9	0.463 ± 0.477[a]	1.435 ± 1.063[a]	2.259 ± 1.772[a]	2.935 ± 1.543[a]	3.336 ± 1.876[a]	4.146 ± 1.849[a]	4.298 ± 1.876[a]	4.706 ± 1.834[a]
T10	0.394 ± 0.525[a]	1.226 ± 1.054[a]	1.988 ± 1.415[a]	3.081 ± 0.955[a]	3.759 ± 1.247[a]	4.893 ± 1.251[a]	5.215 ± 1.648[a]	5.760 ± 1.294[a]
T11	0.975 ± 0.985[a]	3.066 ± 3.788[a]	4.502 ± 5.364[a]	4.802 ± 5.159[a]	5.164 ± 5.153[a]	5.634 ± 4.830[a]	5.897 ± 4.928[a]	6.201 ± 5.126[a]

[a-b] Within a column, different superscript lowercase letters denote significant differences ($p<0.05$) among the samples by Tukey's test. Treatments: (T2) control with addition of 0.24 gL^{-1} $CaCl_2$; (T4) 0.5 gL^{-1} MCP; (T9) 0.25 gL^{-1} Blend 1 + 0.12 gL^{-1} $CaCl_2$; (T10) 0.25 gL^{-1} Blend 2 + 0.12 gL^{-1} $CaCl_2$; and (T11) 0.25 gL^{-1} MCP + 0.12 gL^{-1} $CaCl_2$. Blend 1 is a mixture of MCP with polyphosphate and Blend 2 is a mixture of MCP with MKP.

Table 3:

Treatment	T4	T9	T10	T11
Physicochemical	=	=	=	=
Syneresis	=	=	=	=
Yield	=	=	=	=
Hardness	<	=	=	<

Compared to the control treatment (T2): better (>); worse (<); or the same (=). Treatments: (T2) control with addition of 0.24 gL^{-1} $CaCl_2$; (T4) 0.5 gL^{-1} MCP; (T9) 0.25 gL^{-1} Blend 1 + 0.12 gL^{-1} $CaCl_2$; (T10) 0.25 gL^{-1} Blend 2 + 0.12 gL^{-1} $CaCl_2$; and (T11) 0.25 gL^{-1} MCP + 0.12 gL^{-1} $CaCl_2$. Blend 1 is a mixture of MCP with polyphosphate and Blend 2 is a mixture of MCP with MKP.

3.1. Material suplementar do artigo 2

Effects of calcium chloride substitution on the physicochemical properties of Minas Frescal Cheese

Material and Methods

Methods

Preparation of the gels by enzymatic coagulation

The study of gels was carried out with 50 mL of raw milk, pasteurized at 65 ± 1 ºC for 30 minutes and cooled to 38 ± 1 ºC. For step I, the previous evaluation of the best concentrations of total or partial replacement of $CaCl_2$, twelve treatments were carried out: (T1) 0.12 gL^{-1} $CaCl_2$; (T2) 0.24 gL^{-1} $CaCl_2$; (T3) 0.25 gL^{-1} MCP; (T4) 0.50 gL^{-1} MCP; (T5) 0.25 gL^{-1} Blend 1; (T6) 0.50 gL^{-1} Blend; (T7) 0.25 gL^{-1} Blend 2; (T8) 0.50 gL^{-1} Blend 2; (T9) 0.25 gL^{-1} Blend 1 + 0.12 gL^{-1} $CaCl_2$; (T10) 0.25 gL^{-1} Blend 2 + 0.12 gL^{-1} $CaCl_2$; (T11) 0.25 gL^{-1} MCP + 0.12 gL^{-1} $CaCl_2$; and (T12) only with raw milk. After adding the agents according to the concentration of each treatment, the milk was stirred for 3 minutes to homogenize and 0.16 mLL^{-1} diluted lactic acid (10% v/v) was added, followed by stirring for another 2 minute and then the rennet (0.05 mLL^{-1}) was added according to the manufacturer's recommendations, with stirring for an additional 1 minute. After the initial preparation of the cheeses model, they were immediately used for rheological analysis at 38 ± 1 ºC.

Rheological analysis of the gels

Small-amplitude oscillatory measurements were made to monitored the formation of the gels at 38 ºC using a rheometer MCR 301 (Anton Paar, Germany), equipped with thermostatic bath and a stainless steel double gap geometry. The oscillatory mode was employed at a frequency of 1 Hz and 0.1% strain, and the final G' refers to G' values attained after 40 minutes of oscillatory measurements. The deformation properties of gels were determined by applying a single constant shear rate (0.01 s^{-1}) up to the

yielding of the gel. Yield stress (σ yield) was defined as the point when shear stress started to decrease. Yield strain (γ strain) was the strain value at the yield point.

Results and Discussion

Step I: previous evaluation of the total or partial replacement of $CaCl_2$ in model cheese

In our first step, twelve treatments were performed in order to evaluate the total or partial replacement of $CaCl_2$, and Table S1 shows the rheological parameters of the value of the elastic modulus (G') and yield stress (σ stress) obtained during the enzymatic milk coagulation process. Differences were observed in all parameters analyzed among the different treatments ($p<0.05$). Higher values of G' and yield stress were found in treatments with twice the concentrations of each agent, being the case of T1 and T2, T3 and T4, T5 and T6, T7 and T8. In relation to the treatments with the same concentration of $CaCl_2$, but with the addition of Blend 1 (T9), or Blend 2 (T10), or MCP (T11), the highest G' value was T10 with 0.25 gL^{-1} of Blend 2 + 0.12 gL^{-1} of $CaCl_2$; and when we compared these treatments with treatments only with Blend 1, Blend 2 or MCP, a lower G' value was reported. Although treatments without $CaCl_2$ showed lower values, the incorporation of Blend 1 (T9), Blend 2 (T10) and MCP (T11) with $CaCl_2$ demonstrated the potential in increase the G' in the gel formation when we compare to T1 with the same $CaCl_2$ concentration. Regarding yield stress, that is a known physical and rheological property defined as the minimum shear stress applied to initiate the flow process, or as the force per unit area required to break the structure (Sun & Gunasekaran, 2009) (Table 1), the same value profile was found. The values found for the G' were considered as a means of evaluating the gel strength, since G' is the easiest and most direct way to characterize the gel formation during the coagulation process, the increase of the storage modulus on the clotting time implicates in the formation of the gel network (Hussain *et al.* 2013; Leite Júnior *et al.* 2014). Thus, the treatments that presented the highest G' and σ stress values were chosen to produce Minas Frescal cheese, being the following: T2 (control), T4, T9, T10 and T11.

