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METILXANTINAS POR ELETROFORESE CAPILAR: DESENVOLVIMENTO, OTIMIZAÇÃO E VALIDAÇÃO DE MÉTODO. APLICAÇÃO EM CAFÉ DESCAFEINADO E OUTRAS BEBIDAS.

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Resumo

A otimização simultânea de múltiplas respostas foi utilizada no desenvolvimento de um método por eletroforese capilar (EC) para a determinação de cafeína em café descafeinado. O método foi desenvolvido utilizando o planejamento composto central para otimizar a concentração de dodecilsulfato de sódio (SDS), a concentração de carbonato de sódio e a voltagem. Os experimentos foram conduzidos com uma amostra de café descafeínado, onde o resíduo de cafeína foi extraído com clorofórmio, ressuspenso em água e filtrado. As condições otimizadas foram encontradas pela avaliação dos efeitos de seis respostas: separação de interferentes, área, ruído, variação na linha de base, corrente e tempo de análise. Modelos de regressão lineares e quadráticos foram gerados para cada conjunto de respostas. Os modelos de regressão, coeficientes de correlação e a análise de componentes principais (PCA) foram usados na determinação das condições experimentais ótimas. Os melhores resultados foram obtidos usando-se um capilar de 50 μm x 48 cm, tampão contendo 10 mmol. L^{-1} de carbonato de sódio e 50 mmol. L^{-1} de SDS (pH 11,0), 15 kV, 25,0 ºC e detecção a 206 nm. Sob as condições otimizadas, a cafeína foi separada dos interferentes. O tempo de análise foi de 5,5 min. O método desenvolvido foi validado e comparado a um método por CLAE quanto à eficiência de separação, tempo de análise, custo por análise e volume de resíduos gerados. O método por EC foi então aplicado a amostras de café descafeinado torrado moído e instantâneo e amostras de bebida enérgética. A quantidade total de xantinas presentes nas bebidas chimarrão e tererê, a base de erva-mate (*Ilex paraguariensis*), também foi avaliada. Estas

bebidas foram preparadas como são tradicionalmente consumidas. Os valores de cafeína das amostras analisadas tanto por EC quanto por CLAE não foram estatisticamente diferentes a 95% de confiança. A sensibilidade do método por CLAE foi 42 vezes maior que a do método por EC, porém este apresentou um tempo total de análise 30,4% menor. O volume de resíduos gerados foi 33 vezes maior no método por CLAE, e o custo em reagentes foi 76,5 vezes menor por EC. A vantagem mais importante do método por EC foi o uso de tampão aquoso econômico e ecológico no processo de separação. Com relação às amostras analisadas, sete amostras de café de quatro diferentes marcas apresentaram teor de cafeína acima do permitido pela legislação brasileira, e 76% das bebidas energéticas possuíam menos cafeína do que o valor informado no rótulo do produto. O teor de cafeína e teobromina variou nos diferentes tipos comerciais de erva-mate empregados no preparo das bebidas. Além disso, o tererê apresentou níveis de xantinas 2,5 vezes maior que o chimarrão.

Abstract

Multiple response simultaneous optimization was used to develop a micellar electrokinetic chromatography (MECK) method for caffeine determination in decaffeinated coffee samples. The method was developed using a central composite design to optimize the concentration of sodium dodecylsulfate (SDS), sodium carbonate and voltage (V). The experiments were carried out using a sample of Brazilian decaffeinated coffee extracted with chloroform, which was subsequently recovered with water and then filtered. Optimized conditions were found by evaluating the effects of six responses: interferent separation, area, noise, baseline variation, current e analysis time. Each set of response values was regressed on the factor levels of the experimental design using linear and quadratic models. The regression models, correlation coefficients involving both factor levels and response values, and a principal component analysis (PCA) were used to determine the optimum experimental conditions. Successful results were obtained using a fused-silica capillary of 50 μm x 48 cm total length, buffer containing 10 mmol. L^{-1} of sodium carbonate and 50 mmol. L^{-1} of SDS (pH 11.0), voltage of 15 kV, temperature of 25.0 ºC, with detection at 206 nm. Under optimized conditions, caffeine was separated from the interferents. The analysis time was of 5.5 min. After validation, the capillary electrophoresis (CE) method was compared to a HPLC method regarding separation performance, quantification of caffeine in twenty samples, analysis time, costs per analysis and volume of generated residue. CE method was applied to decaffeinated instant and ground-roasted coffee, and energy drinks. The total amount of xanthines in mate (*Ilex*

paraguariensis) beverages, "*chimarrao*" and "*terere*" obtained in the same way as they are traditionally consumed, was also estimated using the CE method. Caffeine content of the samples analyzed with both CE and HPLC methods did not differ statistically at 95% confidence level. HPLC method sensitivity was 42 times greater than by CE method. In another hand, CE presents total analysis time 30.4% lower than HPLC. The volume of generated residues was 33 times greater in HPLC and the cost in reagents for CE was 76.5 times lower than for HPLC. The most important advantage of CE is the use of economical and ecological aqueous buffer in the separation process. Regarding samples analyzed, seven decaffeinated coffee samples of four different brands showed higher levels than the limit specified by Brazilian legislation and 76% of the energy drinks samples had caffeine content lower than the value informed to the consumer. Caffeine and theobromine levels varied in different commercial types of mate employed for the beverages preparation. Besides, *"terere"* presented quantities 2.5 times higher than the "*chimarrao"* beverage.

SUMÁRIO

INTRODUÇÃO GERAL

Os produtos de origem vegetal contendo cafeína são consumidos pelo homem desde os tempos pré-históricos. A maioria da população mundial, independentemente de localização geográfica, idade, sexo ou cultura consome cafeína diariamente (JAMES, 1991).

A cafeína é a principal representante das metilxantinas, encontrada no café, no chá, na erva-mate, produtos a base de cacau, guaraná e bebidas a base de cola. A teobromina e a teofilina são outras substâncias do mesmo grupo, e suas estruturas moleculares são semelhantes à da cafeína (SPILLER, 1998). Os efeitos fisiológicos das xantinas incluem a diurese, estimulação do sistema nervoso central, dilatação das artérias coronárias, estimulação da secreção de ácido gástrico e aumento nos níveis de glicose e ácidos graxos livres (GRAHAM, 1978; BRUNTON, 2007). As bebidas contendo cafeína são populares, em parte, devido à diminuição da fadiga, aumento da acuidade mental e melhora das funções cognitivas, pelo consumo de doses moderadas (MCCUSKER et al., 2006a).

O café é uma das bebidas mais populares e amplamente consumida no mundo (FUJIOKA & SHIBAMOTO, 2008). Para muitos consumidores, o uso de café descafeinado é a solução para evitar os supostos efeitos adversos decorrentes da ingestão de cafeína (COULTATE, 2004), uma vez que até mesmo baixas doses desta xantina, como 9,0 mg, tem propriedades psicoativas (HASKELL et al., 2008). Além disso, os profissionais de saúde recomendam uma dieta livre de cafeína para gestantes e portadores de várias condições médicas, como hipertensão e arritmias (MCCUSKER et al., 2006a). Desta forma, a

quantidade de cafeína residual deste tipo de produto deve ser constantemente monitorada. A organização internacional para padronização (International Standards Organization – ISO 3509-1989) definiu café descafeinado como sendo o "café do qual a cafeína foi extraída" (RAMALAKSHMI & RAGHAVAN, 1999). A legislação brasileira permite um máximo de 0,1% de cafeína em café descafeinado torrado e moído e 0,3% no café descafeinado solúvel (ANVISA, 2005).

Diferentes métodos, utilizando diferentes técnicas analíticas, já foram publicados para análise de cafeína, teobromina e teofilina, em uma ampla variedade de alimentos e bebidas. Os primeiros artigos científicos que abordaram a análise de cafeína e teobromina apresentaram métodos bastante trabalhosos e morosos, devido à impossibilidade do método clássico em separar essas duas xantinas, havendo ainda maior dificuldade em produtos contendo grande teor de açúcares (YABIKU & KIMURA, 1996).

Com o advento da cromatografia líquida de alta eficiência (CLAE), eliminaram-se várias dessas etapas de preparação, permitindo a análise simultânea das xantinas trazendo maior confiabilidade e reprodutibilidade aos resultados (KIRKLAND & MC CORMICK, 1987).

Nas últimas décadas, a eletroforese capilar (EC) vem sendo apontada como uma alternativa bastante atraente para a determinação de compostos, destacando-se das outras técnicas, principalmente, por sua simplicidade e versatilidade. Algumas vantagens, em relação a outras técnicas analíticas, são o baixo custo e durabilidade dos capilares, menor tempo de análise, pequeno volume de amostra utilizado, menor custo final por análise e baixa geração de

resíduos, assumindo assim uma posição de destaque entre as técnicas analíticas de separação (COLOMBARA et al., 1997; RONDA et al., 2008).

Planejamentos experimentais são usados para identificar os fatores que influenciam as respostas, otimizar condições, e avaliar como esses fatores afetam as amostras analisadas. Nas estratégias tradicionais, apenas uma variável é modificada enquanto as outras permanecem constantes. Esta abordagem requer um grande número de experimentos, e não permite o estudo das alterações nas respostas que podem ocorrer em função das interações entre dois ou mais fatores. O planejamento experimental é uma alternativa a essa estratégia, pois permite que vários fatores sejam examinados simultaneamente, gerando dados menos ambíguos e requerendo um menor número de experimentos. Além disso, planejamentos experimentais combinados com a metodologia da superfície de resposta ajudam a visualizar as relações entre as respostas e os níveis dos fatores para encontrar a região de maiores valores de resposta (MONTGOMERY, 1995).

OBJETIVOS

1 Objetivo Geral

Desenvolver, otimizar e validar um método para determinação de metilxantinas em bebidas por EC.

2 Objetivos Específicos

- Comparar dois métodos para determinação de cafeína em café descafeinado, um por EC e outro por CLAE, quanto à eficiência de separação, capacidade de quantificação, custos e volume de resíduos gerados.
- Avaliar o teor de cafeína residual em amostras de café descafeinado comercializadas no Estado de São Paulo.
- Avaliar o teor de cafeína em amostras de bebidas energéticas comercializadas no Estado de São Paulo.
- Avaliar o teor de metilxantinas presentes nas bebidas chimarrão e tererê, ambas à base de erva-mate (*Ilex paraguariensis* Saint Hil.), preparadas como são tradicionalmente consumidas na Região Sul do Brasil.

REVISÃO DE LITERATURA

1 Metilxantinas

1.1 Propriedades fisico-químicas e farmacológicas

As metilxantinas, ou metil-2,6-hidroxipurinas, são os compostos derivados da purina mais interessantes sob os pontos de vista farmacológico, tecnológico e terapêutico (COSTA, 1967; SPILLER, 1998; ALTIMARI et al., 2005; BRUNTON, 2007). Comumente, as xantinas são classificadas como alcalóides verdadeiros (alcalóides purínicos), em razão de sua marcante atividade biológica, distribuição restrita e presença estrutural de nitrogênio heterocíclico (RATES, 2001). Entretanto, por serem provenientes de bases púricas e não de aminoácidos, como também por seu caráter anfótero, são mais propriamente classificadas como pseudoalcalóides (MORAES et al., 2003).

Os principais representantes desta classe de compostos são a cafeína (1,3,7-trimetilxantina), a teobromina (3,7-dimetilxantina) e a teofilina (1,3 dimetilxantina), que apresentam características de solubilidade distintas. A cafeína apresenta solubilidade em água em ebulição, mas em temperatura ambiente é facilmente dissolvida em clorofórmio. A teobromina é muito menos solúvel que a cafeína na maioria dos solventes, mas é prontamente dissolvida em soluções aquosas ácidas ou básicas. A teofilina apresenta solubilidade intermediária entre as duas outras xantinas. Comparando-se as taxas de solubilidade das xantinas, determinou-se que aquelas em que ambos os átomos de nitrogênio no anel pirimidínico estão metilados (cafeína e teofilina) apresentam uma solubilidade

maior em solventes polares que aquelas em que pelo menos um átomo de nitrogênio não está metilado (teobromina), devido à formação de pontes de hidrogênio intermoleculares nessas últimas (TARKA JR & HURST, 1998).

As estruturas das metilxantinas estão representadas na figura 1.

Figura 1. Estrutura molecular da cafeína (1), teofilina (2) e teobromina (3).

A cafeína se comporta como uma base muito fraca e reage com ácidos; os sais produzidos são prontamente hidrolisados. Evidências da protonação da cafeína podem ser observadas pela mudança do espectro UV (ultra-violeta) da cafeína em pH 0. A teobromina e a teofilina têm caráter fracamente anfótero. Isso explica a razão destas substâncias se dissolverem facilmente em soluções ácidas ou básicas, enquanto são praticamente insolúveis em água pura (TARKA JR & HURST, 1998). Na Tabela 1, são mostradas as constantes de equilíbrio ácida (pKa) e básica (pKb) para as metilxantinas.

Tabela 1 – Constantes de equilíbrio ácida (kA) e básica (Kb) das metilxantinas, expressas como pKa e pKb.

Composto	**pKa**	**pKb**
Cafeína	14,2	-
Teobromina	10,0	13,9
Teofilina	8,8	13,7

Fonte: TARKA JR & HURST, 1998.

A cafeína é um estimulante do sistema nervoso central. Em doses suficientemente elevadas, pode provocar rubor, agitação, irritabilidade, perda de apetite, fraqueza e tremor. Em casos de overdose, já foram relatados sintomas como hipertensão, hipotensão, taquicardia, vômito, febre, alucinações, arritmia, coma e até mesmo morte. O principal mecanismo de ação é o antagonismo à adenosina, que possui funções inibitórias (KERRIGAN & LINDSEY, 2005). O organismo responde à presença crônica de cafeína aumentando o número de receptores de adenosina. Esta pode ser uma das razões para a tolerância aumentada à cafeína em consumidores de grandes quantidades de café e chá (SPILLER, 1998).

Deve-se ressaltar que a cafeína não apresenta somente efeitos não benéficos. Estudos epidemiológicos e experimentais demonstraram efeitos positivos do consumo regular de bebidas contendo cafeína em vários aspectos da saúde, como respostas psicoativas (estado de alerta, mudanças de humor),

condições neurológicas (hiperatividade, mal de Parkinson), desordens metabólicas (diabetes) e função renal (FUJIOKA & SHIBAMOTO, 2008; NKONDJOCK, 2009).

A teofilina causa relaxamento na musculatura lisa traqueal, sendo usada farmacologicamente no tratamento da asma. O efeito diurético das metilxantinas é bem conhecido, sendo mais potente na teofilina, seguido pela cafeína e pela teobromina (SPILLER, 1998). A teobromina apresenta comprovada ação antitussígena, através da inibição da ativação do nervo vago (USMANI et al., 2004).

1.2 Metilxantinas em alimentos

A cafeína é um ingrediente presente em muitos alimentos e bebidas amplamente consumidos ao redor do mundo como, por exemplo, café, chá, mate, refrigerantes e chocolate. A popularidade destes produtos muitas vezes se deve justamente ao poder de estimulação do sistema nervoso central pela ação da cafeína (MACKENZIE et al., 2007). A teofilina é encontrada no chá, e as principais fontes de teobromina na dieta são os derivados do cacau e a erva-mate.

O café é uma das bebidas mais populares do mundo. O Brasil é o maior produtor e exportador de grãos de café, sendo responsável por cerca de 30% do mercado internacional. É também o segundo maior mercado consumidor, atrás apenas dos Estados Unidos (FUJIOKA & SHIBAMOTO, 2008, RAMALAKSHMI et al., 2009, SPILLER, 1998). O suprimento mundial de café provém primariamente de dois tipos de grãos, o Arábica (*Coffea arabica*) e o Robusta (*Coffea*

canephora), que diferem entre si em diversas características, bem como o teor de cafeína. Enquanto os grãos de Arabica contêm cerca de 1% de cafeína, os de Robusta contém o dobro desta quantidade (RAMALAKSHMI et al., 2009, SPILLER, 1998).

O café contém concentrações relativamente baixas de teobromina e teofilina, sendo aproximadamente 20 e 5 mg/kg, respectivamente, no café verde (SPILLER, 1998). CHEN et al. (1998) encontrou 320 mg/kg de teobromina em uma amostra de café torrado, não sendo detectada teofilina nesta amostra. Outras xantinas presentes em quantidades traço no café verde são a xantina, hipoxantina, adenina e guanina, mas todas elas estão ausentes no grão torrado (SPILLER, 1998).

O chá (*Camellia sinensis*) é a segunda bebida mais consumida no mundo, atrás apenas da água, com um consumo per capita de 40 L/ano. A quantidade de cafeína encontrada nas folhas de chá é afetada por vários parâmetros, como aplicação de fertilizantes nitrogenados, variações sazonais e origem genética, sendo em média 3% (HICKS et al., 1996, CHEN et al., 1998). O teor de teobromina e teofilina são consideravelmente menores, sendo relatados valores de 0,16 a 0,20% para a teobromina (HICKS et al., 1996) e 0,04% para teofilina (HICKS et al., 1996; ZEN et al., 1999).

Alimentos derivados do cacau têm sido consumidos por humanos há mais de 2000 anos. A teobromina é a xantina predominante no cacau e no chocolate. A cafeína está presente nesses alimentos em cerca de um oitavo da concentração

da teobromina, enquanto que a teofilina se encontra em nivel traço (APGAR & TARKA JR, 1998). A quantidade das xantinas presentes no chocolate varia de acordo com a quantidade de cacau da formulação. A pasta de cacau não adoçada (100% cacau) apresenta de 1,2 a 2% de teobromina e cerca de 0,15% de cafeína. (KREISER & MARTIN, 1980; CHEN et al., 1998). No chocolate meio amargo, esses valores caem para 0,47% e 0,08%, respectivamente, enquanto que o chocolate ao leite apresenta cerca de um terço desses valores (KREISER & MARTIN, 1980).

A erva-mate, *Ilex paraguariensis* Saint Hilaire (Aquifoliaceae), é uma espécie nativa da América do Sul e ocorre naturalmente no Brasil, Paraguai e Argentina (KUSSLER et al., 2004). Segundo o Instituto Brasileiro de Geografia e Estatística, a produção brasileira de erva-mate em 2007 foi de 438.474 toneladas (IBGE, 2007) concentrando-se na região Sul, devido à tradição de consumo do chimarrão. De acordo com a literatura, o teor de metilxantinas na erva-mate é extremamente variável, sendo apontados teores de cafeína entre 0,16 e 1,4% e de teobromina entre 0,02 e 0,27% (SOUZA, 1947; REGINATTO et al., 1999; BERTONI et al., 1992; MAZZAFERA, 1994; CARDOZO Jr. et al., 2007).

Os refrigerantes e bebidas energéticas também são importantes fontes de cafeína na dieta (MCCUSKER et al., 2006b, REISSIG et al., 2009, MALINAUSKAS et al, 2007).

1.3 Café descafeinado

Para muitos consumidores, o consumo de café descafeinado é a solução para evitar os efeitos adversos devidos à ingestão da cafeína (COULTATE, 2004). SHLONSKY et al. (2003) traçaram o perfil dos consumidores de café descafeinado. As análises dos dados revelaram que as pessoas que consomem café descafeinado o fazem devido a algum tipo de doença, principalmente cardiovascular, ou simplesmente porque procuram um estilo de vida mais saudável, com baixa incidência de tabagismo, baixo consumo de bebidas alcoólicas e cafeinadas e aumento do consumo de medicamentos para prevenir doenças.

A descafeinação é realizada nos grãos crus inteiros, antes do processo de torrefação. Geralmente, um sistema de partição água/solvente orgânico é utilizado. O diclorometano é o solvente mais comumente utilizado, embora outros também sejam empregados, como acetato de etila, etanol, metanol, acetona, e dióxido de carbono supercrítico (SPILLER, 1998). Existem dois métodos básicos para a produção de café descafeinado usando solventes. O primeiro utiliza a extração direta dos grãos pelo solvente orgânico; no segundo, os grãos são imersos em água antes do uso de um solvente para extração da cafeína. No café, a cafeína pode estar na forma livre ou associada ao ácido clorogênico. Uma das funções da água é separar a cafeína associada ao ácido clorogênico e permitir que o solvente tenha acesso à cafeína, além de facilitar sua saída pela parede celular do grão (TOCI et al., 2006).

