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APLICAÇÕES DE CE-DAD E HPLC-DAD-ESI/MS NA DETERMINAÇÃO DE COMPOSTOS FENÓLICOS, METILXANTINAS E ÁCIDOS ORGÂNICOS EM BEBIDAS

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RESUMO GERAL

Considerando que os artigos de pesquisa desta tese já estão em inglês e na forma para serem enviados aos periódicos internacionais, nos quais os resumos estão restritos a 100 ou 200 palavras, nesta seção, os resumos estão apresentados com maiores detalhes para uma apreciação melhor dos trabalhos e dos seus resultados. Devido à grande abrangência dos trabalhos, e para facilitar a leitura, os resumos estão apresentados em parágrafos separados, em vez de um único parágrafo.

Os chás verde e preto (*Camellia sinensis*) são bebidas muito consumidas mundialmente. Na última década, estas bebidas têm sido alvo de investigação intensa devido a seus possíveis efeitos benéficos à saúde, atribuídos aos compostos fenólicos. Catequinas e teaflavinas são os fenólicos mais importantes em chá verde e chá preto, respectivamente. Têm sido também atribuídas a infusões de chá mate (*Ilex paraguariensis*), bebidas bastante consumidas no Brasil, atividades biológicas promotoras da saúde, possivelmente devido aos compostos derivados cafeoilquínicos e outros fenólicos. Portanto, o Capítulo 1 é um artigo de revisão que discute os avanços na instrumentação e metodologia analítica aplicadas aos compostos fenólicos em chás. Técnicas analíticas modernas, como cromatografia líquida de alta eficiência, espectrometria de massas e eletroforese capilar têm fornecido dados importantes sobre a composição destes compostos nas bebidas mencionadas.

Compostos fenólicos ocorrem abundantemente em vinhos e têm sido alvo de inúmeras pesquisas científicas nos últimos anos devido aos supostos benefícios à saúde. Ácidos orgânicos exercem influência direta na qualidade e propriedades sensoriais de vinhos, bem como na sua estabilidade e controle microbiológico. A determinação de ambos

compostos é, portanto, de extrema importância. Uma revisão da literatura (Capítulo 2) mostra que a eletroforese capilar vem substituindo gradativamente a cromatografia líquida de alta eficiência com vantagens de baixo custo operacional, baixo consumo de solventes, rapidez, simplificação no preparo das amostras, etc. Com fenólicos, a eletroforese capilar tem sido utilizada com sucesso para a determinação de isômeros do resveratrol, ácidos fenólicos e flavonóides. Em ácidos orgânicos, sua utilização se dá nos modos de detecção direta e indireta.

"Chimarrão" e "tererê" são infusões de erva mate (*Ilex paraguariensis*) largamente consumidas na América do Sul. O artigo do Capítulo 3 descreve a aplicação de HPLC-DAD-ESI/MS na identificação e quantificação de ácidos cafeoilquínicos (CQA), glicosídeos de flavonóis e purina alcalóides nestas bebidas. Infusões foram preparadas de amostras comerciais de *Ilex paraguarienses* do Sul do Brasil. Os ácidos cafeoilquínicos, 4,5-diCQA, 3-CQA, 5-CQA e 4-CQA foram os principais compostos, 238-289, 153-242, 183-263 e 123-188 μg/mL, respectivamente, em chimarrão, e 206-265, 122-218, 164-209, 103-169 μg/mL, respectivamente, em tererê. Cafeína também foi encontrada em altas quantidades. Os glicosídeos de quercetina e kaempferol estavam presentes em níveis baixos. A atividade antioxidante individual foi determinada por um sistema "on-line" que mede a habilidade da substância sequestrar o radical $ABTS^{\cdot+}$, revelando que a capacidade antioxidante não foi proporcional às concentrações dos compostos fenólicos. 3-*O*-CQA, quercetina-3-*O*-ramnosilglucosídeo e quercetina-3-*O*-glucosídeo tiveram maior contribuição para a capacidade antioxidante, embora os glicosídeos de quercetina estavam em teores mais de 10 vezes menores que o 3-*O*-CQA.

O Capítulo 4 teve dois objetivos: (1) comparar a quantificação de flavonóis nas formas glicosidicas e agliconas, e (2) comparar a composição de fenólicos e metilxantinas

de mates retiradas em dois pontos da produção e entre as infusões chimarrão e tererê. O método quantitativo utilizado para as agliconas obtidas por hidrólise e aquele usado para glicosídeos, outros fenólicos e metilxantinas tiveram bom desempenho, medido em termos de linearidade, limites de detecção e quantificação, recuperação e repetitividade. Para as 13 amostras analisadas, nas duas infusões, resultados equivalentes foram obtidos para quercetina e kaempferol, nas formas glicosídicas e agliconas. Os teores dos analitos foram todos maiores no chimarrão em comparação ao tererê. Em ambas infuses, os níveis de fenólicos e metilxantinas foram significativamente maiores nos mates obtidos diretamente dos produtores que nas amostras comerciais, indicando que perdas ocorreram nas etapas adicionais do processamento e durante a estocagem das amostras obtidas diretamente dos produtores.

Um método rápido por RF-MEKC modificado com β-ciclodextrina sulfatada foi desenvolvido e validado para a determinação de cinco catequinas em chá verde (Capítulo 5). O eletrólito otimizado consistiu de 0.2% trietilamina, 50 mmol/L SDS e 0.8% s-ß-CD (pH=2,9). Os padrões e as amostras foram injetados em 0,6 psi por 5 segundos sob voltagem constante de -30 kV. O preparo da amostra envolveu a extração de 2g de chá com 200mL de água a 95^{o}C sob agitação constante por 5 min. O método demonstrou excelente desempenho com limites de detecção e quantificação de 0,02 a 0,1 μg/L e 0.1 a 0,5 μg/mL, respectivamente, e recuperação de 94 a 101%. O método foi utilizado para analisar infusões de chás verdes comercializados no Brasil. Epigalocatequina galato (23,4-112,4 μg/mL) foi o principal componente, seguido por Epigalocatequina (18,4-78,9 μg/mL), Epicatequina galato (5,6-29,6 μg/mL), Epicatequina (4,6-14,5 μg/mL) e Catequina (3,2-8,2 μg/mL).