Calcium has been added to milk as different salts such as calcium carbonate, tricalcium phosphate, calcium chloride, calcium gluconate, and calcium lactate (Vavrusova & Skibsted, 2014). However, for the production of Minas Frescal cheese, the industry has been using $CaCl_2$ in almost 100% of cases, since the easily dissolved in milk and it causes a notable decrease in pH and an increase in free calcium ion (Ca^{2+}) concentration. Which helps in both stages of milk clotting, since the first step requires lowering the pH for the hydrolysis of κ-casein and the second phase requires free calcium for the formation of aggregation to occur (Ong *et al.* 2013; Wang *et al.* 2020). However, it is important to evaluate other sources of calcium to replace calcium chloride in the production of Minas Frescal cheese, since several studies show that the type of calcium salt added influences the salt balance of milk and partition of salts between casein micelles and the serum (Wang *et al.* 2020; Gaucheron, 2015; On-Nom *et al.* 2010), and consequently, can influence the final properties of cheese.

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Figures legends:

Figure S1: Protocol employed for the manufacture of Minas Frescal cheeses T2, T4, T9, T10 and T11.

Blend 1 is a mixture of MCP with polyphosphate and Blend 2 is a mixture of MCP with MKP.

Figure S1:

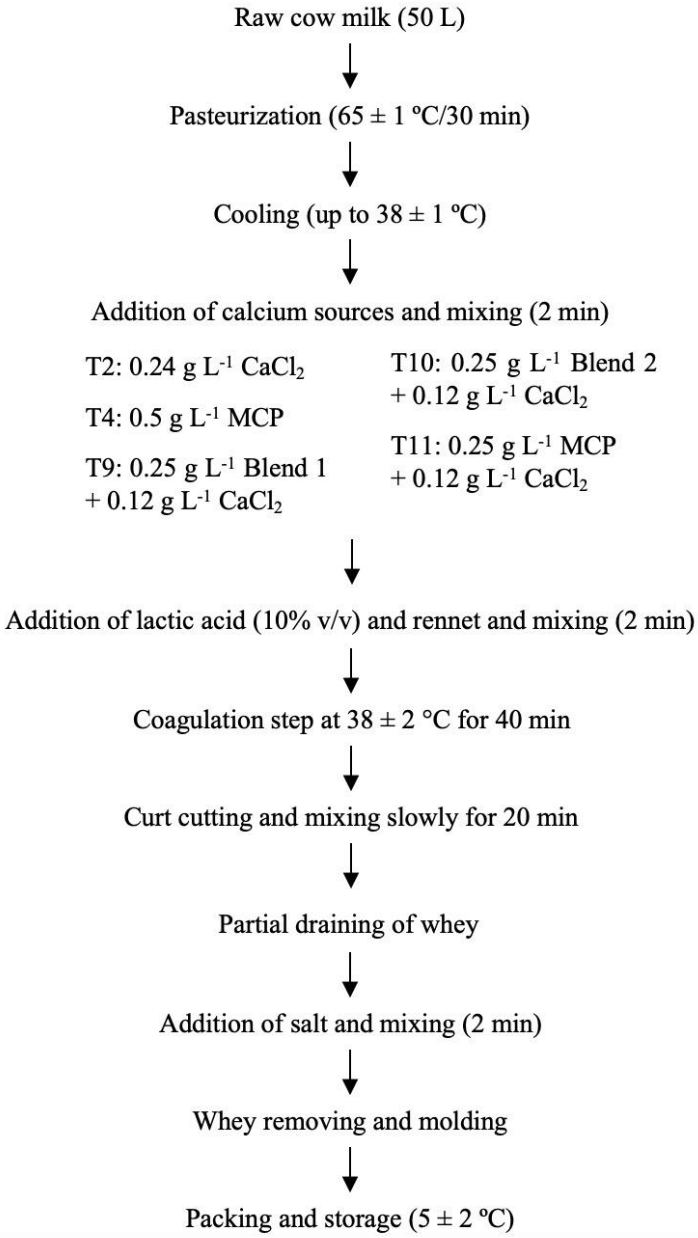
Raw cow milk (50 L)
Pasteurization (65 ± 1 ºC/30 min)
Cooling (up to 38 ± 1 ºC)
Addition of calcium sources and mixing (2 min)
T2: 0.24 g L-1 CaCl2
T4: 0.5 g L-1 MCP
T9: 0.25 g L-1 Blend 1 + 0.12 g L-1 CaCl2
T10: 0.25 g L-1 Blend 2 + 0.12 g L-1 CaCl2
T11: 0.25 g L-1 MCP + 0.12 g L-1 CaCl2
Addition of lactic acid (10% v/v) and rennet and mixing (2 min)
Coagulation step at 38 ± 2 °C for 40 min
Curt cutting and mixing slowly for 20 min
Partial draining of whey
Addition of salt and mixing (2 min)
Whey removing and molding
Packing and storage (5 ± 2 ºC)

Table legends:

Table S1: Evaluation of the rheological parameters obtained during the enzymatic milk coagulation process (n = 2).

Table S2: The effect of $CaCl_2$ substitution on the texture of Minas Frescal cheese (n=3).