Estudos recentes indicam que mesmo doses baixas de cafeína, como as encontradas no café descafeinado, podem apresentar propriedades psicoativas (HASKELL et al., 2008). Por esta razão, os produtores de café descafeinado devem possuir um controle de qualidade adequado, para garantir que os consumidores possam ingerir um produto com baixo teor de cafeína.

A legislação brasileira permite um máximo de 0,1% de cafeína residual em café descafeinado torrado e moído e 0,3% no café descafeinado solúvel (ANVISA, 2005), mesmos limites estabelecidos no Reino Unido e na maioria dos países europeus (RAMALAKSHMI & RAGHAVAN, 1999).

2 Métodos para análise de xantinas

A literatura apresenta vários métodos para a determinação de cafeína, teobromina e teofilina em matrizes alimentares (HURST et al., 1998). Historicamente, eram utilizados métodos espectrofotométricos, gravimétricos, titulométricos e determinação de nitrogênio (Kjeldahl). O método gravimétrico consistia na extração do produto com água ou etanol, limpeza do filtrado com óxido de magnésio e extração subsequente com clorofórmio. Após a evaporação do clorofórmio, a cafeína era determinada por gravimetria (HURST et al., 1998, DE MARIA & MOREIRA, 2007). A teobromina do chocolate era determinada por um processo similar, sendo necessária uma pré-extração com hexano ou éter de petróleo para eliminar a gordura (HURST et al., 1998).

Os métodos espectrofotométricos baseiam-se no fato das xantinas absorverem a radiação UV. No entanto, é necessária uma separação prévia do grande número de substâncias presentes na matriz alimentar que podem interferir na análise (HURST et al., 1998). Assim, embora métodos baseados principalmente na espectrofotometria sejam descritos na literatura (SINGH & SAHU, 2006; KHANCHI et al., 2007) é preferível utilizar esta técnica acoplada a um método de separação, como a cromatografia líquida de alta eficiência (CLAE) ou a eletroforese capilar (EC) (HURST et al., 1998).

A cromatografia em coluna e cromatografia em camada delgada (FENSKE, 2007) são outras técnicas clássicas que foram empregadas na análise de metilxantinas (HURST et al., 1998, DE MARIA & MOREIRA, 2007). No entanto, com o desenvolvimento das metodologias instrumentais, surgiu a possibilidade da utilização de métodos que oferecessem maior reprodutibilidade e sensibilidade, e que pudessem ser aplicados em pequenos volumes de amostra (DE MARIA & MOREIRA, 2007). Assim, a cromatografia gasosa (CG) passou a ser empregada. Inicialmente, eram utilizadas colunas recheadas e detectores de ionização de chama (STRAHL et al., 1977). Posteriormente, as colunas capilares passaram a ser adotadas (ISHIDA et al., 1986; CONTE & BARRY, 1993; MEI & MIN, 1995), aumentando assim a capacidade de separação. Estudos mais recentes relatam o uso da cromatografia gasosa acoplada à espectrometria de massas (GCMS) na determinação de xantinas em amostras ambientais e bebidas (THOMAS & FOSTER, 2004; SHRIVAS & WU, 2007).

No início da década de 70, análises de derivados xantínicos por CLAE passaram a ser usadas (DE MARIA & MOREIRA, 2007). O uso de diversos tipos de coluna já foi descrito na literatura, entre eles a coluna de troca iônica (MURGIA et al., 1973), colunas de permeação em gel (DE MARIA et al., 1995) e coluna de fase reversa (BARBAS et al., 2000; HORIE et a., 2002; FERNANDEZ et al., 2000; KAWAHARA et al.; 2004; GARDINALI & ZHAO, 2002; CASAL et al.1998).

A CLAE com fase reversa e a EC são duas das técnicas de separação mais usadas em análise de alimentos. A CLAE é a técnica melhor estabelecida e aceita no meio científico, nas mais diversas áreas de aplicação, apresentando características de robutez, repetibilidade e reprodutibilidade. Essas características são muito importantes no desenvolvimento de um método analítico com parâmetros de validação adequados. No entanto, a EC se apresenta como uma alternativa real à CLAE em várias ocasiões, com vantagens significativas como simplicidade de operação, maior eficiência de separação, custos de operação reduzidos, baixo uso de solventes, necessidade de menor volume de amostra e tempo de análise mais curto (TZANAVARAS & THEMELIS, 2007; BRUNETTO et al., 2007; KOWALSKI et al., 2005).

2.1 Eletroforese capilar

A eletroforese capilar é uma técnica de separação baseada nas diferenças de mobilidade dos analitos quando submetido à ação de um campo elétrico (SILVA, 2003). A EC está sendo cada vez mais reconhecida como uma importante

técnica de separação por causa de sua velocidade, eficiência, reprodutibilidade, pequeno volume de amostra e baixo consumo de solventes (RONDA et al., 2008).

A eletroforese capilar de zona (CZE) é um dos modos de separação mais usados na prática, provavelmente em razão da facilidade de sua implementação e otimização das condições experimentais (TAVARES, 1997). Nesta técnica, o tubo capilar é simplesmente preenchido com um eletrólito, geralmente com características tamponantes.

A CZE não permite a separação de componentes neutros da amostra, eles simplesmente migram com o fluxo eletrosmótico. Para resolver este problema, a cromatografia eletrocinética micelar (MEKC) pode ser empregada. Esta técnica é baseada na partição do analito em duas fases, as micelas (agregados de moléculas de surfactante) e solução tampão. As micelas são formadas pela adição de um surfactante ao tampão em uma concentração maior que a concentração micelar crítica. A velocidade de migração dos componentes da amostra é influenciada por seu grau de partição na fase micelar (FRAZIER et al., 2000).

A instrumentação usada na MEKC é a mesma empregada na CZE, mas a MEKC é mais versátil, uma vez que diferenças de mobilidade eletroforética podem ser exploradas para separar solutos iônicos, ao mesmo tempo em que diferenças de distribuição entre a fase micelar e o eletrólito podem ser usadas para separar solutos neutros (TAVARES, 1997).

Outras técnicas de separação em EC são a eletroforese capilar em gel (CGE), na qual o capilar é preenchido com gel contendo ligações cruzadas; a

eletroforese capilar de peneiramento (CSE), onde o eletrólito de corrida contém um meio peneirador; a focalização isoelétrica capilar (CIEF); a isotacoforese capilar (CITP) e a eletrocromatografia capilar (CEC) (SILVA et al., 2007).

2.2 Análise de metilxantinas por EC

Vários eletrólitos são usados na separação de xantinas por EC, como tampões fosfato e borato (BONOLI et al., 2003; CHEN et al., 2003; POMILIO et al., 2005). Considerando as curvas de mobilidade das três xantinas mais comumente encontradas em alimentos (cafeína, teobromina e teofilina), recomenda-se o uso de um pH em torno de 10 para sua separação. Por esse motivo, tampões a base de carbonato parecem ser uma alternativa apropriada aos eletrólitos correntemente utilizados. Este tipo de tampão foi usado por GELDART & BROWN (1999) na separação de purinas e pirimidinas por EC, encontrando boa estabilidade, reprodutibilidade e sensibilidade.

Embora a teobromina e a teofilina apresentem caráter anfótero, a cafeína se apresenta na forma neutra na faixa de pH (2 a 12) utilizada nas separações por EC. A separação simultânea das metilxantinas é possível em meio micelar, onde a seletividade é obtida pela interação diferencial entre os compostos neutros e o interior hidrofóbico da micela, usualmente com carga negativa. Assim, a escolha do pH é feita com base no controle do fluxo eletrosmótico, e valores de pH elevados são mais convenientes por proporcionarem determinações mais rápidas (MORAES et al., 2003).

MAESO et al. (2006) analisaram a concentração de cafeína em seis diferentes amostras usando EC. As condições de separação foram: tampão borato 50 mmol/L em pH 9,5, 130 mmol/L de dodecilsulfato de sódio (SDS), 10 kV e detecção a 200 nm. O limite de detecção para este método foi 0,23 µmol/L, e o limite de quantificação 0,76 µmol/L.

A CZE foi empregada por SOMBRA et al. (2005) na análise de cafeína em produtos fitofarmacêuticos à base de guaraná, e os resultados foram comparados com os obtidos por CLAE. Para a CZE, foi utilizado um tampão de 20 mmol/L de tetraborato de sódio (pH 9,2), temperatura de 25 ºC e 25 kV. Os resultados das quantificações obtidas por CZE foram similares às mesmas determinações realizadas por CLAE. Este mesmo modo de separação foi utilizado por WALKER et al. (1997), na determinação de cafeína, aspartame e ácido benzóico em refrigerantes. O tampão utilizado consistiu em uma solução de glicina 20 mmol/L, com pH 9,0.

A separação de cafeína de várias drogas de abuso foi demonstrada por TAGLIARO et al. (1996), que utilizaram dois modos de eletroforese capilar com detecção de absorvância na região do UV: CZE, em meio ácido e básico e MEKC. Os dois modos de separação apresentaram resultados equivalentes para as drogas investigadas, porém, a cafeína (que é um composto neutro) foi resolvida apenas no modo MEKC. ZHAO & LUNTE (1997) demonstraram a separação de metilxantinas por MEKC empregando tampões de fosfato de sódio em meio ácido e básico. Investigaram a influência da concentração do tensoativo empregado,

dodecilssulfato de sódio, e do eletrólito na qualidade da separação, entre outros parâmetros instrumentais. Condições otimizadas para a determinação de metilxantinas em bebidas (refrigerantes, chá e café) e fluidos biológicos foram alcançadas. Outros pesquisadores (VOGT et al., 1997; WATANABE eta al., 1998) desenvolveram metodologias simples empregando MEKC para a determinação de cafeína em amostras de chá, enquanto que POMILIO et al. (2002) investigaram o teor de metilxantinas em 14 amostras comerciais de erva mate. WANG et al. (2000) obtiveram resultados satisfatórios na análise das metilxantinas em preparações farmacêuticas, empregando MEKC com detecção amperométrica. O teor de metilxantinas em extratos comerciais de guaraná e erva-mate foi avaliado por MORAES et al. (2003), utilizando a MECK.

Embora já tenham sido relatados diversos métodos para análise de metilxantinas por EC, nem sempre esses métodos são facilmente reprodutíveis na prática, havendo ainda espaço para o desenvolvimento de novas técnicas analíticas, para a análise de rotina desses compostos em matrizes alimentares, que sejam eficientes, de baixo custo, e gerem baixo volume de resíduos.

3 Otimização de métodos

O desenvolvimento de um método eficiente por eletroforese capilar que permita a separação e quantificação de xantinas em alimentos é um procedimento multivariado, no qual diversos fatores podem afetar a seletividade da separação,

principalmente as características do eletrólito, voltagem e temperatura do capilar (RONDA et al., 2008). Na literatura, é bastante comum encontrar pesquisas onde é aplicado o método clássico para determinação das condições ótimas de separação por meio da modificação de uma variável enquanto as demais permanecem constantes. Entretanto, esta abordagem unidimensional é bastante trabalhosa e com frequência falha em garantir a determinação das condições ótimas (BOX et al., 2005; WERNIMONT, 1985, BRUNS et al., 2006).

Planejamentos experimentais são usados para identificar os fatores que influenciam, otimizar condições, e avaliar como esses fatores afetam as amostras analisadas (CUBAS et al, 2008), sendo uma alternativa à abordagem tradicional univariada. Os planejamentos experimentais permitem que um grande número de fatores possam ser estudados simultaneamente, e podem fornecer dados menos ambíguos (MONTGOMERY, 2005). A abordagem multivariada apresenta vantagens em termos de redução no número de experimentos, melhora nas possibilidades de interpretação estatística dos dados e redução no tempo total de análise (RONDA et al., 2008).

A metodologia da superfície de resposta tem sido empregada com sucesso na melhoria dos resultados analíticos em EC (RONDA et al., 2008). As análises por EC geram uma grande quantidade de dados para expressar a seletividade, sensibilidade, tempo de análise e precisão, e muitas condições da EC influenciam potencialmente essas respostas. Isto requer a otimização das condições experimentais, considerando as respostas simultaneamente, o que pode ser feito

usando métodos multivariados. Contudo, muitos dos métodos de EC encontrados na literatura não utilizam essa abordagem, o que pode ser atribuído ao fato de muitos autores não terem ainda descoberto o potencial inerente das duas disciplinas, EC e otimização multivariada, trabalhando juntas (ALNAJJAR et al., 2007).

4 Validação

A validação de uma metodologia analítica é realizada para garantir que a mesma seja exata, específica e reprodutível. Segundo a USP (2008), a validação de métodos assegura a credibilidade destes durante o uso rotineiro, sendo algumas vezes mencionado como o processo que fornece uma evidência documentada de que o método realiza aquilo para o qual é indicado fazer.

Os parâmetros analíticos normalmente utilzados para a validação de métodos de separação são: seletividade; linearidade e faixa de aplicação; precisão; exatidão; limite de detecção; limite de quantificação e robustez (RIBANI et al., 2004). Estes termos são conhecidos como parâmetros de desempenho analítico, características do desempenho ou figuras analíticas de mérito.

A seletividade, ou especificidade, de um método representa a sua capacidade de avaliar, de forma inequívoca, o analito na presença de componentes que podem interferir com sua determinação em uma amostra complexa. Corresponde ao grau de interferência de espécies, como outro analito,

reagentes, impurezas e produtos de degradação, garantindo que o pico de resposta seja exclusivamente de um componente simples (RIBANI et al., 2004; VIEIRA & LICHTIG, 2004). A seletividade é o primeiro passo no desenvolvimento e validação de um método instrumental de separação e deve ser reavaliada continuamente durante a validação e subsequente uso do método. Para tanto, é conveniente a utilização de testes de pureza de pico com auxílio de detector de arranjo de fotodiodos ou espectrometria de massas (PASCHOAL et al., 2008). Além disso, recomenda-se verificar, em amostras isentas do analito (branco), se não há nenhum interferente com o mesmo tempo de retenção/migração.

A linearidade corresponde à capacidade do método fornecer resultados diretamente proporcionais à concentração da substância dentro de uma determinada faixa de aplicação (GREEN, 1996). A linearidade está relacionada com a variação da inclinação da linha de regressão e é determinada através da curva analítica do analito, obtida a partir de soluções padrões de concentração conhecida (VIEIRA & LICHTIG, 2004).

A precisão corresponde à dispersãode resultados entre ensaios independentes, repetidos de uma mesma amostra, sob condições definidas (RIBANI et al., 2004). É expressa pela medida do desvio padrão relativo (RSD), também conhecido por coeficiente de variação (CV), desta série de ensaios.

A exatidão representa o grau de concordância entre resultados individuais encontrados e um valor aceito como referência, e geralmente é obtida por meio de testes de recuperação (INMETRO, 2003).

O limite de detecção é a menor concentração da substância em exameque pode ser detectada, mas não necessariamente quantificada, por meio de um dado procedimento experimental. Geralmente, é calculado com base na relação sinal-ruído ou baseado em parâmetros da curva analítica (PASCHOAL et al., 2008).

O limite de quantificação representa a menor concentração do analito que pode ser efetivamente determinada num dado procedimente experimental. Os métodos para seu cálculo são semelhantes aos usados para o limite de detecção (RIBANI et al., 2004).

O grau de reprodutibilidade dos resultados odtidos sob uma variação de condições, como diferentes laboratórios, analistas, reagentes, etc., definem a robustez do método (GREEN, 1996).

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CAPÍTULO 1

OPTIMIZATION OF A CE METHOD FOR CAFFEINE ANALYSIS IN DECAFFEINATED COFFEE

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Optimization of a CE method for caffeine analysis in decaffeinated coffee

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ABSTRACT

Multiple response simultaneous optimization was used to develop a micellar electrokinetic chromatography method for caffeine determination in decaffeinated coffees. Buffer composition and voltage were optimized using a central composite design. Six responses were evaluated and each set of response values was regressed on the factor levels of the experimental design using linear and quadratic models. The regression models, correlation coefficients and a principal component analysis (PCA) were used to determine the optimum conditions. Successful results were obtained using a buffer containing 10 mmol.L^{-1} sodium carbonate and 50 mmol.L^{-1} sodium dodecylsulfate, 15 kV voltage, 25°C temperature, 48 cm x 50 μm fused silica capillary, hydrodynamic injection of 50 mBar during 7 s and detection at 206 nm. This optimized method was applied for caffeine analysis in 45 samples of commercial decaffeinated coffee.

Keywords: Capillary Electrophoresis, PCA, central composite design, caffeine analysis.

1. Introduction

Caffeine (1,3,7-trimethylxanthine) is an alkaloid from the xanthine group found in many foods and beverages, including coffee, tea, soft drinks, and chocolate. One reason for the popularity of caffeine-containing beverages is their effect of stimulating the central nervous system (MacKenzie, Comi, Sluss, Keisari, Manwar, Kim, Larson & Baron, 2007), but in high doses caffeine consumption can be associated with the following symptoms: nervousness, anxiety, restlessness, insomnia, gastrointestinal upset, tremors, tachycardia and psychomotor agitation (Reissig, Strain & Griffiths, 2008). Because of this, many consumers avoid caffeine ingestion by consuming decaffeinated coffee (Coultate, 2004). Recent findings indicate that even low doses of caffeine, such as those found in decaffeinated coffee, can have psychoactive properties (Haskell, Kennedy, Milne, Wesnes & Scholey, 2008). For this reason, decaffeinated coffee producers must have good quality control in order to guarantee consumers that the product they are consuming is in fact of low caffeine content.

Brazilian legislation allows at most 0.1% of residual caffeine in decaffeinated ground-roasted (powder) coffee and 0.3% in instant coffee products (ANVISA, 2005).

Many analytical methods for caffeine determination in the most varied matrices have been developed (De Maria & Moreira, 2007), among them: spectrophotometry with UV detection (Khanchi, Mahani, Hajihosseini, Maragheh, Chaloosi & Bani, 2007); thin-layer chromatography (Fenske, 2007); gas chromatography-mass spectrometry (Shrivas & Wu, 2007); ion-exchange

chromatography (Chen, Mou, Hou & Ni, 1998); high-performance liquid chromatography (HPLC) with UV detection (Aresta, Palmisano & Zambonin, 2005; Brunetto, Gutiérrez, Delgado, Gallignani, Zambrano, Gómez, Ramos & Romero, 2007); HPLC-mass spectrometry (Perrone, Donangelo e Farah, 2008); HPLC with gel permeation columns (De Maria, Trugo, Moreira & Petracco, 1995); and capillary electrophoresis with UV detection (Pomilio, Trajtemberg & Vitale, 2005).

Capillary electrophoresis (CE) is increasingly recognized as an important separation technique because of its speed, efficiency, reproducibility, small sample volumes and low solvent consumption (Ronda, Rodríguez-Nogales, Sancho, Oliete & Gómez, 2008).

Each of the analytical techniques for determination of alkylxanthines has advantages and limitations. Large residue generation, compared to CE, is a disadvantage of HPLC technique. The problem with spectroscopic methods is that methylxanthines present high spectral overlap when determined simultaneously, which is why this technique is poorly developed (Regan & Shakalisava, 2005).

The capillary zone electrophoresis (CZE) mode of CE is unable to separate neutral components of a sample, which simply co-migrate with the electroosmotic flow (EOF). To overcome this drawback, micellar electrokinetic chromatography (MEKC) can be used. It can be applied to the separation of not only neutral but also charged analytes. MEKC is based on the partitioning of analytes between two phases, namely, micelles (colloid-sized aggregates of surfactant molecules) and buffer solution. Micelles are formed by the addition of a surfactant to the buffer at a concentration greater than its critical micelle concentration. The most commonly used surfactant is sodium dodecyl sulfate (SDS). The migration velocity of sample

components will be influenced by the degree of partitioning into the micellar phase (Frazier, Ames & Nursten, 2000).