Foi desenvolvido e avaliado um método para determinação simultânea da estilbeno resveratrol, quatro ácidos fenólicos e cinco flavonóides em vinhos por eletroforese capilar

(CE) (Capítulo 6). A composição do eletrólito e as condições instrumentais foram otimizadas, usando um planejamento fatorial 2^{7-3} e análise de superfície de resposta, mostrando TBS (tetraborato de sódio) / metanol como a variável mais influente. As condições eletroforéticas ótimas, minimizando os valores de CRS, consistiram de 17 mmol/L TBS com 20% MeOH como eletrólito, voltagem constante de 25 kV, injeção hidrodinâmica a 50 mBar por 3 segundos e temperatura de 25°C. Os valores de R^2 para linearidade foram igual ou maior que 0,99. Os limites de detecção e quantificação foram de 0,01 a 0,03 e de 0,04 a 0,08 mg/L, respectivamente. Os coeficientes de variação para os tempos de migração e áreas dos picos, obtidos por 10 injeções consecutivas, foram menores de 2%. A recuperação variou de 97 a 102%. O método foi aplicado a 23 diferentes tipos e marcas de vinhos brasileiros.

Um método por eletroforese capilar para os ácidos orgânicos mais importantes em vinho foi desenvolvido e validado (Capítulo 7). O eletrólito otimizado consistiu de 10 mmol/L de ácido 3,5-dinitrobenzóico (DNB) a pH 3,6, com 0,2 mmol/L de brometo de cetiltrimetilamônio para inversão de fluxo. O DNB foi escolhido porque tem mobilidade efetiva semelhante aos analitos, boa capacidade tamponante a pH 3,6 e características cromofóricas para detecção indireta. O preparo da amostra envolveu apenas diluição e filtração. O método demonstrou bom desempenho: linearidade ($R^2 > 0,99$) na faixa de concentração avaliada (6 a 285 mg/L); limites de detecção e quantificação de 0,64 a 1,55 mg/L e de 2,12 a 5,15 mg/L, respectivamente; tempo de análise de 5,5 minutos. Os coeficientes de variação, em relação aos tempos de migração e as áreas dos picos, foram abaixo de 5%. As porcentagens de recuperação variaram de 95 a 102%. O método foi aplicado a 23 tipos e marcas de vinhos brasileiros, confirmando a boa repetibilidade e demostrando a grande variação nas concentrações dos ácidos orgânicos.

GENERAL ABSTRACT

Green and black teas (*Camellia sinensis*) are widely consumed beverages in the entire world. In the last decade, these beverages have been the subject of intense investigations in relation to their possible beneficial effects on health. Catechins and theaflavins are the most important phenolics in green and black tea, respectively. Health promoting have also been attributed to the infusions of mate, beverages widely consumed in Brazil, probably due to caffeoylquinic derivatives and other phenolics. **Chapter 1** reviews advances in instrumentation and analytical methodology applied to phenolic compounds in teas. Modern analytical techniques, such as high performance liquid chromatography (HPLC), mass spectrometry (MS) and capillary electrophoreses (CE), have provided important data about the composition of these compounds in the above mentioned beverages.

Phenolic compounds occur in abundance in wine and have been the object of intense research in recent years because of their potential benefits to health. Organic acids have a direct influence on the quality and sensory properties of wine, as well as in their stability and microbial control. The determination of phenolic compounds and organic acids of wine is, therefore, of paramount importance. A review of the literature (**Chapter 2**) shows that capillary electrophoresis is gradually substituting high performance liquid chromatography, with the advantages of low operational cost, low solvent consumption, short analysis time, minimal sample preparation, etc. With phenolics, CE has been successfully used for the determination of resveratrol isomers, phenolic acids and

flavonoids. With organic acids, this technique has been employed in the direct and indirect mode.

"Chimarrão" and "tererê" are mate (*Ilex paraguariensis*) infusions widely consumed in South America. This paper (**Chapter 3**) describes the application of HPLC-DAD-ESI/MS in the identification and quantification of caffeoylquinic acids (CQA), flavonol glycosides and purine alkaloids in these beverages. Infusions were prepared from commercial samples of *Ilex paraguarienses* from Southern Brazil. The cafeoylquinic acids, 4,5-diCQA, 3-CQA, 5-CQA, and 4-CQA were the major compounds, having 238-289, 153-242, 183-263, and 123-188 μg/mL, respectively, in chimarrão and 206-265, 122-218, 164-209, 103-169 μg/mL, respectively in tererê. Caffeine also had high amounts while glycosides of quercetin and kaempferol were found at much lower levels. The individual antioxidant activity was determined by an on-line system that measured their $ABTS^{\cdot+}$ radical scavenging activity, showing that the antioxidant capacity was not proportional to the concentrations of the phenolic compounds. 3-*O*-CQA, quercetina-3-*O*-ramnosylglucoside, and quercetina-3-*O*-glucoside were the major contributors to the antioxidant capacity, although the quercetin glycosides had concentrations less than 10 times that of 3-*O*-CQA.

Chapter 4 had a two-fold objective: (1) compare quantification of flavonols in the glycosidic and aglycone forms, and (2) compare the phenolic and purine alkaloid composition of mate samples taken from two stages of industrial production and of hot and cold infusions. The HPLC quantitative method used for the quantification of the flavonol aglycones obtained by hydrolysis and that used for quantifying the glycosides, other phenolics and the purine alkaloids performed well. Both methods showed good linearity, limits of detection and quantification, recovery and repeatability. For all thirteen samples,

analyzed as hot or cold infusion, equivalent results were obtained for quercetin and kaempherol, thus these flavonols are amenable to quantification either in the glycoside or aglycone form. The levels of all the analytes in all the samples were lower in the cold infusion. In both infusions the phenolic and purine alkaloid concentrations were significantly higher in the samples obtained from the producers than in the commercial samples, indicating that losses occurred in the additional processing step and during storage of the later samples.