Table S1:

Treatment	Concentration (g L^{-1})	G' (Pa)	Yield stress (σ yield) (Pa)
T1	0.12 $CaCl_2$	86.492 ± 1.527 ^{c,d}	36.968 ± 1.389 ^{b,c}
T2	0.24 $CaCl_2$	98.423 ± 2.994 ^{e}	42.244 ± 3.523 ^{b,c}
T3	0.25 MCP	80.808 ± 1.736 ^{b,c}	33.672 ± 1.150 ^{b,c}
T4	0.50 MCP	93.874 ± 3.717 ^{d,e}	39.496 ± 1.114 ^{b}
T5	0.25 Blend 1	74.643 ± 1.184 ^{b}	33.167 ± 1.163 ^{b,c}
T6	0.50 Blend 1	87.813 ± 0.134 ^{c,d}	36.801 ± 0.343 ^{b,c}
T7	0.25 Blend 2	81.848 ± 0.216 ^{b,c}	34.448 ± 0.410 ^{b,c}
T8	0.50 Blend 2	93.098 ± 0.823 ^{d,e}	37.347 ± 0.532 ^{b,c}
T9	0.25 Blend 1 + 0.12 $CaCl_2$	93.608 ± 2.379 ^{d,e}	39.416 ± 0.680 ^{b,c}
T10	0.25 Blend 2 + 0.12 $CaCl_2$	96.927 ± 3.292 ^{e}	40.935 ± 1.187 ^{c}
T11	0.25 MCP + 0.12 $CaCl_2$	93.876 ± 1.729 ^{d,e}	39.582 ± 1.160 ^{b,c}
T12	Raw milk	62.383 ± 0.000 ^{a}	25.130 ± 0.000 ^{a}

Table S2:

	Treatment				
	T2	T4	T9	T10	T11
Gumminess	5.859 ± 0.299^{c}	4.629 ± 0.377^{b}	5.023 ± 0.354^{b,c}	5.513 ± 0.423^{b,c}	3.503 ± 0.005^{a}
Chewiness	70.305 ± 3.592^{b}	55.556 ± 4.519^{a,b}	60.283 ± 4.254^{b}	66.155 ± 5.079^{b}	44.038 ± 2.773^{a}
Springiness	5.299 ± 0.321^{a}	4.552 ± 0.598^{a}	5.370 ± 0.625^{a}	5.637 ± 0.249^{a}	4.363 ± 0.050^{a}
Cohesiveness	0.914 ± 0.006^{a}	0.923 ± 0.006^{a}	0.914 ± 0.014^{a}	0.913 ± 0.004^{a}	0.919 ± 0.014^{a}

$^{a-b}$ Within a line, different superscript lowercase letters denote significant differences ($P<0.05$) among the samples.

Treatments: (T2) control with addition of 0.24 gL^{-1} $CaCl_2$; (T4) 0.5 gL^{-1} MCP; (T9) 0.25 gL^{-1} Blend 1 + 0.12 gL^{-1} $CaCl_2$; (T10) 0.25 gL^{-1} Blend 2 + 0.12 gL^{-1} $CaCl_2$; and (T11) 0.25 gL^{-1} MCP + 0.12 gL^{-1} $CaCl_2$. Blend 1 is a mixture of MCP with polyphosphate and Blend 2 is a mixture of MCP with MKP.

4. ARTIGO 3

Effect of phosphate salts and varying quantities of casein and whey protein on the syrup characteristics of a sweetened condensed skimmed milk and vegetable fat blend

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Abstract

A blend of sweetened condensed skimmed milk and vegetable fat is a product that can be prepared by mixing milk constituents and/or whey in powder form with the addition of sugar and/or vegetable fat. The aim of this study was to determine the influence of varied casein and whey protein compositions, as well as the use of phosphate salts, on the stability of milk mixture syrup. Syrup production was carried out using a mixture of skimmed milk powder, demineralized whey powder, glucose, vegetable fat, sugar, and phosphate. The casein: whey protein ratios used were 80:20, 70:30, and 56:44, and the phosphates were PQTS and OPSS. The results indicated that the PQTS salt presented the best stability of the mixture, mainly in the 56:44 mixture. In addition, it modified the pH of the mixture to a greater extent than the standard and presented particles with larger sizes at both d_{10} and d_{90}. However, the OPSS salt induced a higher phosphate content in the mixture, consequently lowering the free calcium and total calcium content. The mixture produced with the 56:44 ratio exhibited a reduction in the particle size of the mixtures with phosphates when evaluating the d_{90}. As a result, the industry will select one of these phosphates based on the qualities of the desired end product.

Keywords: phosphate; condensed milk; proteins; particle size, calcium.

Introduction

The blend of sweetened condensed skimmed milk and vegetable fat (SCS) is classified as a product that can be prepared by mixing the milk constituents in powder form with the addition of water or by partially removing the water from the skimmed milk, with the addition of sugar and edible vegetable oil, vegetable fat, or a mixture of these to meet the composition requirements [1].

SCS is classified as a product of the concentrated and dehydrated dairy industry, such as condensed milk, which is a food product that is widely used worldwide [2]. However, condensed milk mixtures are products that have appeared on the market to replace condensed milk [3]. In Brazil, it emerged as a cost-cutting alternative, where milk is partially substituted by whey; in the international market, in addition to the use of whey, animal fat is also substituted by vegetable fat [3].

In Brazil, condensed milk mixture is made from milk or reconstituted milk with whey or skimmed milk with whey and vegetable fat, with the addition of sucrose [4]. According to a study conducted [3] on various condensed milk legislation in Brazil and the world, the variety produced with vegetable fat was not defined in any legislation around the world, despite having already been indicated by CODEX since 1999 [5]; thus, the authors concluded that technological, sensory, or even rheological changes make vegetable fat difficult to use in comparison to fat of animal origin.

The SCS production process involves the same steps as condensed milk production: milk homogenization, sugar addition, concentration, seeding, and cooling [6]. The syrup is a mixture of all the raw materials after the thermal treatment and before carrying out the concentration of the product. This is because the characteristics of the final product will vary according to the technology parameters, equipment, and, in the case of the SCS, the raw materials and proportions of these raw materials used [7]. However, there are no studies showing the influence of the proportion of casein and whey on the characteristics of SCS, as well as the interference in the process of using vegetable fat to replace animal fat.

In a study carried out by [8], it was observed that the addition of partially demineralized whey powder can be used as a partial substitute for milk in the formulation of sweetened condensed milk; however, the product produced was only stable after the use of additives as stabilizers. Thus, a stabilizer must be added to this mixture to

facilitate the technological process, and a good alternative would be the use of phosphate salts. Studies have shown that the effects of these salts in the product can influence viscosity, stability of the protein system in dairy products, and pH control, in addition to being a metal ion complexing, dispersing, and suspending agent [9-12]. In addition, some studies have shown that the interaction between phosphates occurs on the casein molecule, acting as a sequestrant of calcium present in the casein micelle and replacing it with another metal present in the salt molecule, causing the protein to modify its characteristics [9, 13].