Various electrolytes are used for xanthine separation, like phosphate and borate buffers (Bonoli, Colabufalo, Pelillo, Toschi, & Lercker, 2003; Chen, Liang, Lai, Tsai, Tsay, & Lin, 2003; Pomilio, Trajtemberg & Vitale, 2005). Considering the mobility curves for the three xanthines most commonly found in foods (caffeine, theobromine and theophilline), a pH of about 10.6 is recommended for their separation. Due to their pH range, carbonate buffers seemed to be an appropriate alternative to the buffers currently used. Geldart & Brown (1999) used carbonate buffers in purines and pyrimidine analysis by CE, showing good stability, reproducibility and sensibility.

Chloroform was found to be the most suitable solvent to extract caffeine from aqueous solution. The reason is the high caffeine solubility in chloroform, nine times more than in water at room temperature (Shrivas & Wu, 2007). Liquid–liquid extraction is attractive because it operates at atmospheric pressure. It is easy to control and equipment cost is low (Hu, Wan, Bal & Yang, 2003). This extraction is important to eliminate matrix effects caused by hydrosoluble compounds of coffee.

The design of an efficient capillary electrophoretic method that permits the separation and quantification of an analyte is a multivariate process in which many factors can affect separation selectivity, mainly the levels of the running buffer, voltage and capillary temperature. Response surface methodology has been successfully employed to improve analytical results in capillary electrophoresis (Box, Hunter & Hunter, 2005; Bruns, Scarminio & Barros-Neto, 2006; Ronda et al. 2008).

The aim of this study was to optimize an analytical technique for routine caffeine determination in decaffeinated coffee by MEKC with low cost, small residue generation and applicability to industrial decaffeinated coffee. The goal of optimization was the separation of caffeine, theobromine (internal standard) and interferents (SI), baseline variation (BV), allied with higher caffeine area intensity, lower noise, current and analysis time. Moreover, injection conditions were investigated to obtain complete optimization. The optimized method was validated for linearity, repeatability and sensitivity, and subsequently applied to commercial samples.

2. Material and methods

2.1. Instrumentation

All experiments were performed in an Agilent G1600AX (Agilent Technology, Germany) Capillary Electrophoresis system equipped with a diode array detector, at 206 nm. The separation was carried out by using a fused-silica capillary from Agilent Technology (Germany), which had a total length of 48 cm and 50 µm i.d.

2.2. *Chemicals, reagents and samples*

Caffeine and theobromine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Chloroform (pro analysis grade) was from Merck (Rio de Janeiro, Brazil), sodium carbonate (CAR) was from Synth (São Paulo, Brazil) and sodium dodecyl sulfate (SDS) was from Riedel-de-Haën (Germany). All other

reagents and solvents were of analytical grade quality. Ultrapure water (18 MΩ cm) obtained from a Direct-Q 3 UV ultrapure water system (Millipore Corporation, France) was used in all studies.

All the solutions were degassed by ultrasonication (Microsonic SX-20, Arruda Ultra-sons LTDA, Brazil). Stock solutions of caffeine (1000 $mg.L^{-1}$) and theobromine (1000 $mg.L^{-1}$) were maintained under refrigeration until use for preparing standard solutions.

Samples analyzed consisted of eight different brands of decaffeinated instant coffee and eight brands of decaffeinated ground-roasted coffee. Three batches of each brand were analyzed, and the samples were purchased from local market.

2.3. Sample preparation

Caffeine was extracted from the coffee matrix using a liquid-liquid extraction with chloroform. One gram of sample was accurately weighed and transferred to a 125 mL separatory funnel with 10 mL of 0.2 $mol.L^{-1}$ NaOH and 30 mL of chloroform. The system was lightly shaken for 7 min and the organic phase was collected in a 50 mL flask. The aqueous phase was then washed three more times with 5 mL chloroform, always collecting the organic phase. The chloroform from the extracts was evaporated in a water bath at 60℃ and the residue was resuspended in 10 mL of ultrapure water and shaken in a vortex for 2 min. After filtration through a 0.45 µm filter, 800 µL of the recovered residue was added to 100 µL of a 100 $mg.L^{-1}$ theobromine solution (Internal Standard - IS) prior to

injection into the CE system, with the goal of compensating variations in the injection volume. Theobromine was chosen as IS because its structure and physico-chemical behavior are close from those of caffeine, as well as for the fact that theobromine is not present in the sample analyzed.

2.4. Experimental design

The analytical method was optimized by executing a central composite design, with central and axial points (Box et al., 2005; Bruns et al., 2006). The influence of voltage, SDS concentration and CAR concentration was investigated, as well as the interaction between these variables. The experimental design was carried out using a sample of Brazilian decaffeinated coffee with the aim of evaluating matrix interference.

The variable levels studied were defined based on preliminary tests, where the use of SDS at concentrations below 20 $mmol.L^{-1}$ did not promote the separation of caffeine and interferents. The levels used in the factorial design were the following: CAR concentrations from 3.2 (level -1.68) to 36.8 $mmol.L^{-1}$ (level 1.68), SDS concentrations from 23.2 to 56.8 $mmol.L^{-1}$ and voltage from 11.6 to 28.8 kV. All experiments were done in duplicate.

To develop a reliable analytical method, the parameters studied were assessed by means of the six responses that are essential for establishing conditions for qualitative and quantitative analysis by CE: interferent separation (SI), area, noise intensity, baseline variation (BV), analysis time and system current. Baseline variation consisted of the increase in the baseline due to matrix

effects and experimental conditions. BV was measured by the difference in the height of baseline between electroosmotic flow and the end of electropherogram.

Linear and quadratic models were used for regressing each set of response values. The responses were characterized by correlation coefficients and principal component analysis (PCA).

As the last step in the optimization procedure, the optimized conditions found by analysis of the multivariate design were investigated using a 2^2 factorial design to achieve the best system repeatability. The levels consisted in time variations (between 5 and 7 seconds) and pressure variations (from 40 to 50 mBar). The same sample used in the previous design was injected with 6 replicates for each factorial condition and the response was assessed by the coefficient of variation.

Experimental design, data analysis and desirability function calculations were performed using the Statistica version 7.0 software (Statsoft, USA).

2.5. Electrophoretic procedure

At the beginning of each day, the capillary was conditioned by a flush (1000 mBar) with basic solution (5 min with 1 mol.L^{-1} NaOH), followed by a flush of deionized water (5 min) and running buffer (10 min).

To avoid solute adsorption between identical conditions (replicates) of the factorial design, the capillary was washed with the running buffer for 1 min. Before performing each set of conditions of the experimental design, the capillary was washed with running buffer (5 min). For commercial sample analysis, the washing time was 0.5 min. Running electrolytes were filtered through a 0.45 µm filter and

centrifuged at 3000 rpm for 10 min (Excelsa II, mod. 206 BL, Fanem, SP, Brazil) for removal of air bubbles.

Detection was performed at 206 nm. This wavelength was selected in order to increase the sensitivity for caffeine. Injections for the experimental design experiments were made using the hydrodynamic mode for 5 s at 40 mBar. The capillary was maintained at constant temperature of 25ºC.

2.6. Validation

The method was validated according to the US Pharmacopeia (2008) and Brazilian Legislation requirements (INMETRO, 2003; Ribani, Bottoli, Collins & Jardim, 2004). The following validation characteristics were addressed: linearity, limit of detection, limit of quantification, precision and accuracy.

Appropriate aliquots of caffeine and theobromine (IS) standard stock solutions were transferred separately into 10 mL volumetric flasks and diluted to volume with ultra-pure water. Concentration ranging from 1 to 200 $mg.L^{-1}$ of caffeine and 11.1 $mg.L^{-1}$ of IS were obtained. The solutions were filtered using a 0.45 μm filter and injected on the CE instrument. Each solution was injected in triplicate. Peak area ratios (caffeine/theobromine) were plotted versus the respective concentrations of caffeine, in order to study method linearity.

Limits of detection (LOD) and quantification (LOQ) were the caffeine concentrations that lead to signal-to-noise ratios of 3:1 and 10:1, respectively.

Eight determinations of a coffee sample extract were performed to establish the intraday repeatability. The intermediate precision was evaluated by injecting

the same sample solutions prepared for the intraday precision determination, in five consecutive days.

In order to study the efficiency of the extraction at the optimal point, recovery tests were performed spiking decaffeinated coffee samples with caffeine standard at three levels: 5.0, 10.0 and 100.0 $mg.g^{-1}$.

2.7. Application in decaffeinated coffee samples

The commercial samples were analyzed using the validated method, and caffeine content of the samples was submitted to Analysis of Variance (ANOVA), and the means were compared for statistically significant differences by the Tukey test ($P < 0.05$), using Statistica 7.0 software (Statsoft, USA).

3. Results and Discussion

3.1. Statistical analysis

Interferent separations, area, noise intensity, baseline variation, analysis time and system current data results are presented in Table 1 for the fifteen experiments of the central composite design.

Table 1

Levels of factorial design and mean response values.

Exp.	Conditions			SI	Area	Noise	BV	Time	Current
	SDS	CAR	V			(mAU)	(mAU)	(min)	(mA)
1	-1	-1	-1	9.0	26.9	0.11	0.60	5.2	28.2
2	-1	-1	1	4.4	13.1	0.11	1.15	3.0	53.5
3	-1	1	-1	11.6	30.7	0.08	0.83	6.9	54.3
4	-1	1	1	8.2	14.1	0.09	1.63	3.5	113.5
5	1	-1	-1	205.0	29.5	0.10	0.33	5.7	37.1
6	1	-1	1	4.3	18.9	0.23	1.45	3.2	72.3
7	1	1	-1	17.3	35.2	0.11	1.85	7.2	64.4
8	1	1	1	36.0	12.0	0.07	1.43	3.4	138.5
9	-1.68	0	0	45.0	18.5	0.04	0.83	4.6	55.4
10	1.68	0	0	165.0	22.1	0.14	0.90	5.1	79.4
11	0	-1.68	0	44.5	15.4	0.17	0.75	3.8	32.5
12	0	1.68	0	3.5	24.8	0.15	2.55	5.7	104.5
13	0	0	-1.68	11.5	36.8	0.07	0.95	9.1	35.0
14	0	0	1.68	42.5	11.6	0.16	2.50	2.8	116.5
15	0	0	0	7.0	21.3	0.11	2.01	4.9	68.0

SDS (Sodium Dodecyl Sulfate concentration); CAR (Sodium Carbonate concentration); V (Voltage); SI (Interferent Separation); BV (Baseline Variation).

Each set of response values was regressed on the factor levels of the experimental design using linear and quadratic models given below. Linear models, consisting of the first four terms in the equation below, were found to be inadequate to represent the response values (Eq. 1).

(Eq. 1) $\hat{y} = b_0 + b_S x_S + b_C x_C + b_V x_V + b_{SS} x_s^2 + b_{CC} x_C^2 + b_{VV} x_v^2 + b_{SC} x_S x_C + b_{SV} x_S x_V$,

where ŷ is the predict response, b are the model coefficients and x are the variable values. The index S corresponds to the SDS concentration, C corresponds to CAR concentration and V corresponds to the voltage.

Results for the quadratic model coefficients are given in Table 2 along with the mean square lack of fit and mean square pure error ratios for each response.

Table 2

Quadratic model coefficients obtained for the investigated responses and ANOVA mean square ratios for lack of fit and pure error.

Model Coefficients	SI	Area	Noise	BV	Time	Current
b_0	10.48	21.12	0.112	2.04	5.02	68.05
b_S	31.58	1.22	0.021	0.07	0.12	7.57
b_C	-16.05	1.41	-0.018	0.38	0.52	22.03
b_V	-10.10	-7.81	0.019	0.34	-1.64	24.24
b_{SS}	-	-	-0.10	-0.46	-0.14	-0.33
b_{CC}	-	-	0.014	-0.18	-0.19	0.06
b_{VV}	-	1.28	-	-0.16	0.25	2.62
b_{SC}	-20.30	-0.74	-0.012	0.10	-0.04	0.93
b_{SV}	-21.76	-0.43	0.012	-0.08	-0.09	3.08
b_{CV}	27.60	-1.93	-0.019	-0.16	-0.33	9.11
MS_{lof} / MS_{pe}	35.23	54.28	11.40	79.4	461.46	18.62

95% confidence critical value is $F_{5,15,\ 0.05} = 2.90$.

S, C and V represent SDS, Carbonate and voltage.

Calculate MS_{lof}/MS_{pe} (Mean square lack of fit/mean square pure error ratios).

All experiments were performed in duplicates.

The corresponding F 95% critical value for the experimental design (5 degrees of freedom for lack of fit and 15 for pure error) of 2.9 is smaller than any of the calculated ratios indicating that all the quadratic models also suffer from lack of fit. Since the experiments and duplicates were not performed randomly owing to operational difficulties, underestimation of the pure error estimates could result in inflation of the mean square ratios and an erroneous indication of significant model lack of fit. Even though the models cannot be rigorously used to make quantitative predictions, the model coefficients, especially the linear ones, are useful for understanding systematic behavior in the response values as a function of the factor levels. Correlation coefficients were also calculated for all the response values in Table 1 in order to facilitate interpretation of these models.

The model coefficients are consistent with the correlation coefficients. The most important linear coefficient in the model for the SI response is b_s = 31.58. Data for a model with this positive coefficient for SDS, along with the positive quadratic SDS coefficient, b_{ss} = 29.96, would be expected to show a positive correlation coefficient between the SI response and the SDS factor, which is +0.51. This also holds for the noise response for which the most important linear model coefficient corresponds to the SDS concentration. It is positive as is the +0.42 correlation coefficient between noise and SDS.

The most important linear terms are positive and occur for the carbonate and voltage factors of both the BV and current models. This indicates the existence of positive correlation coefficients between carbonate and current (+0.64), carbonate and BV (+0.56), voltage and BV (+0.50), and voltage and current (+0.70) values. The most important negative linear regression coefficients in Table

2 occur for the voltage factor in the area and time models and are consistent with the large negative correlation coefficients between the voltage factor levels and the area and time response values, -0.92 and -0.91, respectively.

There do exist some interesting correlations between the responses. A +0.94 correlation coefficient occurs between the area and the time values. This occurs since both responses are negatively correlated with the voltage levels and that the most important model coefficients in the area and time models are negative. As such it is not possible to maximize area and minimize time by varying the voltage factor, at least for the range of levels investigated in the experimental design. The BV and current values are positively correlated, +0.69. Both these responses are positively correlated with the carbonate levels. Since both responses have positive b_c model coefficients, their values can be decreased by decreasing the carbonate concentrations. Alternatively the voltage levels could be decreased to minimize BV and the current values, which would be accompanied by increasing area and time values. Moderate negative correlation coefficients exist between area and current (-0.56), noise and time (-0.41), and current and time (-0.45). They also provide useful information for attempts to optimize all these factors simultaneously.

Figure 1A shows a loading graph for the first two principal components that represent 69% of the total data variance. The points for area and time are very close to each other since they are related by a high positive correlation coefficient. On the other hand these variables with points on the left in Fig. 1A have negative correlations with the noise, current and BV variables on the right hand side of the

graph. Current and BV are positively correlated while SI, in the upper part of the graph, is negatively correlated with BV at the bottom of the graph.

The first principal component is positively correlated with the V levels used in the experimental design. This can be seen in the score graph in Fig. 1B where low levels of voltage occur for points on the left of the graph, zero levels for those in the center and positive values on the right of the graph. Low levels of voltage result in larger areas, decreasing noise levels, smaller current and BV values but with simultaneously increasing time values.

The second principal component is correlated with the carbonate levels. Although the trend here is not as strong as the one observed for the first component, score points with lower values of carbonate tend to be concentrated in the upper portion of the graph while the higher CAR levels are towards the bottom. As such maximization of SI is favored by employing lower carbonate concentrations.

The third principal component corresponds to 15.6% of the total data variance and shows similar tendencies for SI as does the second principal component. However the fourth principal component accounting for 12.5% of the variance is negatively correlated with the SDS factor levels. The negative loading for the SI response on the fourth principal component and the predominantly negative scores for experiments with high SDS levels are consistent with the positive correlation between SI values and the SDS levels.

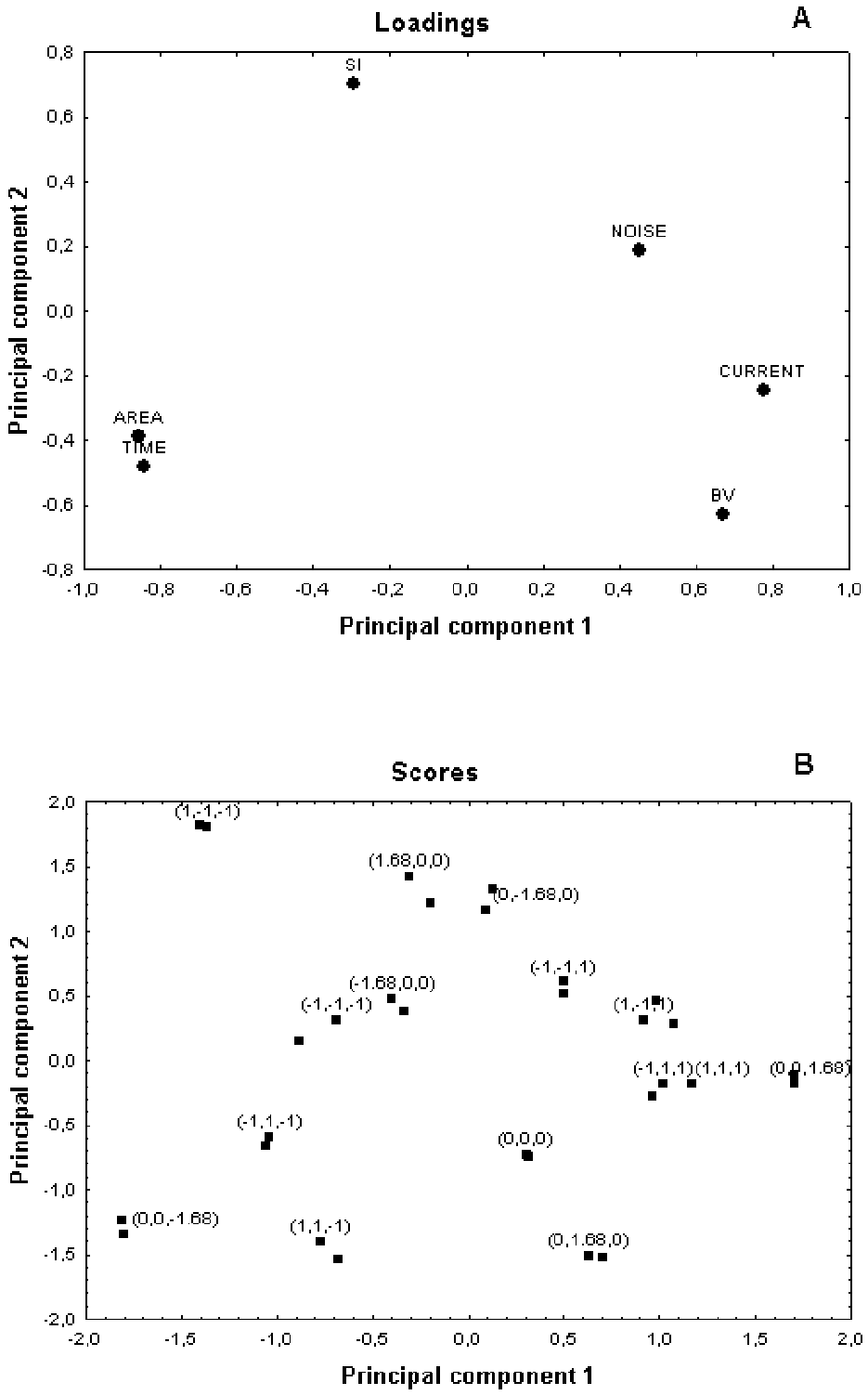

Fig. 1. (A) Loading plot showing first and second principal components for response values. (B) Score showing first and second principal components for the response values. Factor levels are given in parenthesis (SDS, CAR, V).