A fast RF-MEKC method modified by sulfated-ß-cyclodextrin was developed and validated for the determination of catechins in green tea (**Chapter 5**). The optimal electrolyte consisted of 0.2% triethylamine, 50 mmol/L SDS and 0.8% s-β-CD (pH=2.9). The samples and standards were injected at 0.6 psi for 5 s under constant voltage of -30 kV. Sample preparation involved extraction of 2 g of tea with 200 mL water at 95^{o}C under constant stirring for 5 min. The method demonstrated excellent performance, with LOD and LOQ of 0.02 to 0.1 and 0.1 to 0.5 µg/mL, respectively, and recovery percentages of 94 to 101%. The method was applied to six samples of Brazilian green tea infusions. Epigallocatechin gallate (23.4-112.4 µg/mL) was the major component, followed by epigallocatechin (18.4-78.9 µg/mL), epicatechin gallate (5.6-29.6 µg/mL), epicatechin (4.6-14.5 µg/mL) and catechin (3.2-8.2 µg/mL).

In **Chapter 6**, a method for simultaneously determining the stilbene resveratrol, four phenolic acids and five flavonoids in wine by capillary electrophoresis (CE) was developed and evaluated. The CE electrolyte composition and instrumental conditions were optimized using 2^{7-3} factorial design and response surface analysis, showing TBS (tetraborate)/MeOH as the most influential variables. The optimal electrophoretic conditions, minimizing the chromatographic resolution statistic (CRS) values, consisted of

17 mmol/L TBS with 20% methanol as electrolyte, constant voltage of 25 kV, hydrodynamic injection at 50 mbar for 3s and temperature of 25^{o}C. The R^2 values for linearity were equal to or higher than 0.99; limits of detection and quantification were 0.01 to 0.03 and 0.04 to 0.08, respectively. Coefficients of variation for migration time and peak area obtained from ten consecutive injections were less than 2% and recoveries varied from 97 to 102%. The method was applied to 23 different samples of Brazilian wines.

A capillary electrophoresis method for six organic acids in wine was developed and validated (Chapter 7). The optimal electrolyte consisted of 10 mmol/L 3,5-dinitrobenzoic acid (DNB) at pH 3.6 with 0.2 mmol/L cetyltrimethylammonium bromide as flow reverser. DNB was chosen because it had effective mobility similar to the organic acids, good buffering capacity at pH 3.6 and good chromophoric characteristics for indirect detection. Sample preparation involved dilution and filtration. The method showed good performance characteristics: linearity at 6 to 285 mg/L ($r > 0.99$); detection and quantification limits of 0.64 to 1.55 and 2.12 to 5.15 mg/L, respectively; separation time of less than 5.5 min. Coefficients of variation for ten injections was less than 5% and recoveries varied from 95 to 102%. Application to 23 samples of Brazilian wine confirmed good repeatability and demonstrated wide variation in the organic acid concentrations.

INTRODUÇÃO GERAL

Os avanços em análise de alimentos espelham tanto o refinamento da instrumentação e metodologia analítica como o aprofundamento dos conhecimentos sobre a contribuição dos componentes alimentícios na qualidade, segurança e efeitos benéficos à saúde. A aplicação aprimorada e inovadora das técnicas analíticas modernas à análise de alimentos tem proporcionado maior entendimento das propriedades dos compostos alimentícios. Por outro lado, a necessidade de conhecer melhor as funções e ações destes compostos vem incentivando o desenvolvimento de metodologias analíticas mais poderosas, capazes de determinar tanto os macro como os microcomponentes nas suas diversas formas.

Entre as técnicas analíticas disponíveis atualmente, a eletroforese capilar nas suas diferentes modalidades e a cromatografia líquida de alta eficiência com detectores de arranjo de diodos e de massas (CLAE-DAD-EM) destacam-se pelas suas grandes utilidades no estudo de alimentos. Utilizando estas técnicas, portanto, compostos fenólicos foram estudados em chá verde, infusões de erva mate e vinho. No último, foram também investigados os ácidos orgânicos.

Os compostos fenólicos estão bem destacados na literatura internacional pelos seus possíveis benefícios na saúde, principalmente na diminuição do risco de doenças cardiovasculares e de câncer. A determinação da composição de fenólicos, portanto, é considerada uma prioridade mundial. São também importantes na qualidade de bebidas, influenciando as propriedades sensoriais como cor e sabor. Os ácidos orgânicos influem na estabilidade físico-química e microbiológica do vinho e também nas propriedades

sensoriais como cor e sabor. A determinação destes ácidos é, portanto, importante no monitoramento dos processos de fermentação e no controle de qualidade de vinhos.

CAPÍTULO 1

Métodos para a Determinação de Flavonóides em Chás: Uma Revisão

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Métodos para a determinação de flavonóides em chás: Uma revisão

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Resumo

Os chás verde e preto (*Camellia sinensis*) são bebidas muito consumidas no mundo todo, principalmente em países orientais e europeus. Na última década, estas bebidas têm sido alvo de investigação intensa quanto a seus possíveis efeitos benéficos à saúde. Catequinas e teaflavinas são os fenólicos mais importantes em chá verde e chá preto, respectivamente. Possíveis aplicações terapêuticas e farmacológicas têm sido também atribuídas a infusões de chá mate (*Ilex paraguariensis*), bebidas bastante consumidas no Brasil, possivelmente devido a compostos derivados de cafeoilquínicos e outros fenólicos. Técnicas analíticas modernas, como cromatografia líquida de alta eficiência (HPLC), espectrometria de massas (MS) e eletroforese capilar (CE) têm fornecido dados importantes sobre a composição de fenólicos nas bebidas mencionadas. O presente artigo de revisão discute os avanços na instrumentação e metodologia analítica aplicadas aos compostos fenólicos em chás.

Palavras-chave: chá, polifenóis, flavonóides, CLAE, EC, EM

Introdução

Embora tradicionalmente associado a países da Ásia e Europa, o consumo do chá tem aumentado em todo o mundo nos últimos anos, em boa parte devido às evidências ligadas à saúde.

Os chás verde e preto são os mais consumidos, sendo que o chá preto responde a cerca de 80% do consumo mundial enquanto que o chá verde a 20%, com maior concentração de consumo na Europa e Ásia, respectivamente. Ambos são produzidos a partir de folhas de *Camellia sinensis* e suas diferenças principais estão no modo de processamento. No chá verde, ocorre a inativação da enzima polifenoloxidase por injeção direta de vapor ou aplicação de calor indireto com posterior secagem e tostagem. No chá preto, ocorre primeiramente a secagem das folhas, seguida de trituração em rolos para o rompimento das paredes celulares e exposição ao oxigênio e à enzima polifenoloxidase. Este processo é conhecido por fermentação, embora não exista um processo fermentativo envolvido. O termo possivelmente surgiu devido a produção de gás carbônico no processo oxidativo, que foi associada a uma suposta fermentação[75]. Por fim, as folhas são tostadas, e o processo oxidativo é interrompido pela aplicação de calor.