Thus, the objective of this study is to evaluate the influence of PQTS and OPSS phosphate salts on the thermal stability of SCS syrup produced with different proportions of proteins (casein: whey protein).

Material and methods

This study was divided into two stages: the first, containing 12 treatments, is described in the supplementary material, and the second, containing nine treatments, is described in this manuscript. The difference between these steps is the phosphate salts; in the first stage, there are three different phosphate salts and in the second, only two. Because one of the phosphates did not present the desired characteristics, it was eliminated from the study. In addition, in the first stage, the mixture homogenization process was not adopted, that is only mechanical mixing was performed.

Material

Analytical grade chemical reagents were used for the physicochemical analyses. The following ingredients were used for blending sweetened condensed skimmed milk and vegetable fat: Camponesa skim milk powder (Lagoa da Prata, MG, Brazil), Porto Alegre demineralized whey powder (Ponte Nova, MG, Brazil), Alvinho crystal sugar (Urucânia, MG, Brazil), Marvi liquid glucose (Ourinhos, SP, Brazil), palm vegetable fat Akomix NH80 AAK (Jundiaí, SP, Brazil), and commercial phosphate salts: PQTS, OPSS, and DPTS.

Phosphate salt mixtures were composed of orthophosphate (E 339), sodium phosphates (E 339), diphosphate (E 450), polyphosphate (E 452), and sodium citrate (E 331). These mixtures differ in P_2O_5 content, chain length and pH (1% solution) and are as follows: PQTS, phosphate salt composed of sodium phosphate, and sodium

citrates, 20.5 ± 1.0% P_2O_5, pH 9.0 ± 0.3; OPSS, phosphate salt composed of orthophosphate, polyphosphate, and sodium citrate, 40.4 ± 1.0% P_2O_5, pH 11.3 ± 0.3; and DPTS, phosphate salt composed of diphosphate, 58.2 ± 1.0% P_2O_5, pH 7.1 ± 0.3.

Methods

Preparation of the syrup – stage 2

In the experimental design, nine treatments were carried out with syrup with varying proportions of proteins and the addition of different phosphate salts, as described in Table 1. The ratios of casein:whey proteins were 80:20 (treatment 1), 70:30 (treatment 2), and 56:44 (treatment 3). In addition, three levels of phosphate salts were used: without the addition of phosphate (Treatment A), with the addition of PQTS (Treatment B), and with the addition of OPSS (Treatment C).

As it is a bench study, it started with a syrup of 600 g, where the process for rehydrating the powders was performed under constant agitation (100 ± 3 rpm) for 30 min and heating at 70 ± 2 °C (Ethiktechnology Fermentator) and then homogenized under a pressure of 210 bar (APV homogenizer). Subsequently, heat treatment was performed at 82 ± 2 °C for 5 min. The SCS was cooled to room temperature (23 ± 1 °C) for analysis. Three independent replicates of each treatment were performed. The syrup samples were subjected to physicochemical analyses, alcohol tests, thermal stability tests (HCT), particle size distribution, calcium analysis, and determination of phosphate content. The supplementary material presents details of the adopted methodology.

Alcohol test

The samples from each treatment group were subjected to alcohol testing. Ethanol concentrations of 78% (v/v) to 98% (v/v) were used, in intervals of two percentage units; for the evaluation of the microparticles resulting from the alcohol test, two different observation methods were used: direct optical visualization in petri dishes and visualization by digital optical microscopy magnified at 200x (ProScope). A previously described methodology was followed [14], in which the same amount of sample and alcohol were added to the petri dish and homogenized for a standard time for all samples. An aliquot was then placed on a slide for visualization in the ProScope and

visual assessment was performed. The amount of microparticles in each alcoholic content was evaluated.

Determination of heat coagulation time (HCT)

The SCS syrup was subjected to determination of the heat coagulation time (HCT) following a previously described procedure [15]. Aliquots (5 mL) of the samples were transferred to glass tubes with rubber-coated lids and immersed in an oil bath that was thermostatically controlled at 140 ± 2 °C with a constant rocking speed (8 rev/min). The heat coagulation time (HCT) was recorded as the time elapsed between immersing the sample in the oil bath and the onset of visible aggregation in the sample within the test tubes.

Physicochemical analyses

These samples were then subjected to moisture, protein, and pH analyses. The moisture content was determined gravimetrically by drying 5 g of the samples at 105 ± 2 °C until a constant mass was obtained [16]. Protein content was calculated by determination of total nitrogen using the Kjeldahl method, using a conversion factor of 6.38 [17]. The total ash content was determined gravimetrically by incineration at 550 ºC [18]. The pH of the samples was determined directly using a pH meter (Hanna Instruments Ltd., Leighton Buzzard, UK).

Particle size analysis (LS)

Aliquots were obtained from each treatment and subjected to particle size analysis using a Beckman Coulter LS 13 320 laser diffraction particle size analyzer (Beckman Coulter, Miami, FL, USA) coupled to an aqueous liquid module (Beckman Coulter, Miami, FL, USA). The samples taken were slowly added to the reservoir of the liquid analysis module containing water at room temperature, aiming to obtain a level of 47 ± 5% in the PIDS (Polarization Intensity Differential Scattering System) photodetectors. Data were collected in the region from 0.04 to 2000 µm with a collection time set at 90 seconds. The results were obtained using a refractive index of 1.332 for the dispersing medium (water), 1.57 for the casein region, and 1.45 for the palm fat region [19, 20] and were represented by the % of volume occupied by the particles as a function of their size. Beckman Coulter software version 5.03 was applied to obtain statistical data [21]. The readings were performed in duplicate.

Determination of calcium and phosphate

Calcium determination was performed using a LAQUAtwin ionic calcium measuring equipment (Horiba, Japan) for free calcium and calcium at pH 4.0, as described by the manufacturer. The device was calibrated, and the free calcium was measured with the addition of the sample directly to the device. Calcium was also measured at pH 4.0, using HCl p.a. until the sample reached pH 4.0 ± 0.1, and then the reading was carried out on the equipment.

Phosphate content was determined by colorimetric analysis at 420 nm using a conversion factor of 2.29 to obtain phosphate content based on phosphorus content [22].