3.2. Optimal conditions

It was possible to find the optimal point based on the above statistical analyses and practical considerations. The most important response is SI and therefore it was necessary to use the SDS at higher concentrations (level +1 or more). A concentration of 50 $mmol.L^{-1}$ (+1) was chosen because at this level separation occurred and the noise was not too high. The second most important response is BV since when it is very high, it is not possible to quantify the analyte with adequate precision. Thus carbonate and voltage were set at lower levels (10 $mmol.L^{-1}$ and 15 kV) that also provided higher area values, improving method sensitivity. The recorded current was kept low (38 μA), facilitating the stability of the system. The buffer pH showed good stability without needing corrections during the preparation. Analysis time was 5.2 min.

In the light of these practical observations, the statistical interpretation and the response values obtained for the central composite design experiments the (+1, -1, -1) or (50 $mmol.L^{-1}$, 10 $mmol.L^{-1}$, 15 kV) level was selected as the set of conditions furnishing the best electropherogram.

Regarding injection optimization, the relative standard deviations (RSD) for six injection replications were lower then 2.0% for all factor levels, providing good resolution and interferent separation. For this reason, a 50 mBar for 7 s condition was chosen since it resulted in better method sensitivity.

3.3. Validation

The validation results are summarized in Table 3. The results demonstrated that the correlation between caffeine/IS ratios and resulting peak areas in the electropherograms was linear, with a high value for the correlation coefficient (R = 0.9998).

Table 3

Validation of an electrophoretic method for the analysis of caffeine in decaffeinated coffee samples.

Parameter	Result
Slope	0.0767
Intercept	-0.0194
Correlation coefficient	0.9998
LOD (mg .100 g^{-1})	0.87
LOQ (mg .100 g^{-1})	2.88
Repeatability (RSD, %)	1.42
Intermediate precision (RSD, %)	1.96

The limits of detection (LOD-S/N ratio 3:1) and quantification (LOQ-S/N ratio 10:1) were 0.87 and 2.88 mg .100 g^{-1}, respectively, for caffeine in samples of decaffeinated coffee.

The intraday repeatability was determined by performing replicate injections (n = 8) of a same decaffeinated coffee extract. The RSD value for peak area was 1.42% and for the migration times, 0.48%, which indicates acceptable method repeatability.

Intermediate precision was evaluated over 5 days using the same sample injected daily under the same conditions. The RSD value of 1.96% for peak area indicates that the intermediate precision is acceptable.

The accuracy of extraction was evaluated by calculating the percentage of recovery. The results ranged from 96.3 to 107.4% for ground-roasted coffee and from 94.9 to 102.6% for instant coffee. This shows that it was not possible to detect material loss in the procedure.

3.4. Application in decaffeinated coffee samples

The optimum condition showed good results for the practical analysis of decaffeinated coffee, both for ground samples as well as for soluble ones, as can be seen in Figure 2. The liquid-liquid extraction with chloroform was efficient to remove hydrosoluble interferents, showing a reduced matrix effect.

Table 4 shows the caffeine contents of the different coffee samples. The caffeine contents in decaffeinated ground-roasted coffee ranged from 12.4 ± 0.4 mg.100 g^{-1} (Coffee B) to 925.6 ± 16.1 mg.100 g^{-1} (Coffee G). Fugioka & Shibamoto (2008) found similar levels of caffeine in Japanese decaffeinated coffee samples. The caffeine contents in decaffeinated instant coffee ranged from 57.7 ± 0.4 mg.100 g^{-1} (Coffee P) to 414.4 ± 4.9 mg.100 g^{-1} (Coffee N). In two of the eight brands of ground-roasted coffee analyzed (one batch of one brand and one batch of another), and in four out of the 21 instant coffees analyzed (one batch of one brand and all 3 batches of another), the caffeine content was above the maximum

limit specified in the Brazilian Legislation (ANVISA, 2005), which is 100 mg.100 g^{-1} for ground-roasted coffee and 300 mg.100 g^{-1} for instant coffee.

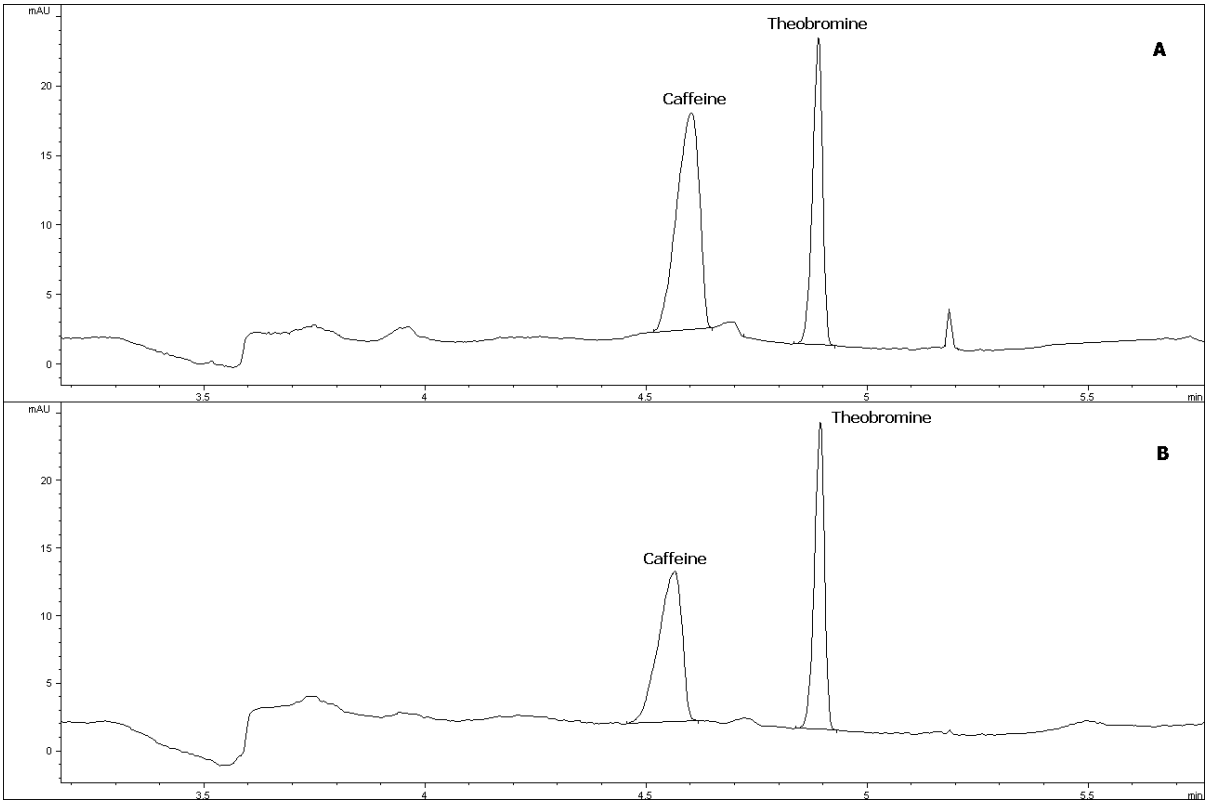

Fig. 2. Electropherograms for the optimum conditions applied to decaffeinated ground-roasted (A) and instant (B) coffee samples. Conditions: Buffer with 50 mM SDS and 10 mM CAR (pH=11.0), at 15 kV, 25ºC, employing a fused-silica capillary of 50 μm x 48 cm total length, hydrodynamic injection of 50 mBar during 7 s, with detection at 206 nm wavelength.

Table 4

Caffeine content of decaffeinated coffee samples

Ground Roasted			Instant		
Brand	**Batch**	**Mean ± SD (mg .100 g^{-1})**	**Brand**	**Batch**	**Mean ± SD (mg .100 g^{-1})**
A	1	13.6 ± 0.12 ^{n}	I	1	65.8 ± 0.92 ^{i,j}
	2	26.1 ± 0.11 ^{j,k,l}		2	80.4 ± 1.40 ^{g,h,i}
	3	20.2 ± 0.14 ^{m}		3	62.7 ± 0.17 ^{j}
B	1	13.3 ± 0.21 ^{n}	J	1	64.8 ± 0.54 ^{i,j}
	2	12.4 ± 0.36 ^{n}		2	74.6 ± 0.34 ^{h,i,j}
	3	13.9 ± 0.04 ^{n}		3	79.9 ± 1.93 ^{g,h,i}
C	1	22.4 ± 0.17 ^{l,m}	K	1	85.1 ± 1.21 ^{g,h}
	2	15.4 ± 0.12 ^{n}		2	106.8 ± 2.26 ^{f}
	3	48.1 ± 1.48 ^{g}		3	*
D	1	79.6 ± 3.88 ^{e}	L	1	96.4 ± 1.10 ^{f,g}
	2	**246.6** ± 8.02 ^{b}		2	80.5 ± 1.57 ^{g,h,i}
	3	74.5 ± 1.15 ^{f}		3	77.6 ± 1.96 ^{h,i,j}
E	1	26.5 ± 0.84 ^{j,k,l}	M	1	**334.1** ± 5.30 ^{c}
	2	26.7 ± 0.43 ^{j,k}		2	199.2 ± 2.96 ^{e}
	3	15.8 ± 0.38 ^{n}		3	295.9 ± 1.41 ^{d}
F	1	23.0 ± 0.12 ^{k,l,m}	N	1	**414.4** ± 4.87 ^{a}
	2	27.9 ± 1.74 ^{i,j}		2	**372.4** ± 1.12 ^{b}
	3	31.5 ± 1.59 ^{i}		3	**386.4** ± 3.76 ^{a}
G	1	93.1 ± 1.20 ^{d}	O	1	92.2 ± 1.74 ^{f,g}
	2	**925.6** ± 16.14 ^{a}		2	*
	3	**201.8** ± 2.87 ^{c}		3	*
H	1	44.0 ± 2.25 ^{g}	P	1	57.7 ± 0.37 ^{j}
	2	36.7 ± 1.35 ^{h}		2	83.0 ± 0.87 ^{g,h,i}
	3	26.0 ± 0.56 ^{j,k,l}		3	65.2 ± 1.42 ^{i,j}

SD: Standard Deviation (n = 4).
*Not found in the market.
Means followed by the same letter in the row are not statistically different by Tukey test at 95% confidence level.
Bold values were above Brazilian legislation limits.

The caffeine content of all samples was above the limit of quantification of 2.88 mg.100 g^{-1}, which represents about 1% and 3% of the maximum content

specified in Brazilian legislation for decaffeinated instant and ground-roasted coffees, respectively.

4. Conclusions

In this study, a simple, direct, rapid and robust MEKC method was developed for quantification of caffeine in decaffeinated coffee. Factorial and central composite designs and their statistical analyses have been used successfully for the optimization of the buffer composition, running voltage and injection conditions.

Adequate multiple-responses of the CE method were obtained by optimizing the relevant factors and applying experimental design methods while performing a minimum number of experiments.

Excellent separation of interferents and analyte area, low BV, noise and current were achieved using a buffer composition of 50 $mmol.L^{-1}$ SDS, 10 $mmol.L^{-1}$ CAR, at 15 kV, 25 ºC, employing a fused-silica capillary of 50 μm x 48 cm total length, hydrodynamic injection of 50 mBar during 7 s, with detection at 206 nm and analysis time of 5.2 min.

The method was linear in the 1-200 $mg.L^{-1}$ range, with a correlation coefficient above 0.9997. The LOQ for caffeine measured in the sample was 2.88 $mg.100\ g^{-1}$, which was about 35 and 105 times lower than the legislation limit for decaffeinated ground-roasted and instant coffees, respectively. The intermediate precision of the quantitative analyses for caffeine did not exceed 1.96%.

The main advantages of the developed method were the good stability of the buffer pH and the low residue generation, which allows its application in routine analysis in laboratories and industries.

Optimum conditions were successfully applied for caffeine quantification in 45 samples of decaffeinated coffee. Seven samples of four different brands showed higher levels than the limit specified by Brazilian legislation.

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CAPÍTULO 2

MULTIVARIATE OPTIMIZATION OF CAFFEINE RESIDUE EXTRACTION FROM DECAFFEINATED COFFEE

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(Manuscrito a ser submetido à Química Nova)

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Abstract

In this work, a 2^3 factorial design was performed to optimize a liquid-liquid extraction of caffeine from a decaffeinated coffee matrix, in order to allow its quantification by capillary eletrophoresis. The parameters analyzed in the factorial design were the ratio solvent:sample, extraction time and filtration or not after extraction. Optimum extraction point was defined using the amount of caffeine extracted as the response factor. The best extraction condition was achieved when using 30 mL of chloroform, 7 min of extraction and without filtration. This condition showed good repeatability (RSD = 2.8%; n = 7) and recovery of 96.7-107.4 %.

Keywords: factorial design optimization, caffeine extraction, capillary electrophoresis

INTRODUCTION

Caffeine (1,3,7-trimethylxanthine) is an alkaloid from the xanthines group, occurring in coffee, tea, mate, cocoa products, and cola beverages. The caffeine content of Arabica and Robusta roasted beans is 1% and 2%, respectively. There are others substances from this group, as theobromine and theophylline, and their molecular structures are very similar to that of caffeine.[1,2]

Caffeine is a mild central nervous system stimulant. At sufficiently high doses, it may produce flushing, chills, agitation, irritability, loss of appetite, weakness and tremor. In cases of overdose, hypertension, hypotension, tachycardia, vomiting, fever, delusions, hallucinations, arrhythmia, cardiac arrest, coma and even death have already been reported. The principal mechanism of action is antagonism related to adenosine, which has inhibitory functions (caffeine has the potential to occupy adenosine receptor sites).[3] It should be noted that caffeine does not always have exclusively non-beneficial effects. Epidemiological and experimental studies have shown positive effects of regular coffee drinking on various aspects of health, such as psychoactive responses (alertness, mood change), neurological conditions (infant hyperactivity, Parkinson's disease), metabolic disorders (diabetes), and gonad and liver function.[3]

For many consumers, the answer to supposed or true adverse effects of caffeine ingestion is to consume decaffeinated coffee.[4] Even so, the established idea that levels of caffeine typically found in decaffeinated coffee have no behavioural effects has been revised. On the basis of studies developed by Haskell

et al.[5], it was discovered that even low doses, as 9 mg, have psychoactive properties which can endure for several hours.

Brazilian legislation allows at most 0.1% of residual caffeine in decaffeinated roasted and ground (powder) coffee and 0.3% in instant coffee products.[6]

Caffeine determination in foods and beverages can be made by various methods, such as UV spectrophotometry, gas chromatography (GC), high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), among others.[7]

The use of CE is growing up in many studies. The main advantages of CE are the high efficiency of separation, good resolution and selectivity, as well as fast analysis (until 20 min), low cost of the reagents and the need of few amounts of sample. The instrument is versatile and shows fast stabilization to the analysis conditions. It should be noted that waste generation is minimum and, in the most of the cases, they don't need a specific treatment because they are constituted by simple buffers.[8,9]

Chloroform was found to be the most suitable solvent to extract caffeine from aqueous solution. The reason is the high caffeine solubility in chloroform, nine times more than in water at room temperature.[10] Liquid–liquid extraction is attractive because it operates at atmospheric pressure. It is easy to control and equipment cost is low.[11] This extraction is important to eliminate matrix effects caused by hydrosoluble compounds of coffee.

Therefore, the aim of this study was to optimize a procedure for caffeine extraction, separating water soluble matrix interferents, allowing this compound quantification by CE.

EXPERIMENTAL

Sample and chemicals

Sample analyzed consisted of a commercial decaffeinated ground-roasted coffee purchased from Campinas local market.

The water used in all studies was ultrapure (18 MΩ cm) obtained from a Direct-Q 3 UV ultrapure water system (Millipore Corporation, France). Caffeine and theobromine were from Sigma Chemical Co. (St. Louis, MO). Chloroform (pro analysis grade) was from Merck (Rio de Janeiro, Brazil), sodium carbonate was from Synth (São Paulo, Brazil) and SDS was from Riedel-deHaën (Germany). All other reagents and solvents were of analytical grade quality.

All the solutions were degassed by ultrasonication (Microsonic SX-20, Arruda Ultra-sons LTDA, Brazil). Running electrolytes and samples were filtered through a 0.45 µm Millex filters (Millipore corporation, France). Running buffer was centrifuged at 3000 rpm for 3min (Excelsa II, mod. 206 BL, Fanem, SP, Brazil).

Stock solutions of caffeine (1000 $mg.L^{-1}$) and theobromine (1000 $mg.L^{-1}$) were maintained under refrigeration until use in preparation of standard solutions and spike analysis.

CE apparatus and operating conditions

An Agilent G1600AX instrument (Agilent Technology, Germany) equipped with a diode array detector. The fused-silica capillary was obtained from Agilent Technology (Germany) and had the following dimension: 48 cm total length, 50 µm i.d.

The CE operation parameters were as follows: the detection was performed at 206 nm, the capillary temperature was maintained at 25 ^{o}C, and voltage was set at 15 kV. Samples were pressure-injected at the anodic side at 50 mbar for 7 s.

To achieve high reproducibility of migration times and to avoid solute adsorption, the capillary was washed between analyses with the running buffer, consisting of 50 mM sodium dodecyl sulfate (SDS) and 10mM sodium carbonate buffer (pH 11.0) for 1 min. Buffer pH was stable during analysis, without needing to adjust it.

A calibration curve, using theobromine as internal standard, was used to quantify the caffeine in samples.

The method was previously optimized and validated.[12]

Experimental design

A liquid-liquid extraction with chloroform was used to separate caffeine from the coffee matrix. The extraction was optimized by a 2^3 factorial design, in witch the amount of chloroform, the time of extraction and the use of filter were the parameters analyzed (Table 1). Optimum extraction point was defined using the amount of caffeine extracted as the response factor.

Table 1. Factorial design parameters for caffeine extraction

Variables	**Studied levels**	
	-1	**1**
Amount of chloroform	20 mL	30 mL
Time of extraction	4 min	7 min
Use of filter	without filter	with filter

One gram of a decaffeinated roasted and ground coffee sample was accurately weighed and transferred to a 125 mL separatory funnel with 10 mL of 0.2 mol.L^{-1} NaOH and 20 or 30 mL of chloroform. The system was lightly shaken for 4 or 7 min and the organic phase was collected in a 50 mL flask, filtering it or not in a common filter paper. The aqueous phase was then washed three more times with 5 mL chloroform, always collecting the organic phase. Chloroform from extracts was evaporated in water bath at 60℃ and the residue was resuspended in 10 mL of ultrapure water and shaken in a vortex for 2 min. After filtration through a 0.45 µm Millex filter (Millipore corporation, France), 800 µL of the recovered residue was added to 100 µL of a 100 g.L^{-1} theobromine solution (Internal Standard) prior to inject in CE system.

After defining the optimum point, the extraction was tested using 40 and 50 mL of chloroform in order to compare with factorial results.

Experimental design, data analysis and desirability function calculations were performed using the Statistica version 7.0 software (Statsoft, USA).

Extraction validation

The repeatability was determined by performing seven replicated extractions of a same decaffeinated coffe sample.

In order to study the efficiency of the extraction in the optimal point, recovery tests were performed spiking decaffeinated coffee sample with caffeine standard in three levels: 5.0, 10.0 and 100.0 $mg.g^{-1}$, before the extraction.

RESULTS AND DISCUSSION

Extraction conditions

Figure 1 shows the results obtained in the factorial design. For each level, the results represent the average of two extractions. None of the studied parameters was significant at 95% of confidence, by the analysis of variance (ANOVA) study. The linear model did not show lack of fit (MQ_{Faj}/ MQ_{Ep} = 0.005, < $F_{Critic\ (1,\ 7)}$ = 5.59). It means that the two populations can be described with the same parameters and used for residue calculation. The residue graphic shown normal distribution and the regression equation was significant (MQ_{Reg}/ MQ_{Res} = 10.933 > $F_{critic\ (6,\ 8)}$ = 3.58).