No Brasil, o chá mais consumido é o chá mate que é obtido a partir de folhas de *Ilex paraguarensis*, encontrada principalmente no Brasil, Paraguai e Argentina. A erva mate, também bastante consumida, difere em sua produção do chá mate somente pela ausência da etapa de tostagem. A matéria-prima (folhas, ramos verdes) é beneficiada em diversas formas pela indústria e comercializada como chás, pó solúvel, essências e erva para chimarrão e tererê. Esta produção está concentrada nos estados da região Sul e Mato Grosso do Sul.

A erva mate é consumida em três formas principais: (1) a forma tradicional na qual a erva é colocada em copo com água quente (70-85^{o}C), conhecido como chimarrão; (2) similar a tradicional, com temperatura da água mais baixa (5-10^{o}C), mais consumida em regiões quentes e conhecido como Tererê; (3) por infusão de maneira similar aos chás verde e preto[20,65].

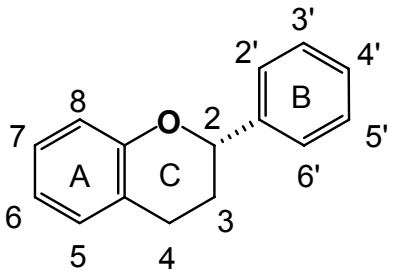
Polifenóis de chá

O efeito benéfico do consumo de chás levou em anos recentes a um aumento significativo do número de trabalhos científicos que tem tentado demonstrar a provável ação biológica, atribuída principalmente aos polifenóis.

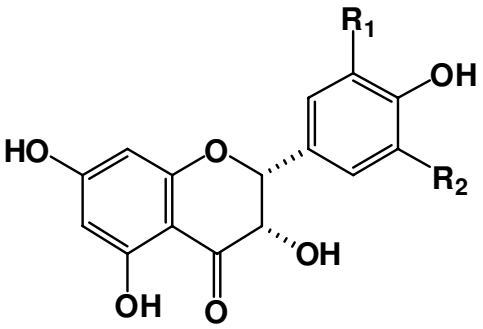
Entre os polifenóis, os mais largamente encontrados em alimentos são os flavonóides. Estes são metabólitos secundários sintetizados por plantas, nas quais possuem propriedades que vão desde a pigmentação a importantes funções na sinalização entre as plantas, fertilidade, defesa e proteção à radiação ultravioleta[71,88].

Os flavonóides podem ser classificados em grupos como: flavonas, flavanonas, isoflavonas, antocianinas, flavonóis e flavanóis. A estrutura dos flavonóides é baseada no núcleo que consiste de dois anéis benzênicos A e B e um anel C (*Figura 1*), que pode ser um pirano heterocíclico, como no caso de flavanóis (catequinas) e antocianidinas, ou pirona, como no caso dos flavonóis, flavonas, isoflavonas e flavanonas, que possuem um grupo carbonila na posição C_4 do anel C. Os flavonóis são geralmente encontrados na natureza em sua forma glicosídica, com exceção das catequinas. Os açúcares ligados na posição 3 do anel C, são geralmente glicose, ramnose ou galactose e sua presença confere caráter mais hidrossolúvel aos flavonóides.

(A)



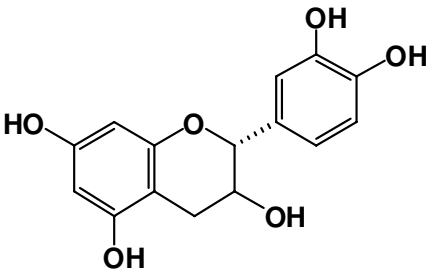
(B)



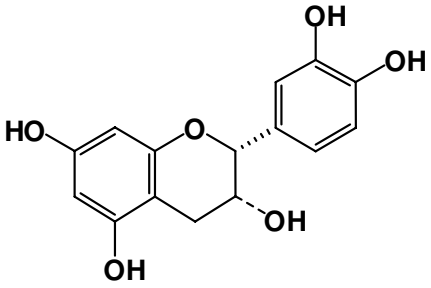
	R_1	R_2
Miricetina	**OH**	**OH**
Quercetina	**OH**	**H**
Kaempferol	**H**	**H**

Figura 1. Estrutura básica de flavonóides (A) e de flavonóis agliconas presentes em chás (B).

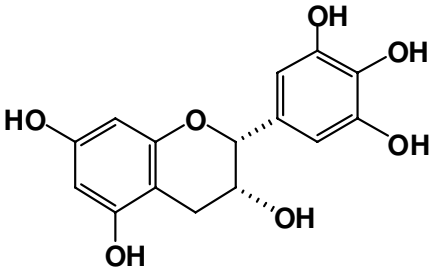
O destaque no chá verde são os flavanóis ou catequinas (*Figura 2*) e no chá preto as teaflavinas (*Figura 3*), que são formados pela condensação de catequinas duas a duas. Em chá verde, as catequinas podem chegar a 75% do conteúdo de flavonóides e podem influenciar o sabor da bebida, podendo servir como um indicativo da qualidade do produto. Podem ocorrer reações químicas conhecidas como epimerização durante o processamento ou preparação do chá, transformando as catequinas majoritárias em seus isômeros[81].