Statistical analyses

For statistical analyses, the results were compared using Tukey's test ($p<0.05$) to identify significant differences at the 95% confidence level using SISVAR® software system version 5.6 [23].

Results and Discussion

The results obtained are shown in Table 2. The alcohol test indicated that for the syrup without the phosphates, as the amount of casein in the blend was reduced, the alcohol cut-off point decreased, leaving an alcohol 84% (v/v) for the 80:20 syrup, decreasing to 82% (v/v) for the 70:30 mixture, and reaching 80% (v/v) for the 56:44. The same behavior was observed for HCT, in which there was a reduction in the clotting time of the blends without the phosphates when reducing the casein content in the syrup, as observed in Table 2. This shows that as the amount of casein in the SCS decreases, thermal stability decreases, which is also shown in Table 2, and with an increase in protein content in the mixture, product stability is decreased. In a study carried out by [24], the relationship between the ratio of protein to total solids (TS) for the thermal stability of skim milk concentrate was investigated, where when the TS ratio decreased, there was an increase in the HCT. When verifying the same TS ratio for the studied treatments, the same relationship was observed, as shown in Table 3.

Another result found was that when phosphate salts were added, an increase in alcohol stability of all syrup was observed, with the phosphate PQTS showing better stability, including in the SCS. The proportion 56:44 did not show coagulation in any alcohol content in all repetitions. Likewise, if we observe the results obtained for the HCT (Table 2), this treatment presented the highest value (50:23 min), differing from the others. Thus, the PQTS salt has greater thermal stability, especially when it contains 56% casein and 44% whey protein in syrup.

For the particle size analysis, we observed an increase in d_{10} when we added phosphates in all treatments (Table 2), which can be explained by the fact that the presence of phosphate induces the formation of larger aggregates as a result of the interaction of these salts with proteins [9]. In general, it was observed that the PQTS salt increased the particle size in all treatments. However, when observing the values obtained for d_{90} in the syrup with a 56:44 ratio, a reduction in the size of the particles was observed when phosphates were added, with the treatment without phosphates having the largest size (43,662 µm). This result is not expected given what was reported above and what is found in the literature; however, further studies are needed to understand what may have happened in this treatment. When evaluating the particle sizes in relation to the content of casein and whey protein present in the SCS, it was observed that the reduction of casein in the mixture led to an increase in both d_{10} and d_{90}. Researchers previously evaluated the particle size of commercial condensed milk samples [20] and obtained a similar profile for the d_{10} and d_{90} values as those found in this study for samples without phosphate (T1A, T2A, and T3A) and T1A. The authors also verified that the dissolution of the sample in a dispersing medium containing a lactose solution instead of water allows slower dissolution of the substance and, therefore, a more reliable measurement of the particle size [20], which may have also influenced the high standard deviation of the samples in this study, as the dispersing medium used was water.

The PQTS salt has a greater ability to modify the pH of the syrup, causing an increase from 0.2 to 0.4, while the OPSS increased pH of the blend from 0.2 to 0.3, upon using the SCS without addition of phosphate as the standard for each treatment (Table 2). In addition, we observed, in relation to the protein ratio, that when reducing the casein content in the SCS, we started with a lower pH, since the syrup with 80:20 had a pH of 6.7, while the blend with 70:30 had a pH of 6.6, and the 56:44 had a pH of 6.5.

The calcium and phosphate contents of the SCS showed that the OPSS phosphate exhibited higher phosphate contents; thus, it presented lower calcium contents, as it contained more available phosphate groups and thus a greater sequestering power, reducing the content of free calcium and calcium at pH 4.0 present in the mixture in all treatments. This is because the interaction of phosphates occurs on the casein molecule, acting as a sequestrant of the calcium present in the casein micelle and replacing it with another metal present in the molecule of the salt, causing the protein to modify its characteristics [9, 13]. The amount of calcium and phosphate present in the product can influence several factors in dairy products, as already reported [25], including the coagulation, mainly the aggregation reaction in the manufacture of cheese, and the content of each of these minerals can influence the coagulation time, and the texture and the insoluble calcium phosphate salt can influence the buffering power of the cheese. However, an excess of these elements can also lead to the formation of precipitates, which can affect the technological aspects. Researchers studied the molar ratio of Ca/P in solutions to observe its effect on the precipitation and the characteristics of these precipitates [10]. This is because the formation of calcium phosphate precipitates causes scaling. The authors concluded that with a Ca/P molar ratio of 1.50 Ca/P there was greater precipitation efficiency, whereas with a ratio of 1.00, calcium was a limiting factor and with a ratio of 2.00, the phosphate concentration was a limiting factor. Thus, when evaluating the molar ratio of Ca/P present in the samples studied (Table 3), it is seen that the vast majority are close to 1.00; thus, the calcium concentration is a limiting factor for the formation of these precipitates. An excess of phosphate may also be present in these samples. Samples T1A and T3A have a molar ratio closer to 1.50, which is precisely the ratio at which there is greater efficiency in the precipitation of calcium phosphates. This indicates that the use of phosphates also helps reduce incrustation in equipment. However, future studies on deposit formation are needed to better understand this dynamic, as the aforementioned evaluation was performed in standard solutions, and the behavior may be different when evaluating the product.

Conclusion

This study shows that phosphate salts modify the stability characteristics and particle sizes of the syrup for dairy mixtures, and among the evaluated salts, the PQTS salt presented the greatest stabilizing power of the SCS, especially with a ratio of 56:44

casein: whey protein. In addition, it has the potential to further increase the pH of the mixture and form larger aggregates as it has a larger particle size. However, the OPSS salt has a higher phosphate content, and therefore, has a greater calcium sequestration power in the mixture. Thus, the choice between which salt to use in the industry should consider which criterion is the most important for the technological process of producing a concentrated dairy mixture.

Other studies should be carried out to better understand what may have happened with the treatment containing 56% casein and 44% whey protein, given the reduction in particle sizes observed when adding phosphates.

This was a preliminary study to evaluate the behavior of syrup with different proportions of casein and whey protein as well as the effect of adding phosphates to this mixture. Future studies should seek to understand the effects of the concentration process on obtaining a condensed milk mixture.