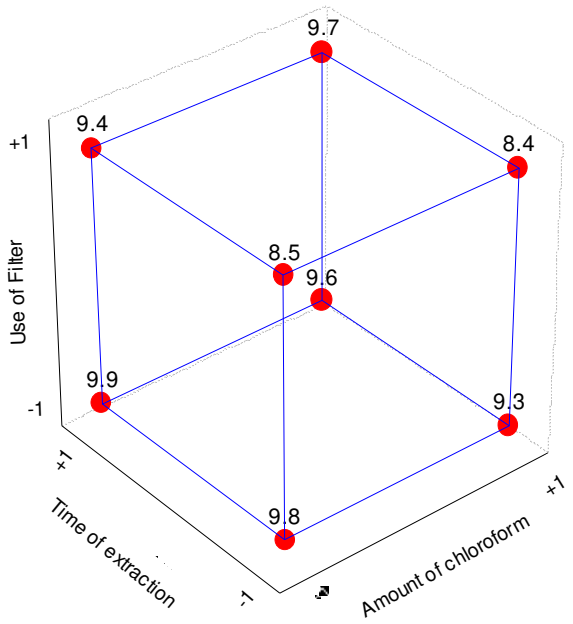

***Figure 1**. Concentration of caffeine (mg.100 g^{-1}) extracted in the levels of the factorial design.*

The regression coefficients of the model are in Table 2. These coefficients were not significant in 95% of confidence. However, with 90% of confidence, the variable time of extraction was significant, indicating that the extraction was more effective when the time of extraction is the highest (7 min). Some tests with time of extraction higher than 7 min were performed, but the formation of emulsion did not allow the liquid-liquid partition. In the experiments without filter, a better repeatability was achieved, once the retention of caffeine residues in the filter was avoided.

Table 2. Regression Coefficients

	Regression	Standard Error	t(7)	p
Mean/Interc.	9.351	0.175	53.497	0.000
(1)Chloroform (mL)	-0.063	0.175	-0.365	0.73
(2)Time of extraction (min)	0.326	0.175	1.867	0.10
(3) Use of filter	-0.323	0.175	-1.851	0.11
1 by 2	0.086	0.175	0.493	0.637
1 by 3	0.136	0.175	0.779	0.462
2 by 3	0.226	0.175	1.295	0.236

During the extractions from factorial design, it was observed the use of chloroform in low level (20 mL) was prejudicial to repeatability of extraction, probably due to saturation of organic phase by matrix components. When 30 mL of chloroform was used, the repeatability was good. High amounts of chloroform were tested (40 mL), but it was obtained the same repeatability and concentration of extracted caffeine.

The best extraction condition was achieved when using 30 mL of chloroform, 7 min of extraction and without filtration.

Extraction validation

The repeatability was determined by performing replicated extractions (n=7) of a same decaffeinated coffee sample. The RSD value for caffeine content was 2.80%.

The accuracy of extraction was evaluated by calculating the percentage of recovery. The results, summarized in Table 3, show that it is not possible to detect loss of material in the procedure.

Table 3 - Recovery test of caffeine in decaffeinated coffee samples

Coffee sample	Spiking level ($mg.100g^{-1}$)	Recovery (%)
Roasted and ground	5.0	96.3
	10.0	107.4
	100.0	99.7

Tfouni et al. [13] obtained 71.5 to 80.5 % of caffeine recovery from guarana seeds using aquous extraction. However, Camargo and Toledo [14], using the same aquous method, found about 100% of caffeine recovery from coffee matrices. Gnoatto at al. [15] compared different methods for caffeine extraction from mate (*Ilex paraguariensis*), and found that, among the studied methods, the most efficient used alcalinization and organic extraction with chloroform, like in the present work.

CONCLUSION

The extraction method optimized in this work allowed to remove matrix interferences that were prejudicial to the resolution of the caffeine peak and, consequently, to the quantification. The experimental design proved to be an

efficient tool for the extraction optimization, with reduced number of experiments and, consequently, shorter analysis time. CE separation method was rapid, efficient and simple.

ACKNOWLEDGEMENT

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CAPÍTULO 3

COMPARISON OF HPLC AND CE METHODS FOR CAFFEINE RESIDUE DETERMINATION IN DECAFFEINATED COFFEE

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Comparison of HPLC and CE methods for caffeine residue determination in decaffeinated coffee

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RESUMO

Este artigo apresenta uma comparação entre métodos para análise de cafeína em café descafeinado por EC e CLAE. Os métodos foram validados para linearidade, sensibilidade, precisão, LOD e LOQ e comparados quanto à eficiência de separação, quantificação de cafeína em 20 amostras, tempo de análise, custo e volume de resíduos gerado. Os métodos apresentaram boa linearidade, repetibilidade (CV < 1,96%) e separação eficientes. O teor de cafeína das amostras determinado por ambos os métodos não apresentou diferença significativa a 95% de confiança. A sensibilidade do método por CLAE foi 42 vezes maior que a do método por EC, porém, EC apresentou tempo total de análise 30,4% menor. O volume de resíduos gerado foi 33 vezes maior em CLAE e os custos dos reagentes para EC foram 76,5 vezes menor. Uma importante vantagem da EC em relação à CLAE é o uso de eletrólitos em solução aquosa, econômicos e ecológicos, no processo de separação.

ABSTRACT

This article presents a comparison of CE and HPLC methods for caffeine residue quantification in samples of decaffeinated coffee. Methods were validated for linearity, sensitivity, precision, LOD and LOQ and were compared regarding separation performance, quantification of caffeine in twenty samples, analysis time, costs per analysis and volume of generated residue. Both methods presented good linearity, appropriate repeatability (RSD < 1.96%), and efficient separation. Caffeine content of the samples analyzed with both methods did not differ statistically at 95% confidence level. HPLC method sensitivity was 42 times greater than by CE method. In another hand, CE presents total analysis time 30.4% lower than HPLC. The volume of generated residues was 33 times greater in HPLC and the cost in reagents for CE was 76.5 times lower than for HPLC. One important advantage of CE over HPLC is the use of economical and ecological aqueous buffer in the separation process.

Keywords: caffeine, CE, decaffeinated coffee, HPLC, method comparison

Introduction

Coffee is one of the world's most popular and widely consumed beverages. Brazil is the largest worldwide producer and exporter of coffee beans, responsible for around 30% of international market. It is also the second consumer market, following the United States.[1-3]

The world's coffee supply comes primarily from two major types of coffee beans, Arabica (*Coffea arabica*) and Robusta (*Coffea canephora*), which differ in several characteristics as well as caffeine content. Arabica is favored for its finer aroma, flavor, and body, and contains 1% caffeine. Robusta is neutral and contains twice as much caffeine.[1,2,4]

Coffee is one of the two largest sources of caffeine throughout most of the world, the other being tea, and many investigations of caffeine and serum cholesterol have used coffee as a source for caffeine. Coffee is known to exhibit a number of bioactivities, such as antioxidant, anti-carcinogenic and anti-mutagenic activity.[1,2] In general, coffee is graded based on the size, colour and percentage of imperfections. The relationship between coffee, caffeine, and serum cholesterol has been studied extensively over the past two decades. Reports from both observational and experimental investigations have shown associations between coffee and serum cholesterol which have ranged from being strong and significant to having little or no effect. These conflicting results are likely attributable to confounding associations between coffee consumption, its preparation methods, variability in the chemical composition of different types of coffee beans, the degree of coffee bean roasting, and potential interactions with other dietary factors

such as increased saturated fat intake from the addition of dairy products to coffee.[1,2]

Caffeine and its metabolites are widely found in the human diet and pharmaceutical formulations. It may be the most popular drug in the world, often present in coffee, tea, cocoa, chocolate, some soft drinks and some drugs. It is also a central nervous system stimulant and used as a probe drug for the assessment of metabolic capacity. In moderate doses, it can increase alertness, reduce fine motor coordination, cause insomnia, headaches, nervousness and dizziness. In massive doses caffeine is lethal. A fatal dose of caffeine has been calculated to be more than 10 g (about 170 mg kg^{-1} body weight). Some studies have shown that caffeine causes physical dependence.[5-7] In order to extend the target consumers of their products, the manufactures have commercialized the decaffeinated coffee. In Brazilian legislation, for example, the maximum level of caffeine in the decaffeinated coffee is 0.1%, regulated by Brazilian Health Ministry.[8]

Since 1996, several techniques have been reported for the analysis of caffeine in various sample matrices (environmental, biological, plants, food, etc.) cover a broad spectrum of instrumental analysis, as described by Tzanavaras and Themelis.[9] These include batch UV–Vis spectrophotometry,[10-13] TLC,[14-16] ion chromatography,[17,18] Fourier transform Raman spectrometry,[19] square-wave anodic stripping voltammetry,[20] FTIR spectrometry,[21-24] near infrared spectrometry,[25] oscillating chemical reactions,[26] flow injection analysis,[13,27-32] CE,[6,33-35] and HPLC.[5,9,25,34,36-52]

HPLC with reversed phase and CE are two of the most frequently used modern separation techniques in different applying of the food analysis. As both techniques have different functional principles, sometimes it is quite easy to evaluate what one of them is indicated for analysis in some specific occasion in demand.

HPLC is the technique well established and with significant acceptance and confidence degree for the scientific public in many different areas of applying. HPLC present well recognized characteristics as robustness, repeatability and reprodutibility (precision). These qualifications are very important to the development of an analytical method with adequate validation parameters. However, CE offers a real and attractive alternative to HPLC in many instances. CE can present significant advantages over HPLC in terms of relative simplicity of operation, higher efficiency of separation, reduced cost of the operation, no need for organic solvents in mobile phases, smaller sample volume requirements and shorter analysis times.[9,27,36,53] Concentration sensitivity is typically an order of magnitude less than in HPLC, but peak efficiencies are much higher. Higher peak efficiencies generally mean greater resolution in short run times by CE.[54,55] The range of CE applications in biomedical and pharmaceutical analysis is at least as extensive as that of HPLC.[53] CE has been developed as a technique for the analysis of charged molecules, including proteins, peptides and oligonucleotides.[27] Many of the common problems associated with HPLC, such as high pressure, solvent leakage and the high cost of column may be eliminated.[53]

In order to compare the quantitative performance of both analytical techniques to quantify substances in food at residual levels, this paper describes two analytical methods to determine caffeine in decaffeinated coffee.

Experimental

Decaffeinated coffee samples

Samples analyzed consisted of twenty different commercial decaffeinated ground-roasted coffees, purchased from Campinas (SP, Brazil) local market.

Chemicals

Caffeine and theobromine (internal standard – IS) standards were from Sigma Chemical Co. (St. Louis, MO, USA), chloroform (pro analysis grade) was obtained from Merck (Rio de Janeiro, Brazil), sodium dodecylsulfate (SDS) from Riedel-de-Haën (Germany) and sodium carbonate from Synth (São Paulo, Brazil). All the other reagents and solvents were of analytical grade quality. The water used in all studies was ultrapure water (18 MΩ.cm) obtained from a Direct-Q 3 UV ultrapure water system (Millipore Corporation, France).

Preparation of standard solutions

Stock solutions of caffeine and theobromine were prepared by dissolving the compound in ultrapure water, at concentrations of 1000 $mg.L^{-1}$ and 100 $mg.L^{-1}$, respectively. Caffeine working solutions for calibration were prepared with final concentrations ranged from 1 to 100 $mg.L^{-1}$.

All solutions were filtered through 0.45 μm filter and stored under refrigeration. Before use, the solutions were degassed by ultrasonication for 5 minutes (Microsonic SX-20, Arruda Ultra-sons Ltda., Brazil).

Sample preparation

Caffeine was extracted from samples according to the procedure described by Meinhart et al.[56] One gram of coffee sample was added to a separatory funnel with 10 mL of 0.2 mol.L^{-1} NaOH and 30 mL of chloroform. The system was lightly shaken for 7 min and the organic phase was collected in a 50 mL flask. The aqueous phase was then washed three times with 5 mL of chloroform, always collecting the organic phase. Chloroform was evaporated from the extracts in a water bath at 60 ℃. The residue was resuspended in 10 mL of ultrapure water and filtered through a 0.45 μm filter.

In CE analysis, theobromine was added as IS in a ratio of 100 μL theobromine stock solution to 800 μL of sample. The purpose of IS addition was correcting possible variations of injection volume by the equipment. The samples were transferred for vials, degassed by ultrasonication for 5 minutes and analyzed by the two methods.

Instrumental parameters and conditions

Capillary electrophoresis

CE separation was performed in an Agilent G1600AX system (Agilent Technology, Germany) equipped with a DAD. The methodology was accomplished

according to the study of Meinhart et al.[56] Separation was carried out by using a fused-silica capillary which had a total length of 48 cm and 50 µm i.d.. CE operation parameters were as follows: detection was performed at 206 nm, capillary temperature was maintained at 25 ºC, voltage was set at +15 kV, and hydrodynamic injection at 50 mBar for 7 s.

The buffer solution consisted in 10 $mmol.L^{-1}$ sodium carbonate and 50 $mmol.L^{-1}$ SDS. Buffer resulting pH was 11.0, providing a good separation in the CE system. After filtration through a 0.45 µm filter, the buffer was centrifuged at 5000 for 10 min (for removal of air bubbles). Capillary was washed between runs with buffer for just 0.5 min. At the beginning of each day, capillary was washed for 5 min with 1 $mol.L^{-1}$ sodium hydroxide, 5 min with ultrapure water and finally with running buffer (10 min). In the end of the day, the capillary was clean for 5 min with 1 $mol.L^{-1}$ sodium hydroxide and 5 min with ultrapure water, and stored in water. The time for each electrophoretic run was 5.2 min.

Caffeine quantification in samples was done using an internal calibration curve and it was positively identified by comparing to the migration time and spectrum obtained for caffeine standard.

High-performance liquid chromatography

HPLC analysis was performed in an Agilent instrument (Hewlett Packard, 1100 series), with quaternary pumping, column oven at 25 ºC, DAD at 274 nm and automatic injector. Compounds were separated using a 15 cm x 4.6 mm i.d. and 5 µm particle C_{18} reversed-phase column (Varian).

HPLC method used was developed basing on the studies of Gnoatto and Bassani[57] and De Maria and Moreira,[58] with modifications. The mobile phase was a binary mixture of water:methanol 60:40 (v/v). Isocratic elution was performed at a flow rate of 0.7 $mL.min^{-1}$. The injection volume was 20 μL. At the beginning of each day, the column was conditioned with mobile phase for 60 min, with a flow of 0.5 $mL.min^{-1}$. After analytes elution, the flow of mobile phase was maintained by one minute for interferents elution and column regeneration. At the end of daily activities, the column was cleaned with mobile phase (flow of 0.7 $mL.min^{-1}$, for 30 minutes), in order to avoid solutes adsorption, staying in mobile phase until the following day. The analysis time was 6.5 min.

Caffeine quantification in samples was performed by external calibration curve and it was positively identified by comparing to the retention time and spectrum obtained for caffeine standard.

Validation and comparison of analytical methods

The methods were validated taking account the guidelines of the main international agencies such as United States Pharmacopeia (USP),[59] European Commission,[60] International Union of Pure and Applied Chemistry (IUPAC),[61] Brazilian agencies such as National Health Surveillance Agency (ANVISA)[62] and National Institute of Metrology, Standardization and Industrial Quality (INMETRO),[63] as well as the considerations published by Ribani et al.[64] and Paschoal et al.[65]

The following validation characteristics were addressed: linearity, sensitivity, precision (intra and inter-day repeatabilities). Moreover, quantification of caffeine in 20 samples, analysis time, costs per analysis and generated organic waste were compared for both methods.

Linearity of both methods was tested in the range of 1 - 100 $mg.L^{-1}$ concentrations. The LOD and LOQ were estimated as three and ten times the signal-to-noise ratio, respectively. Intraday repeatability was determined through caffeine quantification in a same extract 8 times consecutively. Intermediate precision assays were performed analyzing this same sample 8 times in 5 different days.

The quantification of caffeine content was performed in twenty commercial samples, extracted in triplicate, using both methods. The results of each sample, obtained by CE and HPLC, were analyzed through analysis of variance (ANOVA, $P < 0.05$) to verify if there was difference among them, using software Statistica 7.0 (Statsoft, USA).

For comparison of analysis time, amount of used reagents, reagents costs and generated residues by the two methods employed, the following parameters were considered: capillary and column initial stabilization, injection of a calibration curve (7 concentrations, injected in triplicate), analysis of 10 samples (extracted in triplicate and injected three times) and capillary or column cleaning after the total sequence analysis (111 runs).

Moreover, it was also considered the waste treatment. For those residues that needed just neutralization, the treatment was performed at the laboratory. However, for those that needed incineration treatments, it was considered the

costs to sign out tercerized services. Reagents costs were calculated with basis in the mean of three budgets of Brazilian companies, except for ultrapure water, which costs were estimated from financial reports of the laboratory's water purification system. Sample extraction phase was not considered because it was the same for both methods.

Results and Discussion

Validation parameters

Both methods presented good linearity in the analyzed range. All correlation coefficients were better than 0.9997. Regression data for calibration plots are listed in Table 1.

Table 1. Quantitative parameters of the analysis obtained with CE and HPLC

Parameter	CE	HPLC
Linearity range ($mg.L^{-1}$)	1.0 – 100.0	1.0 – 100.0
Slope	0.0767	81,42
Intercept	-0.0194	-39.902
Correlation coefficient (r^2)	0.9997	0.9999
LOD ($mg.100.g^{-1}$) – S/N ratio 3:1	0.87	0.021
LOQ ($mg.100.g^{-1}$) – S/N ratio 10:1	2.88	0.071
Separation time (min)	5.2	6.5
Repeatability (RSD, %), n = 8	1.42	0.31
Intermediate precision (RSD, %), n = 5	1.96	1.53

The LOD for HPLC and CE were 0.021 and 0.87 mg of caffeine in 100 g of sample, respectively. The LOQ were 0.07 and 2.88 mg in 100 g, respectively. It was verified that HPLC sensitivity was about 42 times better than the obtained by CE, what is a great advantage of HPLC method. It is noteworthy that detectability of CE methods can be improved by means of on-line concentration techniques.[66-67] In this study, it was not necessary to employ these strategies, since sensibility obtained by CE was adequate for analyte quantification in the samples assayed, even being lower than for the HPLC.

In CE, optical path length and injected volumes were considerable smaller than for HPLC, leading to larger detection limits. In spite of that, the caffeine content present in the samples allowed their analysis by CE with safety.

The intraday precision, determined by repeated injections of the same sample (n = 8), was 1.42% for CE and 0.31% for HPLC. Intermediate precision evaluated over 5 days, was 1.96% for CE and 1.53% for HPLC.

HPLC chromatogram and CE electropherogram obtained from extracts of decaffeinated coffee are shown in Fig. 1. CE presented better selectivity than HPLC, since it was not observed interferents in the electrophoretic run.

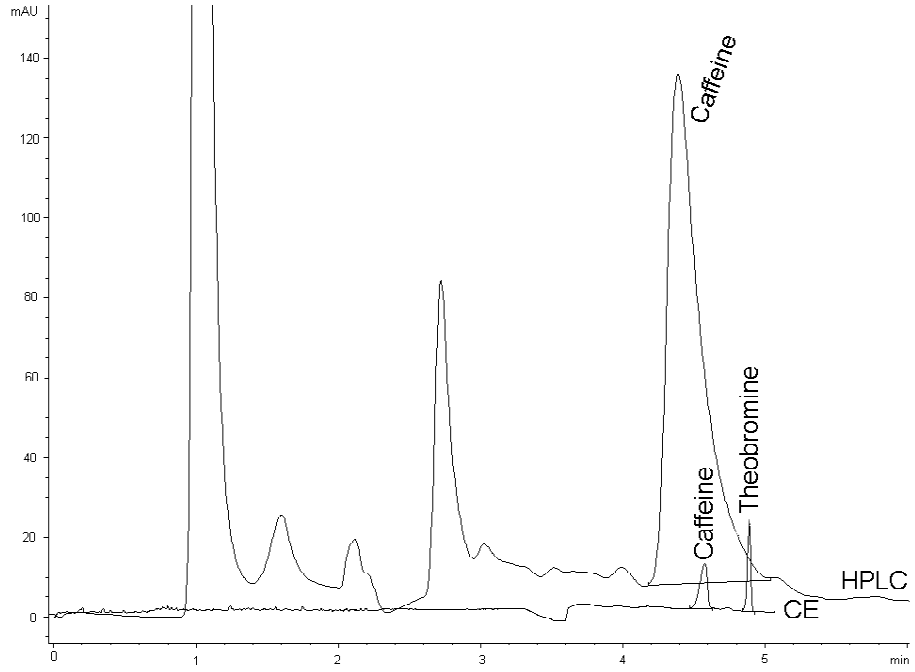

Figure 1. HPLC chromatogram and CE electropherogram overlapped for an extract of decaffeinated coffee. Analysis conditions are described in the text.