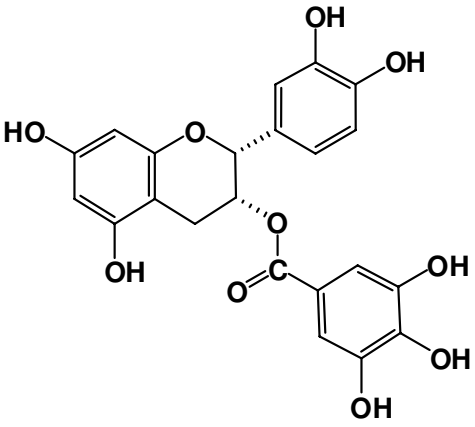
(+) - Catequina



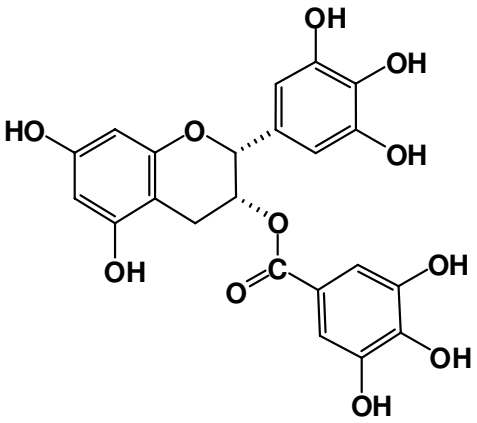
(-)-Epicatequina



(-)-Epigalocatequina

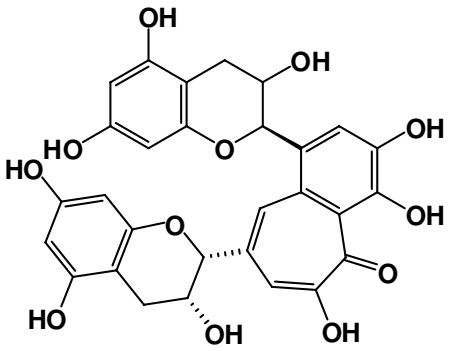


(-)-Epicatequina galato

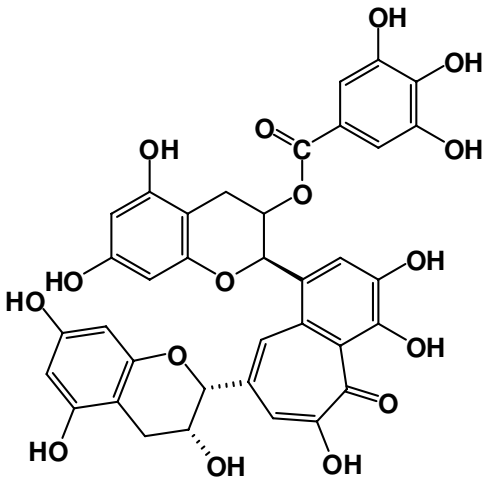


(-)-Epigalocatequina galato

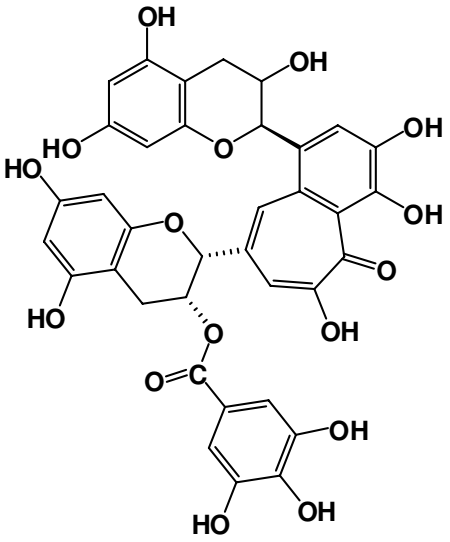
Figura 2. Estruturas das principais catequinas encontradas em chás.



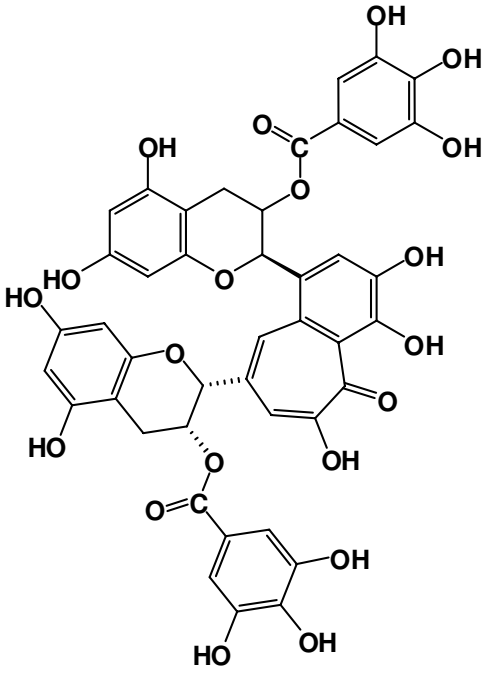
Teaflavina (TF1)
Epicatequina + Epigalocatequina



Teaflavina 3'-galato (TF3)
Epicatequina + Epigalocatequina Galato



Teaflavina 3-galato (TF2)
Epicatequina Galato + Epigalocatquina



Teaflavina 3,3'-digalato (TF4)
Epicatequina galato + Epigalocatequina galato

Figura 3. Estruturas das teaflavinas encontradas em chás.

Em chá preto as teaflavinas são responsáveis pelo desenvolvimento de sabor na infusão e também contribuem para o desenvolvimento de cor, variando de amarelo a marrom[8].

Efeitos benéficos à saúde

Os polifenóis, até pouco tempo, eram considerados como anti-nutrientes. Posteriormente, a constatação da sua ação antioxidante promoveu novo interesse em relação ao seu papel benéfico à saúde humana[12].

Vários estudos foram publicados nos últimos anos sobre os benefícios dos polifenóis na saúde humana[19,23,28,29,49,68,76].

Estudos *in vitro* encontram-se bem avançados, e mostram possíveis mecanismos de ação de catequinas e teaflavinas nas diversas etapas do desenvolvimento do câncer. Resumidamente, na etapa de iniciação, os mecanismos são: redução da formação[72] e ativação do carcinógeno[66], aumento da detoxificação do carcinógeno[44], redução da ligação carcinógeno-DNA[43], atividade antioxidante[91] e redução da alquilação do DNA[70]. Na etapa de promoção, os mecanismos são os seguintes: redução na ligação do promotor[1,21,92], proteção da comunicação célula-célula[46] e redução da lipoperoxidação[30]. Na etapa de progressão, há uma diminuição da conversão da célula benigna a maligna[42] e diminuição do crescimento de tumores[5,58].