Conflict of Interest

On behalf of all the authors, the corresponding author states that there are no conflicts of interest.

Authors' contributions

AFC was responsible for conceptualization, funding acquisition, and supervision of the activities. FLS, EFM, ITP, RS, and AFC designed the study. FLS, ILP, JAC, and NSC conducted laboratory analysis and validated the results. FLS and AFC were responsible for data analysis. FLS wrote the original manuscript draft. FLS, EFM, ITP, RS, and AFC reviewed and edited the manuscript.

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Table legends:

Table 1: Proportion of ingredients used in each treatment.

Table 2: Effect of the addition of phosphate salts in the syrup of the blend of sweetened condensed skimmed milk and vegetable fat (n=3)

Table 3: Relation of thermal stability, protein and total solids ratio, and Ca/P for samples with phosphate addition in different proportions of casein and whey protein (n=3)

Table 1:

Treatment	Casein: whey protein ratio	Skimmed milk powder (g/100 g)	Demineralized whey powder (g/100 g)	Sucrose (g/100 g)	Glucose (g/100 g)	Vegetable fat (g/100 g)	Phosphate (g/100 g)	Water (g/100 g)
1	80:20	2,54	0	19,73	4,62	4,07	0,1	69,04
2	70:30	2,42	0,35	19,73	4,35	4,07	0,1	69,09
3	56:44	2,2	0,94	19,73	3,9	4,07	0,1	69,16

[a-b] Within a row, different superscript lowercase letters denote significant differences ($p<0.05$) among the samples.

Table 2:

Treatment		Description	HCT (min)	d_{10} (µm)	d_{90} (µm)	pH	Moisture (g/100g)	Protein (g/100g)	Free calcium (ppm)	Calcium in pH 4.0 (ppm)	Phosphate (P_2O_5) (mg/100g)
1-A	80:20	No phosphate	33:40 ± 04:28^{a}	0.122 ± 0.003^{a}	2.939 ± 0.451^{a}	6.7 ± 0.2$^{a.b.c}$	73.05 ± 2.29^{a}	1.29 ± 0.07^{a}	41.77 ± 18.90$^{b.c}$	255.00 ± 85.44$^{a.b}$	66.97 ± 3.68^{a}
1-B		PQTS	32:42 ± 05:02^{a}	0.331 ± 0.287^{a}	34.397 ± 52.594^{a}	7.1 ± 0.2^{c}	71.58 ± 0.38^{a}	1.48 ± 0.37^{a}	21.00 ± 7.86$^{a.b}$	233.33 ± 95.70$^{a.b}$	92.45 ± 2.84$^{b.c}$
1-C		OPSS	31:36 ± 05:14^{a}	0.281 ± 0.083^{a}	4.514 ± 1.023^{a}	6.9 ± 0.4$^{b.c}$	71.00 ± 2.77^{a}	1.51 ± 0.46^{a}	13.17 ± 3.21^{a}	218.33 ± 67.14$^{a.b}$	113.53 ± 5.89$^{d.e.f}$
2-A	70:30	No phosphate	32:34 ± 08:02^{a}	0.140 ± 0.028^{a}	7.390 ± 3.738^{a}	6.6 ± 0.2$^{a.b}$	72.42 ± 0.85^{a}	1.46 ± 0.23^{a}	56.80 ± 15.14^{c}	263.33 ± 66.58$^{a.b}$	82.18 ± 9.87$^{a.b.c}$
2-B		PQTS	31:11 ± 03:25^{a}	0.571 ± 0.230^{a}	65.840 ± 22.114^{a}	6.8 ± 0.3$^{a.b.c}$	72.18 ± 1.59^{a}	1.83 ± 0.03$^{a.b}$	21.50 ± 5.68$^{a.b}$	201.67 ± 68.25^{a}	96.82 ± 4.09$^{c.d}$
2-C		OPSS	37:34 ± 02:36$^{a.b}$	0.493 ± 0.071^{a}	11.377 ± 10.619^{a}	6.9 ± 0.2$^{b.c}$	73.88 ± 1.21^{a}	1.64 ± 0.22^{a}	13.50 ± 1.32^{a}	218.33 ± 57.95$^{a.b}$	116.59 ± 9.61^{f}
3-A	56:44	No phosphate	30:56 ± 03:35^{a}	0.157 ± 0.013^{a}	43.662 ± 31.608^{a}	6.5 ± 0.1^{a}	72.12 ± 0.23^{a}	3.56 ± 0.14^{d}	38.83 ± 13.82$^{a.b.c}$	286.67 ± 104.92^{b}	76.84 ± 3.83$^{a.b}$
3-B		PQTS	50:23 ± 00:20^{b}	0.172 ± 0.060^{a}	29.139 ± 43.101^{a}	6.8 ± 0.0$^{a.b.c}$	72.38 ± 0.50^{a}	2.35 ± 0.01$^{b.c}$	19.50 ± 4.92$^{a.b}$	251.67 ± 59.65$^{a.b}$	97.43 ± 3.63$^{c.d.e}$
3-C		OPSS	36:38 ± 02:45^{a}	0.328 ± 0.233^{a}	12.822 ± 18.204^{a}	6.8 ± 0.0$^{a.b.c}$	73.06 ± 0.86^{a}	2.70 ± 0.02^{c}	12.67 ± 2.75^{a}	231.67 ± 54.85$^{a.b}$	115.48 ± 8.70$^{e.f}$

$^{a-b}$ Within a column, different superscript lowercase letters denote significant differences ($p<0.05$) among the samples.