Sample analysis

In Table 2, caffeine content is presented for 20 samples of decaffeinated coffee, obtained by CE and HPLC methods. Through the analysis of variance, with 95% confidence level, there was not significant difference among the results for HPLC or CE. It could be verified that differences between the results obtained by the two methods exhibited randomized distribution. Moreover, it was possible to observe that three of the twenty samples analyzed presented caffeine levels above 0.1%, maximum limit allowed by the Brazilian legislation.[8]

Table 2. Caffeine quantification in the samples by HPLC and CE.

Sample	Concentration (mg 100 g^{-1}) ± SD[a]		*p*[b]	Difference (mg)
	HPLC	CE		
1	13.8 ± 0.35	13.6 ± 0.13	0.229	0.2
2	26.8 ± 0.49	26.3 ± 0.18	0.088	0.5
3	20.7 ± 0.40	20.2 ± 0.14	0.066	0.5
4	14.0 ± 0.30	13.5 ± 0.31	0.063	0.5
5	12.1 ± 0.20	12.5 ± 0.28	0.058	-0.4
6	14.0 ± 0.15	13.9 ± 0.04	0.326	0.1
7	21.6 ± 0.12	21.8 ± 0.32	0.230	-0.2
8	15.2 ± 0.27	15.4 ± 0.12	0.215	-0.2
9	47.1 ± 0.93	47.4 ± 0.66	0.607	-0.3
10	76.7 ± 1.27	75.5 ± 0.93	0.174	1.2
11	242.8 ± 0.21	242.5 ± 4.44	0.894	0.3
12	26.7 ± 0.46	26.4 ± 0.41	0.329	0.3
13	26.5 ± 0.43	26.6 ± 0.62	0.828	-0.1
14	15.8 ± 0.13	15.9 ± 0.08	0.175	-0.1
15	93.2 ± 0.71	92.7 ± 0.93	0.500	0.5
16	931.6 ± 0.77	930.9 ± 13.66	0.929	0.7
17	204.4 ± 0.74	201.8 ± 2.87	0.132	2.8
18	46.2 ± 0.77	45.4 ± 0.75	0.220	0.8
19	37.4 ± 0.35	37.4 ± 0.44	0.877	0.0
20	25.8 ± 0.22	26.0 ± 0.56	0.589	-0.2

[a]Standard deviation (n = 4).

[b]$p < 0.05$ indicates significantly differences between the results obtained by both methods.

Analysis time, reagents, costs and wastes

Table 3 presents the results of analysis time, use of solutions and reagents, costs and generated residues for the two techniques. The results of this

comparison referred only to the methods and equipments described in this work and to the costs of local market.

Table 3. Comparison of analysis time, use of reagents, costs and generated residues[a]

Description	HPLC	CE
Analysis time	14.6 hours	11.2 hours
Generated residues	Methanol, water: 602.5 mL	SDS[b], Na_2CO_3, NaOH, water: 18 mL
Residues treatment	Incineration	Neutralization and discard
Cost in reagents[c]	U$ 3.81	U$ 0.05

[a] For calibration curve and 10 samples (extracted and injected in triplicate)

[b] Sodium dodecylsulfate

[c] Mean of three budgets obtained in October 2008

HPLC method presented analysis time 30.4% higher than CE method. So, CE instrument stays available to perform a high number of analysis, optimizing laboratory work and improving analysts productivity.

Moreover, CE method employed non-toxic solutions, while HPLC method contain methanol in mobile phase. This solvent requires specific cares in its use, because it can be harmful to analyst health under prolonged exposition.[68-70]

The CE method generated a residual volume about 33 times lower than the HPLC method. Besides, the solutions used by CE just need neutralization before its discard in conformity with demands of Brazilian regulatory agencies.[71-73] Unlike that, methanol and water residues of HPLC demand incineration treatment. In this way, residues of analyses performed by the HPLC method demand larger storage

space, increase the costs of the laboratory with the incineration and the necessary human resources (there are many activities involving the withdrawal, transport and incineration stages) and, very important too, incineration affects the environment, that is one of the largest current discussions about the quality of future life.

The low consumption of solutions and reagents in CE was already verified in several other works and it is due to the low internal volume of the capillary and to the fact that the method allows to accomplish 20 electrophoretic runs with the same buffer vials. It is important to comment that already exist ultra-performance liquid chromatography equipments, where are used smaller columns and systems more compacted, reducing the used mobile phase volume and the organic residues generated, when compared to HPLC.[74,75]

In summary, HPLC method presented better sensitivity while in CE the analysis time and the generation of residues were smaller, which was also observed by other authors.[34,76,77]

Conclusions

Both analytic procedures presented good results. Moreover, for routine analyses in matrices where the analytes concentrations were in order of $mg.L^{-1}$, capillary electrophoresis method presented significant advantages over HPLC method.

Sensitivity, linear range, quantification limits and precision of the methods were satisfactory for the samples evaluated. Short analysis time and appropriate resolution were achieved for both techniques.

Organic solvent consumption by CE method was much lower than by HPLC, which is of great economic benefit for decaffeinated coffee analysis, although sensitivity was better for HPLC. CE seems to be more convenient for routine analysis, because in the long term it will be more cost-effective than HPLC.

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CAPÍTULO 4

ANÁLISE DE CAFEÍNA EM BEBIDAS ENERGÉTICAS POR CROMATOGRAFIA ELETROCINÉTICA MICELAR

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Análise de cafeína em bebidas energéticas por Cromatografia Eletrocinética Micelar

Resumo

As bebidas energéticas tornaram-se populares devido às suas propriedades estimulantes. Sua composição constitui-se de vitaminas, carboidratos, cafeína e taurina. O objetivo deste trabalho foi validar um método para determinação de cafeína nessas bebidas por cromatografia eletrocinética micelar (MEKC). Foram analisadas sete marcas, cada uma em três lotes. As amostras foram degaseificadas, filtradas e injetadas em sistema de eletroforese capilar com capilar de silica fundida (50 µm x 48 cm), a 30 kV durante 3,0 min, temperatura de 25ºC, injeção a 50 mbar por 5 s e detecção em 274 nm. O eletrólito consistiu em 50 mmol L^{-1} de dodecil sulfato de sódio (SDS) e 10 mmol.L^{-1} de carbonato de sódio (pH 11,0). O limite de detecção foi de 4,06 mg.L^{-1}, enquanto que o limite de quantificação foi de 13,55 mg.L^{-1}. Os coeficientes de variação para o teor de cafeína foram 1,36% (no dia) e 1,89% (entre dias). A recuperação em dois níveis de adição ficou entre 98,9 e 102,9%. A concentração média de cafeína variou entre 122,8 e 318,6 mg.L^{-1}. Considerando os intervalos de confiança (95%), verificou-se que 76% das amostras possuiam teor de cafeína menor que o informado aos consumidores.

Palavras-chave: cafeína; bebidas energéticas; validação; cromatografia eletrocinética micelar

Abstract

Energy drinks have gained popularity due to the attribution of energy-giving properties. Their composition is based on vitamins, carbohydrates, caffeine and taurine. The aim of this study was to validate a method for caffeine determination in energy drinks by micellar electrokinetic chromatography (MEKC). Seven brands of energy drinks, each one in three batches, were analyzed. The samples were degassed and filtered before injection into a capillary electrophoresis system. Separations were performed using uncoated fused-silica capillary (50 µm x 48 cm) at 30 kV during, 3.0 min temperature of 25ºC, injection at 50 mbar for 5 s, and detection at 274 nm. The running buffer consisted of 50 mmol.L^{-1} sodium dodecyl sulfate (SDS) and 10 mmol.L^{-1} sodium carbonate (pH 11.0). The limit of detection was 4.06 mg.L^{-1}, while the limit of quantification was 13.55 mg.L^{-1}. The relative standard deviation values for caffeine content were 1.36% (intra-day) and 1.89% (inter-days). Recovery in two spiking levels resulted between 98.9 and 102.9%. Mean caffeine concentration varied between 122.8 and 318.6 mg.L^{-1}. Considering confidence intervals (95%), it was found that 76% of the samples had caffeine content lower than the value informed to the consumer.

Keywords: caffeine; energy drinks; validation; micellar electrokinetic chromatography

1 Introdução

Desde seu lançamento, em 1987, as bebidas com adição de componentes estimulantes, mais conhecidas como bebidas energéticas, vêm ganhando mercado no Brasil e no mundo (CARVALHO et al., 2006; FINNEGAN, 2003; REISSIG et al, 2009). Essas bebidas são tipicamente compostas por cafeína, taurina e vitaminas, podendo ou não conter uma fonte de energia (carboidratos) e outras substâncias, e são comercializadas com o propósito de promover real ou perceptiva melhoria psicológica ou efeitos no desempenho (SAFEFOOD, 2002).

No Brasil, a Agência Nacional de Vigilância Sanitária (ANVISA) define "composto líquido pronto para o consumo" como sendo o "produto que contém como ingrediente(s) principal(is): inositol e ou glucoronolactona e ou taurina e ou cafeína, podendo ser adicionado de vitaminas e ou minerais até 100% da Ingestão Diária Recomendada (IDR) na porção do produto", sendo permitidas as expressões "bebida energética" ou "energy drink". A quantidade máxima de cafeína permitida para este tipo de produto é 35 mg/100 mL (ANVISA, 2005). Mesmo antes da atual legislação, o termo bebida energética já era comum na denominação destes produtos no Brasil, pois dentre os efeitos associados a estas bebidas estão reações como aumento da sensação subjetiva de alerta ou vigor, sensações relacionadas a um estado de "maior energia" e disposição no indivíduo (CARVALHO et al., 2006).

Devido a cafeína ser uma das substâncias mais largamente utilizadas em todo o mundo, os estudos sobre as implicações para a saúde resultantes do consumo desta substância são de interesse dos consumidores de alimentos,

bebidas e medicamentos (BORSTEL, 1983). A cafeína é um estimulante do sistema nervoso central e, dependendo em parte da quantidade consumida, pode produzir uma variedade de efeitos em outros órgãos (REISSIG et al, 2009). Uma pesquisa feita na Irlanda, em 2001, constatou que consumidores habituais de bebidas energéticas chegam a ingerir 8 latas numa única noite, associadas ou não a bebidas alcoólicas, correspondendo em média a cerca de 640 mg de cafeína. A ampla ingestão de cafeína por alguns consumidores é motivo para preocupação, particularmente em relação aos potenciais efeitos agudos da cafeína, como taquicardia, aumento da pressão sanguínea e desidratação, bem como efeitos cognitivos e comportamentais. Os efeitos do consumo crônico ou habitual de cafeína ainda não estão completamente elucidados (SAFEFOOD, 2002).

A eletroforese capilar (EC) é uma técnica de separação considerada rápida, eficiente, reprodutível e econonômica, utilizando pequeno volume de amostra e baixo consumo de solventes (RONDA et al., 2008). Esta técnica, particularmente a cromatografia eletrocinética micelar (MEKC), tem sido empregada com sucesso na determinação de metilxantinas em alimentos e produtos de origem vegetal (MEINHART et al., 2009; FRAZIER et al., 2000; WALKER et al., 1997; MAESO et al., 2006; THOMPSON et al., 1995)

O objetivo deste trabalho foi validar um método para determinação de cafeína em bebidas energéticas por cromatografia eletrocinética micelar (MEKC) e avaliar amostras comerciais dessas bebidas para verificar se o seu teor de cafeína está de acordo com o valor apresentado no rótulo.

2 Materiais e métodos

2.1 Amostras

As amostras analisadas consistiram de sete diferentes marcas de bebida energética, cada qual em três lotes, adquiridas no comércio local de Campinas.

2.2 Reagentes

O padrão de cafeína foi obtido da Sigma Chemical Co. (EUA). O carbonato de sódio foi da Synth (São Paulo, Brasil) e o dodecilsulfato de sódio (SDS) da Riedel-de-Haën (Alemanha). Em todos os experimentos, utilizou-se água ultrapura obtida em um sistema Direct-Q 3 UV (Millipore Corporation, França).

Todas as soluções foram degaseificadas por ultrassonicação (Microsonic SX-20, Arruda Ultra-sons LTDA, Brasil). Solução estoque de cafeína (1000 $mg.L^{-1}$) foi mantida sob refrigeração até o uso no preparo de soluções padrão.

2.3 Instrumentação

Os experimentos foram realizados utilizando um sistema de eletroforese capilar (EC) Agilent G1600AX (Agilent Technology, Alemanha), equipado com detector de arranjo de diodos. A separação ocorreu em uma coluna de sílica fundida da Agilent Technology (Alemanha), com comprimento total de 48 cm e 50 µm de diâmetro interno.

2.4 Preparo da amostra

Uma alíquota de cada amostra homogeneizada foi degaseificada por ultrassonicação durante 20 min e filtrada em membrana de 0,45 μm. As amostras foram então injetadas no sistema de eletroforese capilar. Todas as amostras foram analisadas em duplicata.

2.5 Eletroforese capilar

As análises foram realizadas de acordo com o método desenvolvido por MEINHART et al. (2010), com modificações para reduzir o tempo de corrida. Os parâmetros de operação de EC foram os seguintes: detecção a 274 nm, temperatura do capilar mantida em 25 ºC, voltagem de +30 kV e injeção hidrodinâmica de 50 mbar por 5 s.

O tampão consistiu em uma solução contendo 10 mmol.L^{-1} de carbonato de sódio e 50 mmol.L^{-1} de SDS. O pH resultante do tampão foi 11,0, propiciando uma boa separação no sistema de EC. Após filtração em membrana de 0,45 μm, o tampão foi centrifugado a 5000 rpm por 10 min, para remoção de bolhas de ar. O capilar foi lavado por 0,5 min com tampão entre as corridas. No início de cada dia, o capilar foi condicionado por 5 min com solução de hidróxido de sódio 1 mol.L^{-1} , 5 min com água ultrapura e finalmente 10 min com o eletrólito de corrida (tampão). O tempo de cada corrida eletroforética foi de 2,0 min.

A cafeína das amostras foi quantificada utilizando-se uma curva de calibração externa com sete pontos, em triplicata, na faixa de concentração de 50 a 450 mg.L^{-1}.

2.6 Validação

O método foi validado nos seguintes parâmetros: linearidade, limites de detecção e quantificação, repetibilidade, precisão intermediária e recuperação.

A linearidade foi testada na faixa de 15 a 3000 $mg.L^{-1}$. Os limites de detecção e quantificação foram estimados em três e dez vezes a relação sinal:ruído, respectivamente. A repetibilidade foi determinada por meio da quantificação de cafeína na mesma amostra por 10 vezes consecutivas. Os ensaios de precisão intermediária foram feitos analizando a mesma amostra em três dias diferentes.

Os testes de recuperação foram feitos adicionando-se padrão de cafeína em amostras de bebidas energéticas em dois níveis: 200,4 e 300,6 $mg.g^{-1}$, e comparando-se as concentrações da cafeína obtidas com a concentração original de cafeína amostra. Os testes de recuperação foram realizados em triplicata.

2.7 Aplicação em amostras de bebida energética

As amostras comerciais de bebida energética foram analisadas usando o método validado. O teor de cafeína das amostras foi avaliado através da análise variância (ANOVA) e as médias comparadas estatisticamente pelo teste de Tukey ($p < 0,05$), usando o programa Statistica 7.0 (Statsoft, USA).

3 Resultados e discussão

3.1 Validação

Na Tabela 1 são apresentadas as figuras de mérito do método. Pode-se observar que o método mostrou-se adequado para a análise de cafeína em bebidas energéticas, sendo linear na faixa de concentrações estudada e apresentando baixos coeficientes de variação.

Tabela 1. Parâmetros analíticos do método.

Parâmetro		**Valor**
Faixa de linearidade ($mg.L^{-1}$)		15,0 - 3000,0
Equação da reta		y = 0,2171 x + 3,5414
r^2		0,9998
Limite de detecção ($mg.L^{-1}$)		4,06
Limite de quantificação ($mg.L^{-1}$)		13,55
CV (%) intra-dias (n = 10)		1,18
CV (%) entre dias (n = 3)		1,89
Recuperação (%)	200,4 $mg.g^{-1}$	98,93
	300,6 $mg.g^{-1}$	102,85

CV: coeficiente de variação

3.2 Teor de cafeína nas amostras de bebida energética

A Figura 1 apresenta o eletroferograma de uma amostra de bebida energética.

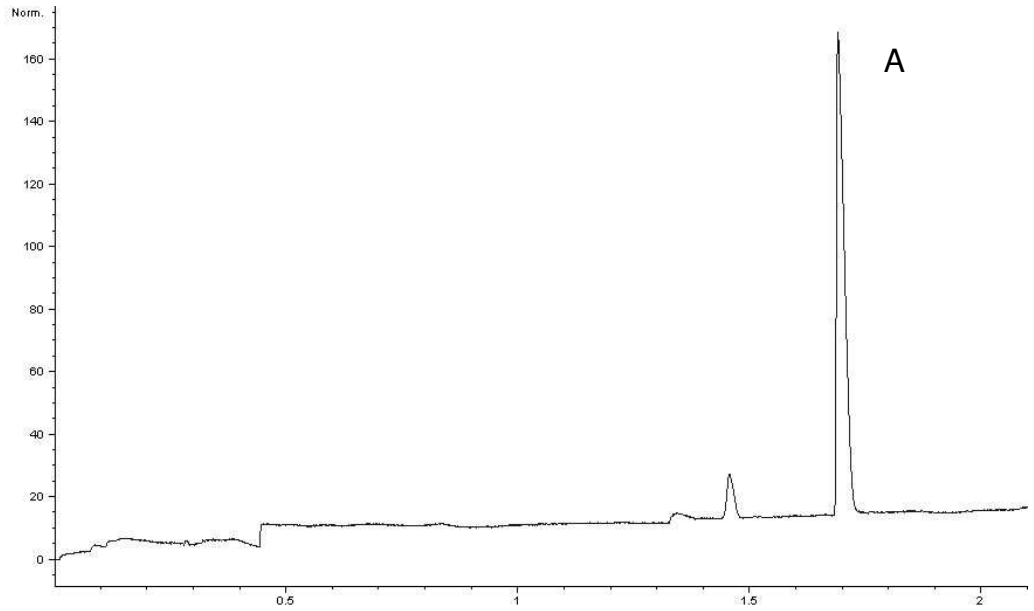

Figura 1. Eletroferograma da determinação de cafeína (A) em amostra de bebida energética. As condições eletroforéticas estão descritas no texto.

O conteúdo de cafeína nas amostras de bebida energética analisadas está apresentado na Tabela 2.

Tabela 2. Conteúdo médio de cafeína ($mg.L^{-1}$) das amostras de bebida energética analisadas.

Marca	**Lotes**			**Valor declarado**
	1	**2**	**3**	
A	267,8 ^{a} ± 2,13	272,6 ^{a} ± 3,21	271,0 ^{a} ± 1,75	320
B	256,8 ^{a} ± 1,18	276,0 ^{a} ± 5,93	263,9 ^{a} ± 8,86	320
C	115,4 ^{c} ± 1,59	130,3 ^{a} ± 0,84	122,9 ^{b} ± 0,17	148
D	327,7 ^{a} ± 2,21	295,9 ^{b} ± 2,25	315,5 ^{a} ± 6,02	320
E	288,5 ^{a,b} ± 1,79	273,2 ^{b} ± 3,51	291,6 ^{a} ± 6,02	320
F	279,0 ^{a} ± 2,44	267,5 ^{a} ± 5,86	289,7 ^{a} ± 11,20	320
G	309,7 ^{a} ± 3,15	320,7 ^{a} ± 3,57	325,4 ^{a} ± 9,34	320

Médias ± desvio padrão. Letras iguais, numa mesma linha, indicam que não há diferença significativa entre os lotes pelo teste de Tukey (95% de confiança).