Em animais (ratos) catequinas e teaflavinas reduziram a proliferação ou número de tumores gastrointestinais[22] ,do estômago[79,93] ,do pulmão[79,80,93] e da pele[54] em experimentos induzidos por compostos como teleocidina, azometano, nitrosamina, luz ultravioleta B, entre outros.

Papilomas provocados por nitrosamina, acetato de tetradecanoiforbiol, antraceno e luz ultravioleta regrediram ou foram inibidos quanto ao seu crescimento em peles de ratos[80]. Extratos de chá verde tiveram ação em dois importantes estágios (iniciação e propagação) de tumores de estômago e pulmão em ratos[42].

Os estudos epidemiológicos, porém, vêm fornecendo resultados não conclusivos ou inconsistentes. Kono e colaboradores[48] no Japão, relataram a correlação inversa entre o consumo de chá verde e a incidência de câncer gástrico em 2.991 indivíduos. Outro estudo no Japão, conduzido com 8.000 pessoas, revelou uma correlação direta entre o consumo de chá preto e câncer no reto, enquanto que para o de próstata a correlação foi inversa[25]. Tajima e Tominaga[74] não constataram uma correlação significativa entre o consumo de chás verde e preto com o risco de câncer de estomago e cólon em 550 japoneses. Outro estudo, com 12.763 homens, não encontrou relação entre o consumo de flavonóis e flavonas com a mortalidade por câncer de pulmão e colo-retal[27]. Outros estudos foram conduzidos e todos eles tiveram resultados promissores, porém, ainda não conclusivos sobre o efeito benéfico do consumo de chás em relação à incidência de várias doenças[39,73,76].

Os estudos epidemiológicos, portanto, ainda não fornecem suficiente suporte à idéia do aumento do consumo de chá para a proteção contra câncer, embora exista alguma evidencia que o chá verde em grandes quantidades pode proporcionar algum benefício na prevenção do câncer do trato digestivo, especialmente câncer gástrico.

Métodos analíticos

Cromatografia líquida de alta eficiência

A técnica analítica mais utilizada para as análises de polifenóis em chá tem sido a cromatografia líquida de alta eficiência (HPLC). O primeiro trabalho para este fim foi publicado em 1976 por Hoefler e Coggon[31], envolvendo a identificação de 5 catequinas na infusão de chá verde. A separação foi pobre em termos cromatográficos, mas como marco inicial, merece destaque, pois representa o início dos trabalhos com catequinas em chá. A *Tabela 1* apresenta os principais métodos relatados na literatura, bem como suas características principais.

A HPLC no modo fase reversa tem sido o mais usado para a separação dos polifenóis, com a fase estacionária C_{18}, sendo a preferida pela grande maioria dos autores.

As fases móveis mais comumente empregadas consistem de acetonitrila ou metanol em uma mistura ácida diluída com ácido acético[18,63] ou acido fórmico[40,53] em concentrações que não ultrapassam 1.0%. Em alguns casos se utiliza solução tampão fosfato pH=2,4, como no caso do trabalho de Hertog et al.[26] ou mesmo com a utilização de tampão acetato como no caso do trabalho de Karakaya e Ei[41]. Dalluge e colaboradores[16] compararam várias fases estacionárias e condições de eluição e observaram que a utilização de fases estacionárias com máxima cobertura e sílica ultrapura melhorou a separação cromatográfica, e a presença do ácido na fase móvel foi essencial para a separação. As fases móveis utilizando metanol foram piores que aquelas com acetonitrila como solvente base.

Tabela 1. Métodos por cromatografia líquida de alta eficiência para a análise de polifenóis em chás.

Analitos	***Coluna (comp. x diametro interno (mm)***	***Fase móvel***	***Temperatura (°C)***	***Detecção (nm)***	***Padrão interno***	***Referência***
Catequinas	Hypersil ODS 3 µm 250 x 4,0	5% ácido acético, MeOH		PCR		77
EGCg, ECg, EGC, EC, TB, cafeína	Tosoh ODS-80Ts 250 x 4,6	20 mmol/L fosfato (pH 3.0) ACN	45	207		86
EGCg, Ecg, EGC, EC, C, TF	Hypersil 3µm ODS 100 x 4.6	0.5% ácido acético, 30% ACN 0.5% ácido acético, 69.5% água		280 (450 TF)		69
EGCg, EcCg, EGC, EC, GC, C, GCg, Cg, cafeína	Develosil ODS-HG 150 x 4.6	94.5% água, 4.5% ACN 0.05% ácido fosfórico, 49.95% água, 50.00% ACN, 0.05% ácido fosfórico	40	231		24
EGCg, ECg, EGC, EC, cafeína	Capcell pak C18 UG 120 200 x 4,6	6% ACN,11% MeOH, 0.5% ácido fosfórico, 82.5% água	40	270		34
EGCg, ECg, EGC, EC, cafeína	Hypersil ODS 5 µm 250 x 4,6	12% acetonitrila, 2% acetato de etila,0.04% H_2SO_4		280	(+)-catequina	96
EGCg, ECg, EGC, EC, cafeína	Altima C18 250 x 4,6	tampão acetato (1 mmol/L, pH 4.5) ACN		210	naringenina	14
EGCg, Ecg, EGC, GCg, C	Zorbax Eclipse XDB-C18 250 x 4,6	0.05% TFA, 60% MeOH, 40% acetonitrila, 0.05% TFA		210	naringenina	16
EGCg, Ecg, EGC, EC, cafeína, TP, Tb, GCg, Cg	Cosmosil C18-MS 250 x 4,6	20% MeOH, 0.3% ácido fórmico, 79.7% água 99.7% MeOH, 0.3% água		280		53
EGCg, Ecg, EGC, EC, catequinas minoritárias	Nucleosil 100-5 C18 250 x 4,6	0.05 M ácido fosfórico, 60% água, 40% ACN, 0.05 mol/L ácido fosfórico		280		67