Table 3:

Treatment		Description	HCT (min)	Protein (g/100g)	Total solids (g/100g)	TS	Ca/P
1-A	80:20	No phosphate	33:40 ± 04:28[a]	1.29 ± 0.07[a]	73.05 ± 2.29[a]	4.81 ± 0.49[a]	1.35 ± 0.37[d]
1-B		PQTS	32:42 ± 05:02[a]	1.48 ± 0.37[a]	71.58 ± 0.38[a]	5.22 ± 1.37[a,b]	0.89 ± 0.31[a,b,c]
1-C		OPSS	31:36 ± 05:14[a]	1.51 ± 0.46[a]	71.00 ± 2.77[a]	5.34 ± 2.12[c,d]	0.68 ± 0.20[a]
2-A	70:30	No phosphate	32:34 ± 08:02[a]	1.46 ± 0.23[a]	72.42 ± 0.85[a]	5.27 ± 0.67[a]	1.13 ± 0.36[b,c,d]
2-B		PQTS	31:11 ± 03:25[a]	1.83 ± 0.03[a.b]	72.18 ± 1.59[a]	6.58 ± 0.43[a,b]	0.74 ± 0.22[a,b]
2-C		OPSS	37:34 ± 02:36[a.b]	1.64 ± 0.22[a]	73.88 ± 1.21[a]	6.30 ± 1.12[c,d]	0.66 ± 0.17[a]
3-A	56:44	No phosphate	30:56 ± 03:35[a]	3.56 ± 0.14[d]	72.12 ± 0.23[a]	12.77 ± 0.48[a]	1.32 ± 0.43[c,d]
3-B		PQTS	50:23 ± 00:20[b]	2.35 ± 0.01[b.c]	72.38 ± 0.50[a]	8.51 ± 0.15[b,c]	0.91 ± 0.21[a,b,c,d]
3-C		OPSS	36:38 ± 02:45[a]	2.70 ± 0.02[c]	73.06 ± 0.86[a]	10.02 ± 0.34[c,d]	0.71 ± 0.14[a,b]

[a-b] Within a column, different superscript lowercase letters denote significant differences ($p<0.05$) among the samples.

4.1. Material suplementar do artigo 3

Material and Methods

Methods

Preparation of the syrup – stage 1

The study was carried out with 500 g of syrup, prepared by varying the proportion of proteins and addition of different phosphate salts, as described in Table 1 (in the manuscript). There were three levels of protein ratio variation as follows: 80:20 (treatment 1), 70:30 (treatment 2), and 56:44 (treatment 3) considering the casein: whey protein ratio, with a ratio of 80:20 to mimic the ratio present in milk; 56:44, where we have almost the same amount of whey and casein; and 70:30, an intermediate ratio between the two; since the idea of the product is cost reduction with the addition of whey, we evaluated three conditions in which we observed an increase in the proportion of whey in relation to casein. Each Treatment A had four variations of phosphate salts: syrup without the addition of salts as a control (treatment A) and syrup with the addition of PQTS (treatment B), OPSS (treatment C), and DPTS (treatment D). The variations in the proportions of proteins were intended to evaluate which would be the best proportion to work with, as there is no literature informing this. The phosphate salts were evaluated to determine the best capacity for thermal and emulsion stabilization and future shelf life of the product.

In stage I, the previous evaluation that phosphates would have the best thermal stability to proceed with the study, 12 treatments were carried out: (T1A) 80:20/without phosphate; (T1B) 80:20/PQTS; (T1C) 80:20/OPSS; (T1D) 80:20/DPTS; (T2A) 70:30/without phosphate; (T2B) 70:30/PQTS; (T2C) 70:30/OPSS; (T2D) 70:30/DPTS; (T3A) 56:44/without phosphate; (T3B) 56:44/PQTS; (T3C) 56:44/OPSS; and (T3D) 56:44/DPTS. After the addition of the ingredients according to each treatment, the powders were rehydrated with constant agitation for 30 min, and the syrup was heated at 90 ± 2 °C for 5 min. The SCS was cooled to room temperature (23 ± 1 °C) for analysis. Three independent replicates of each treatment were performed. The syrup samples were subjected to an alcohol test, a thermal stability test (HCT), and determination of phosphate content.

In stage II, the same methodology was used as described in the manuscript; however, a homogenization step was added before the heat treatment.

Results and Discussion

Stage I: previous evaluation of the thermal stability of the syrup

In the first stage, 12 treatments were performed to evaluate the effect of phosphates on the stability of the SCS syrup. Table S1 shows the values obtained for the test of HCT, pH, and alcohol and phosphate contents of the mixtures. Differences were observed in all parameters analyzed among the different treatments ($p<0.05$), except for pH, which did not present a significant difference ($p>0.05$) in any of the analyzed treatments. The low HCT values for the treatments containing DPTS phosphate, even lower than those for the control without the addition of phosphate, suggest that the amount of phosphate present in this treatment acted as a destabilizer of the micellar structure. As can also be seen by the values of determination of phosphates in the treatments, the treatments that presented DPTS had the highest values. These factors were observed regardless of the casein-whey protein ratio in the samples. Crowley et al. [1] and Ho et al. [2] found a relationship between the amount of ionic calcium (Ca^{+2}) and the HCT value for rehydrated MCP powders, in which a higher Ca^{+2} content lowers the HCT value. Perhaps the same behavior can be observed when there is a greater number of phosphates in the product, which reduces the HCT of the samples containing DPTS phosphate. Thus, DPTS phosphate was eliminated from stage 2 of this experiment, as we were looking for a phosphate that could provide greater thermal stability to the product during processing (evaluated in this study). Since the study of HCT allows us to evaluate the thermal stability of the product, and as SCS is a product that has not yet been studied, it is important to evaluate this parameter to avoid process variability or product quality losses due to heat-induced destabilization during processing steps [1]. The DPTS phosphate was also the one that presented the lowest stability in relation to the other phosphates for the alcohol test, presenting a medium cutoff point in alcohol 96.

Another observation was that for the control, without the addition of phosphate, the HCT value increased slightly when the casein content was reduced and whey protein content in the sample was increased, unlike the treatments with the addition of

phosphates, in which the HCT increased as the content of whey increased. At this stage, PQTS phosphate showed the highest HCT values for each casein:whey protein ratio studied. PQTS phosphate also had no cut-off point in any alcohol for any sample, showing no clots even in alcohol 98. The phosphate content of the samples was lower than that of the control sample. This reinforces that the amount of P_2O_5 present in the sample is directly related to the thermal stability.