Observa-se que, das sete marcas analisadas, três apresentam conteúdo de cafeína estatisticamente diferente (95% de confiança) entre os lotes. Além disso, calculando-se os intervalos de confiança (n = 3; t_2 = 4,303), apenas duas marcas (D e G) estão de acordo com o teor de cafeína apresentado no rótulo, e 76% das amostras (5 em 21) apresentaram quantidades menores que as informadas. Levando-se em consideração que a legislação brasileira (ANVISA, 2005) permite um máximo de 35 mg de cafeína por 100 mL do produto, todas as amostras estariam dentro do especificado. No entanto, segundo a Resolução RDC nº 360, da Agência Nacional de Vigilância Sanitária (ANVISA, 2003), é admitida uma tolerância de até 20% a mais com relação aos valores de nutrientes declarados no rótulo, não havendo especificação com relação à presença de quantidades menores.

Resultados similares foram encontrados por LAU et al. (1992), utilizando a espectrometria derivada segunda e cromatografia líquida de alta eficiência (CLAE), por MCCUSKER et al. (2006), utilizando cromatografia gasosa com detector nitrogênio-fósforo, e por ARANDA & MORLOCK (2007), que utilizou cromatografia planar acoplada à espectrometria de massas. O método por eletroforese capilar apresentado neste trabalho, no entanto, mostrou-se mais simples, rápido e econômico que estes, podendo ser utilizado no controle de qualidade de bebidas energéticas e outras bebidas contendo cafeína.

4 CONCLUSÃO

O método utilizado para a determinação de cafeína em bebidas energéticas apresentou resultados adequados para os parâmetros de validação, preparo de amostra simples, baixo tempo de corrida (2 min), uso de reagentes de baixo custo e em pequenas quantidades, além da pouca geração de resíduos. Essas características permitem que ele seja utilizado no controle de qualidade de indústrias produtoras deste tipo de bebida.

Com relação às amostras analisadas, apesar do teor de cafeína de todas elas estarem abaixo do limite máximo permitido na legislação brasileira para este tipo de bebida, a maioria (76%) apresentou uma quantidade deste composto estatisticamente inferior à declarada no rótulo do produto, desrespeitando o consumidor. A maioria das marcas apresentou uma boa homogeneidade no teor de cafeína entre os lotes analisados, o que pode ser um indicativo de controle de qualidade eficiente.

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CAPÍTULO 5

METHYLXANTHINES AND PHENOLICS CONTENT EXTRACTED DURING THE CONSUMPTION OF MATE (*ILEX PARAGUARIENSIS* ST. HIL) BEVERAGES

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Short Title: Methylxanthines and phenolics content in Mate beverages

Methylxanthines and Phenolics Content Extracted During the Consumption of Mate (*Ilex paraguariensis* St. Hil) Beverages

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ABSTRACT

"Chimarrao" and *"terere"* are popular beverages consumed in South America prepared using mate (*Ilex paraguariensis* St. Hil.). *"Chimarrao"* consists of a partial infusion where hot water is added, while *"terere"* is a total infusion, with addition of cold water. This study was designed to simulate preparation of these beverages for consumption, in order to estimate the total amount of xanthines and phenolic compounds in aqueous extracts that would be ingested by the consumer. Different commercial types of mate were employed for *"chimarrao"* preparation (native, smooth, traditional and course-ground) and these were compared to *"terere"*. In *"chimarrao"*, beverages from coarse-ground mate showed the highest levels of xanthines. However, *"terere"* presented quantities 2.5 times higher than the beverage of the coarse-ground mate. Considering the total phenolics in *"chimarrao"*, there was no difference between the types of herbs, but, in *"terere"*, the extraction of almost all of the phenolics was observed.

KEYWORDS: *Ilex paraguariensis* St. Hil; mate herb; *"chimarrao"*; *"terere"*; caffeine; theobromine; total phenolic compounds; capillary electrophoresis.

INTRODUCTION

Mate, *Ilex paraguariensis* St. Hilaire (Aquifoliaceae), is a South America native species and grows naturally in Brazil, Paraguay and Argentina *(1)*. About 80% of the occurrence area belongs to Brazil, especially in the states of São Paulo, Mato Grosso do Sul, Rio Grande do Sul, Santa Catarina and Paraná *(2)*. According to the Brazilian Institute of Geography and Statistics, Brazilian production of mate in 2007 was 438,474 tons *(3)* concentrated in the South region, due to the tradition of *"chimarrao"* consumption *(2)*. This crop has high social importance, since much of the production creates jobs and income to small farmers. Brazilian law defines mate as being composed exclusively by branches and leaves of *Ilex paraguariensis* St. Hil., obtained by fragmentation and drying process and used for the preparation of *"chimarrao"* or *"terere"*, to which sugar may be added *(4)*.

Mate is traditionally consumed as two different hot infusions, one with the simple addition of boiling water to the dry material of the plant (tea) and another by repeated additions of hot water (*"chimarrao"*) *(5)*. *"Chimarrao"* is prepared in a container made from a Porongo fruit and its called "gourd", or *cuia* in Portuguese, where mate fills 2/3 of its volume. The free volume is completed with hot water, forming a partial infusion (part of the mate stays dry). The resulting aqueous extract is sipped by the consumer through a metal straw known as a "*bomba*" (in Portuguese) or "*bombilla*" (in Spanish). The addition of water is repeated several times. In South America, approximately 30% of the population ingests more than 1 liter per day of these beverages *(5)*. Mate can also be consumed as *"terere"*, a cold

infusion, made with successive additions of cold water *(6)*. Mate for *"terere"* can be pure (containing only leaves and branches of the plant) or composed (added with other plant species, and may contain flavoring and/or sugar) *(4)*.

The processing from mate to *"chimarrao"* basically consists of three steps: scorching, drying and leaf grinding. The scorching is done with fire and comprises the rapid passage of the branches with leaves over the fire flames. The drying step may be performed in two types of mechanical dryers: rotary or belt. The main difference between the two types of dryers is related to the contact of the raw material with smoke during the drying process. After grinding, the herb is sieved and the final product is called ground herb. This can be used directly as raw material for tea production or, after going through a punch process, as herb for *"chimarrao" (7)*.

Chemical constituents found in mate include tannins, polyphenols, amino acids, saponins, alkaloids, and others *(8,9)*. Among alkaloids, methylxanthines (caffeine and theobromine) are highlighted. Caffeine in humans stimulates the central nervous system and the heart, besides enhancing brain activity and having diuretic action. However, the ingestion of high doses (10 to 15 mg/kg of body weight) can cause undesirable symptoms such as tremors, tachycardia, insomnia, irritability, anxiety, nausea and gastrointestinal discomfort *(10-14)*. Theobromine has diuretic and vasodilator effects, and is regarded as a cardiac stimulant *(15)*. It has been described that the content of methylxanthines in mate is extremely variable and indicates levels of caffeine between 0.16 and 1.4% and between 0.02 and 0.27% theobromine *(16-20)*.

Phenolic compounds commonly found in mate are related to derivatives of chlorogenic and caffeic acids, as well small amounts of flavonoids. Phenolic compounds may posses antimutagenic and antioxidant properties and they are therefore highlighted as important in preventing cancer, stroke and chronic degenerative diseases such as cardiovascular *(21,22)*. It was found (*23*) levels of 9.608% of phenolic derivatives in the dry extract of *Ilex paraguariensis*, especially chlorogenic and caffeic acids and some derivatives of them, and 0.064% of flavonoids rutin, quercetin and kaempferol.

Since *"chimarrao"* is a partial infusion, the fact that the matrix displays high levels of total phenolic and methylxanthines compounds does not necessarily means that the aqueous extract normally ingested by the consumer has the same levels. This study aimed to estimate the amount of caffeine, theobromine and phenolic compounds in aqueous extracts of *"chimarrao"* and *"terere"*, obtained in the same way as they are traditionally consumed. Furthermore, the comparison was made among different commercial types of mate usually employed for *"chimarrao"* preparation (native, smooth, traditional and course-ground) and these were compared to *"terere"*.

MATERIALS AND METHODS

Samples. Samples of mate herbs were acquired in a local mate industry in the Northwest region of Rio Grande do Sul state (Brazil). The herbs analyzed were those commercially known as smooth, traditional, native, course-ground and pure

"terere". **Table 1** describes the composition of each type of mate, as provided by the manufacturer. One package of each herb was used to prepare the beverages.

Table 1. Composition of mate herbs used in the experiments.

Composition	Smooth	Traditional	Native	Course-ground	*"Terere"*
Leaves of Native Plants	25%	39%	60%	41%	-
Leaves of Cultivated Plants	50%	53,5%	22,5%	46%	70%
Branches	15%	7,5%	17,5%	13%	30%
Sugar	10%	-	-	-	-

Apparatus. Determination of xanthines levels was conducted in a G1600AX (Agilent Technology, Germany) capillary electrophoresis system equipped with a diode array detector. The separation occurred in a 37 cm x 50 µm i.d. fused silica capillary (Agilent Technology, Germany). To determine the level of total phenolic compounds, a UV-160 spectrophotometer was used (Pro-Análise, Brazil).

Total xanthines in mate. To assess total xanthines content in mate used for the preparation of *"chimarrao"*, the extraction was carried out based on the study from *(24)*. The sample was ground in a 100 mesh Marconi TE 600 (São Paulo, Brazil) mill. After milling, one gram of the sample was added to 150 mL of water alkalinized with NaOH (pH 11.0). The mixture was kept boiling for 1 hour. After cooling, the volume was set to 200 mL and the sample was filtered. The extract was analyzed by capillary electrophoresis. The extraction method was evaluated by repeatability (n = 10) and recovery (with the addition of 350 and 700 mg of caffeine and 150 and 300 mg of theobromine per 100 g of sample).

Total phenolic in mate. The extraction of phenolic compounds was performed based on the method described in *(25)*. After milling, 20 g of the sample were placed in 100 mL of methanol and kept under constant stirring for 3 hours. The extract was filtered and the solid residue was taken back to shaking with 100 mL of methanol for another 1 hour. The methanolic extracts were evaporated to dryness in rotary evaporator at 38 ± 1 °C and stored under temperature of -18 °C. Before analysis the extract was resuspended in methanol (0.3 mg of extract per mL of methanol).

***"Chimarrao"* preparation.** 85 g of each mate (smooth, traditional, native and course-ground) were weighed in a medium size gourd. This amount is approximately two-thirds the size of the gourd, following the method of preparation indicated on the product label. Mate was packed as shown in **Figure 1B**. Then, 150 mL of water at 75 ± 2 °C was added and, after 30 seconds, the liquid was extracted with the aid of a vacuum pump coupled with the *"chimarrao" bomba* (**Figure 1A**).

After a 2-min interval, the gourd was filled up again with 110 ± 10 mL of hot water and the liquid was extracted again. The gourd was supplemented with water 28 more times, always within a 2-min interval, thus simulating 30 aqueous extracts of *"chimarrao"* drunk successively by consumers. In general, after 30 water additions, mate beverages lose its flavor, and then, a new beverage must be prepared. During the experiments, care was taken to not move the mate, keeping it packed inside the gourd at the same way until the 30th extraction.

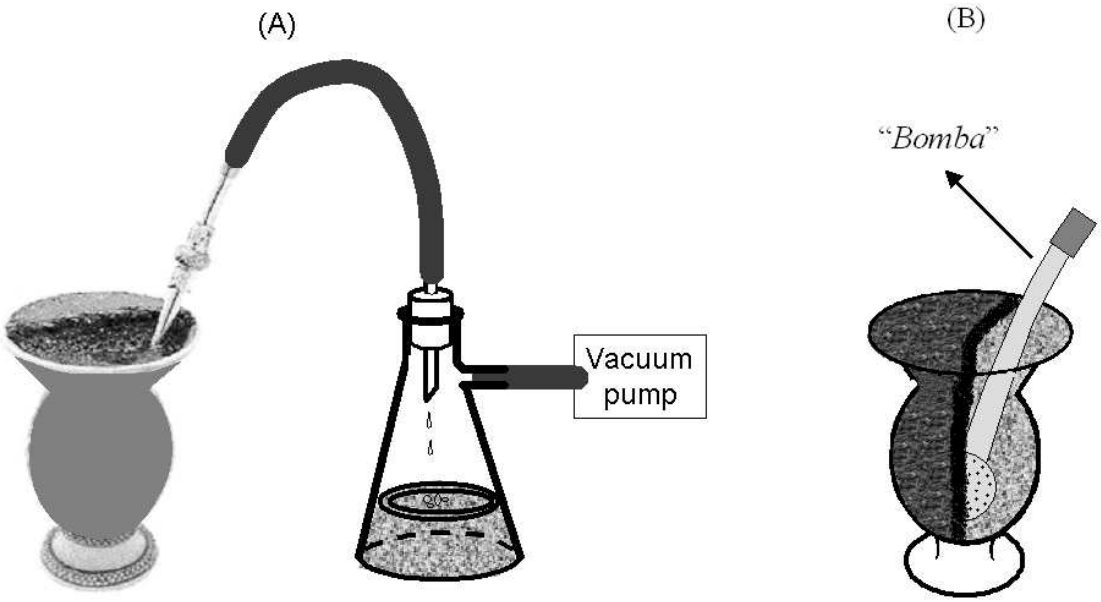

Figure 1. *"Chimarrao"* aqueous extracts obtention (A) and internal view of *"chimarrao"* gourd packed with mate herb (B).

For *"terere"* extraction, 50 g were weighed into a glass. In this case, 180 mL of cold water was added at a temperature of 11 ± 2 ºC, until complete herb infusion has occurred (30 seconds). Like *"chimarrao"*, *"terere"* was extracted 30 times, always within a 2-min interval, adding 100 ± 5 mL of cold water in each subsequent extraction.

In all tests, it was used a metal straw ("*bomba*", **Figure 1B**) with a sieve of 3.5 cm in diameter, 20 cm distant from the nozzle. The sieve contained 340 holes on each side with a 1 mm diameter each. For each type of mate studied,

preparation of either *"chimarrao"* or *"terere"* was conducted in triplicate and, in each replicate, 30 additions of water were followed by vacuum extraction. Each extract had its volume recorded and reserved for phenolic and xanthines analysis (also in triplicate). It was analyzed all the extracts separately from the 1st to the 6th, and then, each one within a 4-extracts interval, until the 30th.

Analysis of caffeine and theobromine content by capillary electrophoresis. The extracts for total xanthines content of mate leaves, as well as the aqueous extracts of each *"chimarrao"* and "*terere*", were filtered and analyzed by capillary electrophoresis. The method was based on *(26)*. Electrolyte composition was 50 mmol/L of sodium dodecyl sulfate and 10 mmol/L sodium carbonate (buffered in pH 11.0). The separation was performed at 25 ℃ with voltage of 30 kV, injection of 50 mbar for 7 seconds and detection at 206 nm. The method was validated for linearity, repeatability (n = 10), intermediate precision (n = 3), limits of detection and quantification.

Analysis of total phenolic content. Phenolic compounds were evaluated according to the method described in *(27)* with modifications, using the Folin-Ciocalteu reagent. Briefly, 0.5 mL of each methanolic extract (to determine the total phenolic content present in mate leaves) and of the aqueous extracts of *"chimarrao"* were taken in test tubes, then added with 2.5 mL of the Folin-Ciocalteu reagent 10% in distilled water. After 5 minutes, 2 mL of sodium carbonate 7.5% solution were added. The tubes were kept for 2 hours protected from light. The absorbance was measured in spectrophotometer at 740 nm. A blank sample was carried out under the same conditions, replacing the extract by the same amount of

solvent. An external calibration curve with gallic acid solutions between 0.003 and 0.100 mg/mL (R^2 = 0.998) was used. The result was expressed in g of gallic acid equivalents (g GAE).

Statistical analysis. The results obtained for caffeine, theobromine and total phenolic compounds content present in the matrices were subjected to analysis of variance (ANOVA) and Tukey test, both with 95% confidence, using the software Statistica 7.0 (Statsoft, USA).

RESULTS AND DISCUSSION

Method validation. The method for extraction of total xanthines present in the matrix showed good repeatability, obtaining a relative standard deviation (RSD) of 2.40% for caffeine and 2.57% for theobromine. Recovery in the two levels studied was 103.7 and 101.0% for caffeine and 101.4% and 99.8% for theobromine. Separation and quantification of caffeine and theobromine were made by capillary electrophoresis. The method was validated for repeatability, where it was observed RSD values of 1.29% for caffeine and 1.88% for theobromine, in ten measurements made on the same day. In the determination of intermediate precision (3 days), the RSD values were 0.93 and 2.95% for caffeine and theobromine, respectively. The method proved to be linear in the ranges from 5 to 200 mg of caffeine/L and 2 to 100 mg theobromine/L (R^2 > 0.998). The method allowed the separation of compounds in 2.7 minutes. The detection limits (3 times S/N - signal/noise) were 0.103 mg/100 g of sample for caffeine and 0.037 mg/100

g for theobromine. The limits of quantification (10 S/N) were 0.342 mg/100 g of sample for caffeine and 0.123 mg/100 g for theobromine.

Methylxanthines and phenolic content in mate leaves. Table 2 shows the total content of caffeine, theobromine and phenolics present in mate leaves used to prepare *"chimarrao"* and *"terere"*, obtained after thorough extraction of the compounds.

Table 2. Total content of caffeine, theobromine and phenolic compounds present in mate leaves, obtained after thorough extraction of the compounds.

Mate herb	Caffeine (g/100 g)[a]	Theobromine (g/100 g)	Phenolic compounds (g GAE/100 g)[b]
Native	0.83 ± 0.01 [b]	0.203 ± 0.003 [a]	5.18 ± 0.30 [a]
Traditional	0.66 ± 0.02 [c]	0.177 ± 0.003 [c]	5.22 ± 0.31 [a]
Smooth	0.61 ± 0.02 [c]	0.166 ± 0.004 [d]	4.41 ± 0.19 [ab]
Course-ground	0.79 ± 0.03 [b]	0.190 ± 0.003 [b]	4.92 ± 0.59 [ab]
Terere	1.02 ± 0.02 [a]	0.212 ± 0.006 [a]	4.18 ± 0.09 [b]

[a]Mean ± Standard Deviation (n = 3)

[b]GAE = Gallic Acid Equivalents

Same letters in a row indicate there is no difference statistically significant (95% confidence level), considering Tukey test.

Caffeine content was higher in *"terere"* mate, followed by native and coarse-ground, which values showed no significant difference. Herbs that contained the lowest amounts were the traditional and smooth. Both *"terere"* and native herb showed the highest contents of theobromine, followed by coarse-ground, traditional and smooth mate. It was observed that native and traditional mate herbs showed the highest levels of total phenolic compounds, with statistically similar values of

about 5.2 g GAE/100 g of sample. For coarse-ground, smooth and *"terere"* mate herbs, levels were similar, being 4.9, 4.4 and 4.2 g GAE/100 g of sample, respectively.

Methylxanthines and phenolic content in mate beverages. Figure 2 shows the amounts of caffeine, theobromine and phenolic compounds in aqueous extracts of *"chimarrao"* and *"terere"* that would normally be ingested by the consumer of these beverages. The extracts from native mate *"chimarrao"* had, in average, volumes of 95 ± 2.6 mL, those of the traditional 92 ± 2.3 mL, smooth 115 ± 5.2 mL, coarse-ground 67 ± 6.5 mL and 100 ± 10 mL of the *"terere"*. Among different types of mate herbs used to prepare *"chimarrao"*, it was observed that extracts from native, traditional and smooth mate showed similar levels of caffeine and theobromine (**Figures 2A** and **2B**). Extracts of coarse-ground mate had the greatest levels of xanthines, in such a way that the 30th mate extract still contained about 4.0 mg of caffeine and 1.0 mg of theobromine.