Cafeína, TB, Ecg, EGCg, ácido clorogênico	Hypersil 5 µm ODS 250 x 4,6	2% ácido acético, ACN		280, 380, 460, 510	7
C,EC,EGC,EGCg,EGC,GC, Caf, GCg	Kingsorb 5um, 150 x 4,6	MeOH /água/ácido fosfórico (20/79.9/0.1)	30	210	82
GC,EC,ECG,EGC,EGCG,Cafeína, adenina,TF,GA,CA	Partsphere 5um C18 110 x 4,6	(A) 5% ACN(0.035%) TFA (B) 50% ACN (0.025%) TFA	32	205	51
Miricetina,Quercetina e Kaempferol	Kingsorb 5um, 150 x 4,6	30% ACN, 0.025MKH_2PO_4 pH=2.5	30	370	83
GA,EGC,EGCG,EC,ECG,CA,Cg	Adsorbosil C18 250 x 4,5	água/ácido acético (97:3) (A) MeOH (B)		280	98
(+)-GC, (-)-GC, EGC, (+)-C, (-)-C, Caf, (-)-EGCG, (-)-EC, GCG, ECG, C	Wakosil-II 5um C18 HG 150 x 3,0	água/MeOH/ácido fosfórico (85/15/0.1)	40	280	59
Cafeína, C, EC, GC, EGC,ECg,EGCg	Phenomenex C18 5um 300 x 4,6			280	84
Cafeína,EGC,C,EC,EGCg,ECg e TF	Xterra Waters RP C18 23.5um 100 x 3,9	água/ACN/TFA (919/80/1) (A) água/ACN/MeOH/TFA (699/270/30/1) (B)	35	280	56
TB,GC,EGC, C,Cafeína,EC,EGCg,GCG,ECg	Mightysil RP-18 5um 150 x 4,6	ACN (A) ACN + 0.05% ácido fosfórico (B)	40	231	94

EGCg, Epigalocatequina galato; ECg, Epicatequina galato; EGC, Epigalocatequina; EC, Epicatequina; C, catequina; GCg, Galocatequina galato; EGCg, Epigalocatequina galato; ACN, acetonitrila; TFA, ácido trifluoroacético

O detector mais usado é, sem dúvida, o arranjo de diodos (DAD), pois os flavonóides apresentam duas bandas características com máximos entre 300 a 550nm (banda I) e 240 a 285nm (banda II). O comprimento de onda utilizado para a detecção varia bastante, sendo que os comprimentos mais utilizados são 280nm[2,9,53,63,69] e 210nm[16,51,82]. Alguns autores justificam o uso de 210nm pela relação sinal:ruído apresentada.

Eletroforese capilar

Recentemente, a eletroforese capilar (CE) tem sido utilizada, oferecendo custo/volume reduzido de reagentes, rapidez nas análises e condicionamento da instrumentação, entre outras vantagens. Vários métodos foram publicados para a separação e quantificação de catequinas em chás. Entre as técnicas, a eletroforese capilar de zona (CZE)[3,34] e a cromatografia micelar eletrocinética (MEKC)[10,11,32,35,57,85] tem sido as mais empregadas, tanto para folhas frescas quanto para infusões. A *Tabela 2* detalha os 18 métodos relatados, com a utilização de CE nos diversos modos de operação.

Lee e Ong[51] compararam HPLC e CE na análise de catequinas e teaflavinas em chá. As condições analíticas foram otimizadas para os dois métodos e ambos mostraram-se confiáveis e aplicáveis ao uso em rotina.

Métodos por MEKC oferecem melhores alternativas em relação aos métodos iniciais por CZE, por sua versatilidade e rapidez, entre outras vantagens. Recentemente, métodos utilizando a técnica de MEEKC (cromatografia eletrocinética micelar em microemulsão)[36,38,62] tem sido utilizados como técnica alternativa a ME

Tabela 2. Métodos eletroforéticos (CZE , MEKC e MEEKC) para análise de polifenois em chás.

Método	*Analitos*	*Dim. Capilar (cm x µm)*	*Eletrólito*	*Detecção (nm)*	*Voltagem (kV)*	*Temeratura (°C)*	*Tempo de corrida (min)*	*Ref*
CZE	EGCg, Ecg, EGC, EC, C tiamina, cafeina, ácido ascórbico	77 x 50	20 mmol/L borato (pH 8,0)	200	30	23	11	34
CZE	EGCg, ECg, EGC EC, C, ácido gálico, adenina	77 x 57	150 mmol/L borato (pH 8,5)	210	20	20	20	3
MEKC	EGCg, Ecg, EGC EC, C, tiamina, cafeína, ácido ascórbico	75 x 57	50 mmol/L ácido bórico, 10 mmol/L fosfato 50 mmol/L SDS, 10% MeOH (pH 8,4)	194 270	25	3	11	32
MEKC	EGCg, Ecg, EGC, EC, tiamina, Cafeína, ácido ascórbico	75 x 57	50 mmol/L borato, 10mmol/L fosfato 50 mmol/L SDS, 10% MeOH (pH 8,2)	200 270	25	20	-	35
MEKC	EGCg, Ecg, EGC, EC, C, Cg, GCg, cafeína, ácido ascórbico	50 x 36	25 mmol/L SDS, 25 mmol/L fosfato 50 mmol/L borato (pH 7,0)	280	20	20	10	85
MEKC	EGCg, Ecg, EGC, EC, C, GCg, cafeína	50 x 85	20 mmol/L borato - fosfato 25mmol/L SDS (pH 7,0)	200	30	21	20	10
MEKC	EGCg, Ecg, EGC, EC, C, Gg	50 X 67	20 mmol/L borato, 110 mmol/L SDS, 14% MeOH 1.5% uréia, 1mmol/L, β-CD (pH 8,0)	280	20	20	30	57
MEKC	EGCg, Ecg, EGC, EC, cafeína, TB, ácido clorogênico	50 x 64,5	20 mmol/L SDS, 50 mmol/L fosfato 50 mmol/L borato, 1.0% acetonitrila	278	-	25	20	50
MEKC	C,CG,EC,EG,EGC,TF,TF2, TF3,TF4,Cafeína, adenina, Q,GA,CA	40 x 50	500 mmol/L borato, 200 mmol/L fosfato, 20 mmol/L β-CD, acetonitrila	205	25	30	10	51
MEKC	C, EC, EG, CG, EGCg, ECG, cafeína	50 x 50	100 mmol/L SDS, 90% fosfato 20 mmol/L, pH 2,5, 10% MeOH	195	-	25	22	90
MEKC	C,EC,EGC,ECg,EGCg, tiamina, GA	58 x 50	25 mmol/L fosfato (pH=7.) 100 mmol/L SDS e 6% MeOH	200	14	25	13	4