References

1. Crowley SV, Megemont M, Gazi I, Kelly AL, Huppertz T, O'Mahony JA (2014) Heat stability of reconstituted milk protein concentrate powders. Int Dairy J 37:104–110. https://doi.org/10.1016/j.idairyj.2014.03.005

2. Ho QT, Murphy KM, Drapala KP, O'Callaghan TF, Fenelon MA, O'Mahony JA, McCarthy NA (2018) Effect of pH and heat treatment on viscosity and heat coagulation properties of milk protein concentrate. Int Dairy J 85**:**219–224. https://doi.org/10.1016/j.idairyj.2018.05.012

Table legend:

Table S1: Effect of the addition of phosphate salts in the syrup of the blend of sweetened condensed skimmed milk and vegetable fat (n=3)

Table S1:

Treatment		Description	HCT (min)	Alcoohol test	pH	Phosphate (P_2O_5) (mg/100g)
1-A	80:20	No phosphate	31:16 ± 01:16^{b}	90 ± 2	6.3 ± 0.2^{a}	54.98 ± 3.44^{a}
1-B		PQTS	64:53 ± 03:22^{e}	-	6.8 ± 0.2^{a}	72.65 ± 5.21^{a,b}
1-C		OPSS	49:44 ± 07:15^{c,d,e}	98 ± 0	6.8 ± 0.1^{a}	94.29 ± 8.14^{c,d}
1-D		DPTS	05:45 ± 00:28^{a}	96 ± 0	6.4 ± 0.3^{a}	108.73 ± 11.67^{c,d}
2-A	70:30	No phosphate	32:48 ± 02:48^{b,c}	94 ± 2	6.4 ± 0.3^{a}	58.88 ± 7.29^{a}
2-B		PQTS	51:52 ± 01:07^{d,e}	-	6.8 ± 0.2^{a}	74.05 ± 10.97^{a,b}
2-C		OPSS	44:57 ± 02:36^{b,c,d}	-	6.6 ± 0.5^{a}	103.39 ± 1.12^{c,d}
2-D		DPTS	06:16 ± 00:21^{a}	95 ± 1	6.5 ± 0.0^{a}	120.59 ± 6.09^{d,e}
3-A	56:44	No phosphate	35:50 ± 06:05^{b,c,d}	89 ± 1	6.6 ± 0.2^{a}	61.20 ± 9.95^{a}
3-B		PQTS	51:00 ± 09:51^{d,e}	-	6.7 ± 0.5^{a}	86.78 ± 1.82^{b,c}
3-C		OPSS	40:19 ± 00:41^{b,c,d}	98 ± 0	6.8 ± 0.1^{a}	107.69 ± 11.31^{c,d}
3-D		DPTS	04:29 ± 00:19^{a}	96 ± 2	6.7 ± 0.3^{a}	138.79 ± 14.75^{e}

$^{a-b}$ Within a column. different superscript lowercase letters denote significant differences ($p<0.05$) among the samples

5. OUTRAS PUBLICAÇÕES DURANTE O DOUTORADO

1. DA SILVA, Fernanda Lopes, *et al*. Monitoramento da distribuição do tamanho das partículas do leite integral e desnatado durante os processos de coagulação ácida ou enzimática. **Research Society and Development**, v. 11, n.1, 7011124438, 2022.
2. PINTO, Vinícius Rodrigues Arruda, *et al*. Proposal for determining valence and arousal thresholds: Compromised pleasure threshold, unpleasure threshold, and arousal threshold. **Journal of Sensory Studies**, v. 37, n. 2, 12726, 2022.

6. CONCLUSÃO GERAL E PERPECTIVAS

Com o aumento regular da demanda por novos produtos mais saudáveis, a indústria tem a necessidade de rever as formulações e tecnologias de processamento. Conforme visto no Artigo 1, o uso de fosfato teve a capacidade de alterar o sabor residual deixado pela stevia em chá preto, mudando assim a percepção do consumidor. Os limiares hedônicos é uma metodologia interessante para aplicação nas indústrias visto a sua relativa facilidade, uma vez que, utiliza a tabela hedônica, e permite que a indústria consiga determinar qual é o limiar em que o consumidor consegue diferenciar o que é aceitável ou não, permitindo o uso nos estudos em que se deseja reduzir os teores de determinado constituinte, como açúcar, sal, de forma mais assertiva. Essa metodologia deve ser mais utilizada pelas indústrias de alimentos. Bem como a utilização de fosfatos para redução do *aftertaste* da stevia em outros tipos de alimentos, visto que stevia é um edulcorante natural, no entanto, o seu ponto negativo é justamente o sabor residual.

Alternativas para substituição de um constituinte devem ser estudada e entendida, visto a possibilidade de escassez ou aumento de preço no mercado. A produção do queijo Minas Frescal é realizada com cloreto de cálcio, que tem entre outras funções, o controle de pH e a reposição de cálcio para a formação de uma coalhada mais firme. No entanto, nenhum estudo demonstra o uso de outras fontes de cálcio para substituir o cloreto de cálcio nessa produção. O Artigo 2 buscou mostrar como a utilização do monofosfato de cálcio agiria na substituição parcial ou total do cloreto de cálcio, e a utilização de uma mistura de outros fosfato com o MCP. E os resultados deste estudo demonstrou ser uma boa alternativa, visto que manteve as características de composição do produto e o rendimento. No entanto, estudos futuros devem ser conduzidos para entender o porquê a dureza dos queijos foram modificadas, e bem como entender melhor como fica a questão do cálcio nas diferentes fases, visto que é um importante mineral na produção de queijos.

Dentro dos estudos de pesquisa e desenvolvimento, o estudo de formulações e testes pilotos é necessário antes de levar o produto para a escala industrial. De forma a reduzir custo, uma vez que na escala piloto, já é possível selecionar uma formulação relativamente pronta para ver como ela se comporta em grande escala. O Artigo 3 buscou entender o processo de produção da calda de uma mistura láctea condensada

antes da aplicação em escala industrial. Com esse trabalho, é possível verificar a utilização de diferentes proporções de soro e caseína na formulação, bem como o uso de diferentes fosfatos. Baseado neste trabalho, é possível ver características diferentes de estabilidade, tamanho de partículas e teores de fosfato e cálcio de acordo com os tratamentos utilizados. No entanto, serviu para ter uma panorama melhor de como esse produto a base de soro e gordura vegetal se comporta, e ter uma ideia de como seria a concentração do mesmo, visto as características de estabilidade determinada. Mas estudos futuros devem buscar entender melhor as características da mistura produzida com uma proporção 56:44 caseína: proteína do soro, e realizar a concentração do produto, para verificar agora a estabilidade do produto ao processo de concentração e armazenamento.