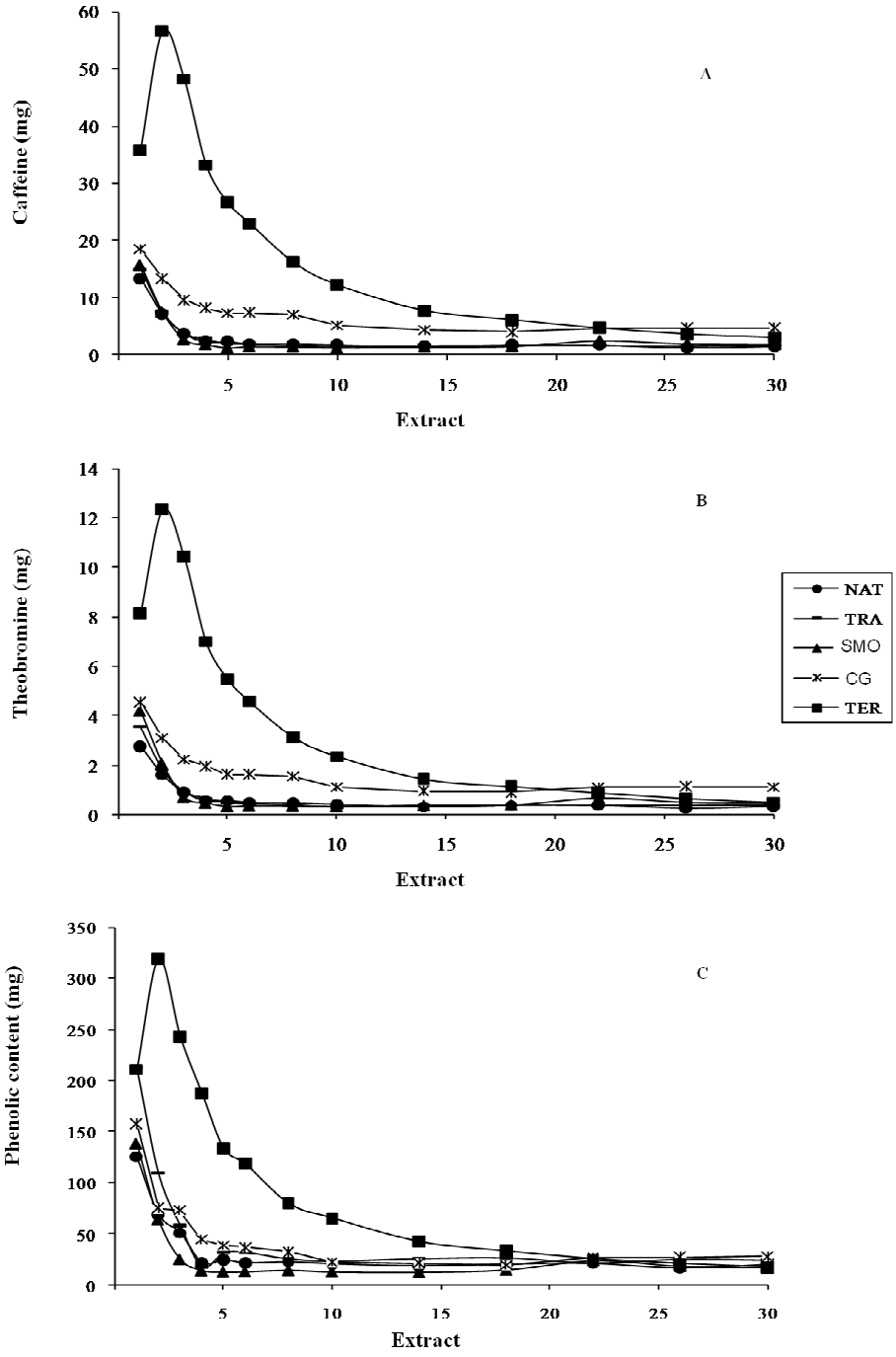

Figure 2. Caffeine (A), theobromine (B) and total phenolic compounds (C) content present in aqueous extracts from *"chimarrao"* and *"terere"*. NAT: native; TRA: traditional; SMO: smooth; CG: course-ground; TER: *"terere"*.

During the withdrawal of the *"chimarrao"* extracts, it was noted that the volume of water added to the gourd were similar for all mate. However, the coarse-ground extracted volumes were lower, indicating that there was a retention of greater amounts of water. *"Terere"* beverage showed levels much higher of methylxanthines and phenolic compounds when compared to the extracts from *"chimarrao"*. It was observed that in the 2nd and 3rd extracts, there was an increase in levels of all the studied compounds in relation to the first extract, probably due to the fact that leaves and branches of *"terere"* mate were not completely immersed during the first extract, while in the second, and after, the mate was completely submerged in water. From the 4th to 10th extract, the levels still remained high, and after the 20th extract a reduction in the content was observed, close to those found in *"chimarrao"* extract of the coarse-ground mate.

Considering total amount of caffeine in the 30 aqueous extracts from *"terere"* beverage, it showed average levels that were 2.5 times higher when compared to the amount found in the *"chimarrao"* extraction of coarse-ground mate. Compared to extractions made with native, traditional and smooth mate herbs, *"terere"* showed levels 6 times higher. The extraction of higher levels in the beverage with cold water could be correlated with the complete infusion of mate, as in hot beverages (*"chimarrao"*) the water is partially in contact with the herb. For total phenolic compounds (**Figure 2C**), it was found that among beverages with hot water, extracts from the traditional mate showed higher content, followed by extracts of coarse-ground, smooth and native mate herbs.

Considering *"terere"* beverage, the first extract showed 210 mg of GAE, a value slightly higher than the obtained in the first extraction of traditional mate herb. But in the second extract, 319 mg GAE were extracted; the third were 243 mg, the fourth 187 mg, fifth and sixth were 133 and 118 mg GAE, respectively. After the eighth extract, values declined from 79 to 17 mg GAE in the thirtieth. The *"terere"* extracts showed greater content of total phenolics, being higher than all the hot beverages extracts, so that the 8th aqueous extract of *"terere"* (79 mg GAE) showed higher amount of phenolics than the 3rd extract of any *"chimarrao"*. Similarly to what occurred with xanthines, this greater efficiency of extraction may be related to the fact that in *"terere"* water is thoroughly in contact with the herb.

Percentage extracted from the matrices. Considering the total amount of xanthines and phenolic compounds present in the whole mate herbs put into the gourd, **Figure 3** shows the percentage extracted at the successive additions of water. It is possible to see that percentages of theobromine and caffeine extracted are similar, indicating that both xanthines are equally solubilized during beverage consumption. It was observed that, despite of native mate presented a superior amount of total xanthines when compared to the traditional and smooth mate herbs, it was not reflected in the percentage of caffeine and theobromine extracted.

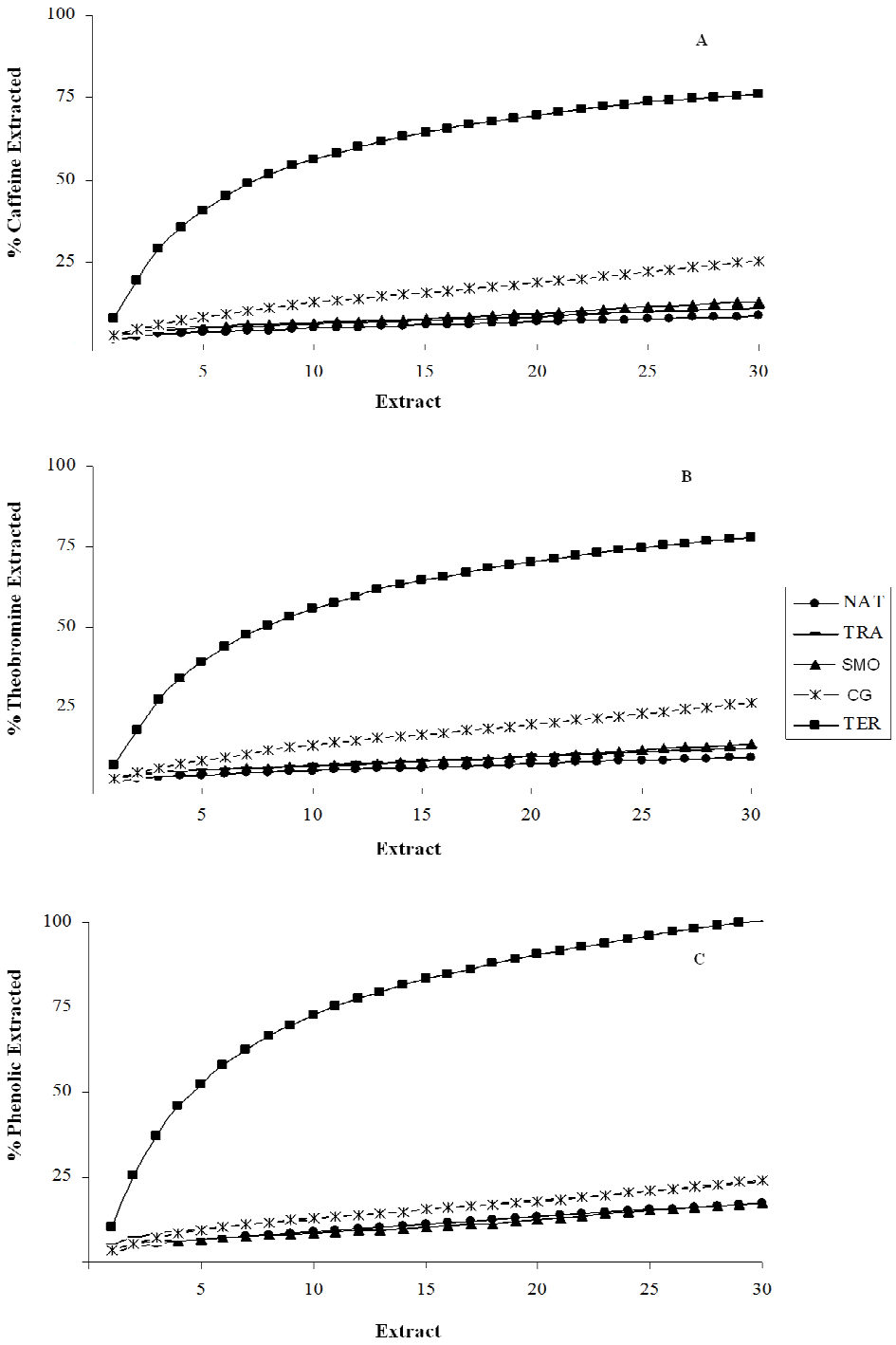

Figure 3. Accumulated percentage of caffeine (A), theobromine (B), and total phenolic compounds (C) extracted during successive water additions. NAT: native; TRA: traditional; SMO: smooth; CG: course-ground; TER: *"terere"*.

The graphs (**Figures 3A** and **3B**) showed close percentages of extraction for the extracts from these mate herbs. Along the 30 aqueous extracts, 9.0% of the total xanthine content were extracted from native mate herb in the gourd, 11.7% from the traditional and 13.3% from the smooth. It is worth mentioning that the first four *"chimarrao"* extracts removed 3.5% of the total caffeine and theobromine content present in the native mate herb, 4.9% in the traditional and 5.3% in the smooth. In extracts of coarse-ground mate *"chimarrao"*, the percentage extracted from the beverage was greater (up to three times) than in other matrices used for hot beverages. Until the fourth mate extract, 7.3% of total caffeine and theobromine were extracted, and until the 30th, 26.0% were removed. This fact could be correlated with better permeability of water in this type of mate herb.

Comparing *"terere"* to *"chimarrao"*, a much higher percentage of extraction was observed for the first beverage. It could be noted that in the fourth *"terere"* extract, 34.8% of total caffeine and theobromine had been extracted and, until 30th, 77.1% had been extracted. It can be observed in **Figure 3** that the phenolic compounds are more efficiently extracted than caffeine and theobromine. For the *"terere"* mate herb, the content of phenolic compounds present in the matrix was the lowest when compared to other mate herbs. However, as observed for caffeine and theobromine, the percentage extracted was higher in this beverage.

Among the native, traditional, smooth and coarse-ground mate herbs, it was observed that extracts of traditional and coarse-ground showed the highest percentages extracted. Over the 30 aqueous extracts, 17.5% of total phenolics present in the matrix from native and smooth mate herbs were extracted, 23.6%

from the traditional mate and 24.23% from the coarse-ground. In *"terere"* it was possible to extract almost all the total phenolic compounds, considering that until 4th extract, 45% had already been extracted. Over the 30 aqueous extracts, approximately 98% of total phenolic compounds were extracted from the herb.

Comparison with other foods and beverages. Since many consumers ingest *"chimarrao"* extracts, a comparison was made between the content of these compounds present in extracts and in other foods or drinks. In the case of mate herbs that showed similar levels in the analyzed extracts, a mean was calculated for comparison (**Table 3**). It was found that the ingestion of the extract from the first *"chimarrao"* of native, traditional or smooth mate herbs, corresponds to caffeine content found in approximately 16 mL of espresso coffee *(28)*. No comparisons were made for the subsequent extracts, because the content extracted was very low when compared to the four first extracts.

By comparison with published studies, it was possible to observe that the 4th *"chimarrao"* extract prepared with coarse-ground mate herb had large amounts of caffeine, similar to those in 10 mL of espresso (*28*), 118 mL of cola type soft drink (*29*), 34 mL of energy drink (*29*) or 0.5 g of guarana powder (*30*). The amount of theobromine in this extract is equivalent to 6.2 grams of milk chocolate (*31*). Besides, the total phenolic compounds present correspond to 45.6 mL of red grape juice (*32*), 11.7 mL of red wine (*33*), 21 mL of green tea infusion (*34*) or 37 mL of orange juice (*34*).

Table 3. Xanthines and phenolic compounds content present in mate beverages.

Caffeine					
Aqueous Extract	*"Chimarrao"* (mg/100 mL)				*"Terere"* (mg/100 mL)
	Native	Traditional	Smooth	Course-ground	
1st	14.0	16.6	13.7	26.4	35.8
2nd	7.5	8.3	6.5	19.0	56.5
3rd	3.9	4.2	2.3	13.6	48.2
4th	2.3	3.0	1.6	11.6	33.2
10th	1.8	1.7	1.0	7.3	12.2
18th	1.8	1.8	1.3	5.7	6.1
30th	1.5	1.8	1.6	6.6	2.9
Theobromine					
Aqueous Extract	*"Chimarrao"* (mg/100 mL)				*"Terere"* (mg/100 mL)
	Native	Traditional	Smooth	Course-ground	
1st	2.8	3.9	3.7	6.4	8.2
2nd	1.7	2.0	1.8	4.4	12.3
3rd	0.9	1.0	0.6	3.1	10.4
4th	0.5	0.8	0.4	2.9	7.0
10th	0.4	0.3	0.3	1.6	2.4
18th	0.4	0.4	0.3	1.3	1.1
30th	0.3	0.4	0.3	1.6	0.5
Phenolic Compounds					
Aqueous Extract	*"Chimarrao"* (mg/100 mL)				*"Terere"* (mg/100 mL)
	Native	Traditional	Smooth	Course-ground	
1st	131.9	235.6	120.5	225.7	235.3
2nd	72.2	121.1	55.5	107.1	350.0
3rd	53.3	64.4	21.9	104.3	268.4
4th	22.3	20.0	12.3	62.9	208.5
10th	21.1	25.6	11.0	31.4	72.3
18th	20.7	29.2	12.8	27.0	36.3
30th	20.4	26.7	14.5	38.6	19.7

Likewise, the 4th *"terere"* aqueous extract is similar in amounts of caffeine as those shown in 24 mL of espresso (*28*), 277 mL of cola type soft drink (*29*), 80 mL of energy drinks (*29*) or 1.1 g of guarana powder (*30*). Theobromine content corresponds to 14.5 g of milk chocolate (*31*). The phenolic compounds present in

the fourth extract of *"terere"* can also be found in 252 mL of red grape juice (*32*), 65 mL of red wine (*33*), 115 mL of green tea infusion (*34*) or 205 mL orange juice (*34*).

To summarize, the capillary electrophoresis method used to determine caffeine and theobromine showed validation parameters adequate to obtain a reliable quantification of these compounds**.** Considering the total content of xanthines and phenolic compounds present in different matrices, the mate herb used to prepare *"terere"* showed the highest levels of caffeine and theobromine, while the traditional mate herb (used for *"chimarrao"*) exhibited the greatest amount of total phenolic compounds.

In the aqueous extracts obtained by simulating the consumption of *"chimarrao"*, the highest levels of xanthines were found in coarse-ground mate herb. For *"terere"*, levels of xanthines extracted were higher than all the *"chimarrao"* extracts. Considering the sum of caffeine amount in the 30 aqueous extracts, *"terere"* beverage showed about 2.5 times more caffeine than the amount found for the coarse-ground mate herb.

The aqueous extracts of *"chimarrao"* made with traditional mate herb showed the highest amount of total phenolic compounds, but the extracted levels were much higher for *"terere"* beverage. For extraction percentage, *"terere"* again exhibited the highest values, since after thirty extractions almost all the phenolic compounds have been removed. Moreover, it could be noted that the phenolic compounds were extracted more efficiently than xanthines, for both beverages.

"Chimarao" had moderate levels of caffeine and theobromine, and relatively high amounts of total phenolic compounds, which makes it an interesting source

for the intake of phenolic compounds in human diet. Extracts from *"terere"* showed even greater levels of phenolics, with significant amounts of caffeine and theobromine. *"Terere"* consumption, besides providing significantly higher amounts of methylxanthines and phenolic compounds than *"chimarrao"*, could prevents the negative effects associated to repetitive ingestion of this very hot drink on the development of oroesophagic cancer.

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CONCLUSÕES GERAIS

O método para análise de metilxantinas por MECK desenvolvido neste estudo mostrou-se simples, direto, rápido e robusto. O planejamento compostos central foi utilizado com sucesso na otimização da composição do eletrólito de corrida e voltagem.

As principais vantagens do método desenvolvido foram a boa estabilidade do pH do tampão e a baixa geração de resíduos, o que permite a sua aplicação rotineira em laboratório de análise e indústrias.

Por meio da otimização das condições de extração de cafeína em café descafeinado, foi possível remover os interferentes da matriz que prejudicavam a resolução do pico e impossibilitavam a quantificação desta xantina.

No que se refere à comparação de métodos, tanto o método por EC quanto o método por CLAE apresentaram parâmetros de validação satisfatórios. Além disso, para análises de rotina em matrizes cuja concentração de cafeína esteja na ordem de $mg.L^{-1}$, a eletroforese capilar apresentou algumas vantagens em relação ao método por CLAE. O consumo de solventes orgânicos foi muito menor em EC do que por HPLC, embora a sensibilidade desta última técnica tenha sido melhor. A EC pode ser mais vantajosa na análise de rotina, pois a longo prazo apresenta uma melhor relação custo-benefício que a CLAE.

As condições ótimas foram aplicadas com sucesso na quantificação de cafeína em 45 amostras de café descafeinado, das quais sete, de quatro marcas diferentes, apresentaram níveis de cafeína superiores ao estabelecido pela legislação brasileira. Com relação às amostras de bebidas energéticas analisadas,

76% apresentaram um conteúdo de cafeína estatisticamente menor do que o informado no rótulo do produto.

Considerando o conteúdo total de xantinas presentes nos diferentes tipos comerciais de erva-mate estudados, a erva-mate para o preparo de tererê apresentou o maior teor de cafeína e teobromina, enquanto que a erva do tipo tradicional, usada no preparo do chimarrão, exibiu as maiores quantidades de compostos fenólicos totais. Nos extratos aquosos obtidos simulando o preparo de chimarrão, o maior conteúdo de xantinas foi encontrado na erva-moída grossa. No entanto, extratos de tererê possuíam cerca de 2,5 vezes mais destas substâncias, considerando a soma de todos os 230 extratos aquosos para cada bebida.

O chimarrão e o tererê possuem níveis moderados de cafeína e teobromina, e quantidade relativamente altas de compostos fenólicos, o que os torna uma fonte interessante desses últimos na dieta humana.