NACE	TF's (chá preto)	40 x 50	71% acetonitrila ,25% MeOH, 0,1M KOH, 4% ácido acético, 90 mmol/L acetato de amônio	380	22.5	18.5	10	89
MEKC	GC, C, EGC, EGCg, EC, ECg, GCg, Teofilina, Teobromina, ácido gálico, cafeína	40 x 50	20 mmol/L fosfato, 50 mmol/L borato 200 mmol/L SDS (pH 7.0)	200	20	29	5	11
MEKC	EC,EGC,C,EGCG,EGC, Cafeína	77x50	25 mmol/L fosfato pH=7.0 20 mmol/L SDS	200	27	-	29	87
MEKC & MEEKC (comparação)	ácido sivingico, ácido *p*-coumárico, ácido vanilico, ácido caféico, GA, 3,4-dihidroxibenzóico, 4-hidroxibenzóico, catequina, epigalocatequina, epicatequina galato, epigalocatequina galato, epicatequina, galocatequina, cafeína e teofilina	48,5 x 50	MEKC: 2.89% SDS, 2% MeOH, e 95.5% fosfato 25 mmol/L pH 2.0 MEEKC: 2.89% SDS. 1.36% heptano, 7.66% ciclohexanol, 2% acetonitrila e 86.1% fosfato (25 mmol/L, pH 2.0)	200	-20 -27	35	14	37
MEKC	(+)-C,(-)-C,(+)-EC,(-)-,CG, (-)-EGC,(-)-EGCG, (-)-ECg, (-)-EGCg,(-)-ECg, Cafeína	64,5 x 50	200 mmol/L borato-20 mmol/L fosfato (pH 6.4) 240 mmol/Le 25 mmol/L 6G-β-CD	210	25	20	35	47
MEEKC	EC,C,EGCg,ECg,EGC,GC	47,5 x 50	50 mmol/L fosfato,PH2.0 SDS (2.89%) co-surfactante (2-propanol, isobutanol ou ciclohexanol (3.66% a 7.66%) e acetato de etila, cliclohexano, heptano ou octano (1.36%) borato 55 mmol/L (pH=8)	200	-20	30	16-24	38
MEEKC	ECg, EGCg, EC, C, EGC, GC, cafeína. teofilina	24 x 50	50 mmol/L fosfato (pH 2.5) surfactante SDS (2.31% a 2.89% e n-heptano (1.36%) e nove alcoois (co-surfactantes): (1-butanol ou tert-butanol ou 1-pentanol ou 2-pentanol ou 3-pentanol ou ciclopentanol ou 1-hexanol ou 2-hexanol e ciclohexanol)	230	-10	40	10-18	62

EGCg, epigalocatequina galato; ECg, epicatequina galato; EGC, epigalocatequina; EC, epicatequina; C, catequina; GCg, galocatequina galato;.Cg, catequina galato; TF, teaflavina; GA, ácido gálico; CA, ácido cafeico; TP, teofilina; TB, teobromina;

MEKC foi comparado a HPLC na análise de catequinas (11) em chá verde sob condições otimizadas, e apresentou vantagens sobre HPLC, principalmente em tempo de análise (separação em menos de 4,5 minutos de corrida para 7 catequinas) e limites de detecção 20 a 100 vezes menores que HPLC. O método por HPLC, no entanto, apresentou-se mais robusto que MEKC.

A separação de catequinas em chá verde foi estudada por CZE[34] e MEKC[32,33] e ambas técnicas mostraram-se dependentes da formação de um complexo com borato, o qual foi usado como um dos componentes do eletrólito.

Espectrometria de massas

A espectrometria de massas (MS) tem se tornado uma ferramenta indispensável em laboratórios analíticos modernos, tanto como instrumento utilizado em separado, ou quanto acoplado a uma técnica de separação, como a cromatografia gasosa de alta resolução, HPLC ou CE. Diferentes sistemas de espectrometria de massas têm sido utilizados, incluindo bombardeamento rápido de átomos (FAB/MS)[78], ionização química a pressão atmosférica (APCI/MS)[95] e ionização eletrospray (ESI/MS)[17] para as análises em chás. A *Tabela 3* traz um resumo dos métodos publicados, utilizando a MS na determinação de polifenóis em chá.

O primeiro trabalho com a utilização de HPLC-MS para a separação e identificação de catequinas em um extrato de chá foi publicado em 1993[52], utilizando-se o modo de ionização "termospray". HPLC-ESI/MS foi utilizada para catequinas em plasma humano e chá verde por Miketova et al.[55]. Neste e no trabalho de Poon[64], a espectrometria de massas foi também diretamente empregada, sem a utilização de HPLC.

A interface APCI-MS[95] forneceu informações sobre a massa molecular e informações sobre as fragmentações produzidas. Todas as catequinas analisadas produziram o íon m/z=139, sendo que este íon foi derivado do anel A destes compostos.

Del Rio et al.[17], utilizaram HPLC-MS para a análise de mais de 30 compostos fenólicos, incluindo catequinas, teaflavinas e derivados glicosilados de flavonóides. Infusões de chá verde e preto foram injetadas em HPLC, usando dois gradientes diferentes,

Tabela 3. Aplicações de espectrometria de massas na análise de fenólicos em chás.

Composto/matriz	*Dimensões coluna (mm)*	*Fase móvel*	*Modo de operação/interface*	*Referência*
EGCg, Ecg, EGC, EC (chá verde)	Zorbax Eclipse C_{18} 300 x 4.6	5 mmol/L acetato de amônio, 0.05% TFA 5 mmol/L acetato de amônio , 0.05% TFA 40% ACN, 60% MeOH (gradiente)	$[M+H]^+$íons ESI	15
EGCg, Ecg, EGC, EC (chá verde)	Waters C_{18} 250 x 2.0	30% MeOH, 0.05% TFA (gradiente)	$[M+H]^+$ íons MS-MS, CID	52
Ácidos cafeoilquínicos, ácidos coumaroilquínicos, TF's e TR's (Chá preto)	Waters C18 250 x 4.6	0.1 mol/L acetato de amônio 10% MeOH	$[M+H]^+$íons TSI	45
27 Compostos fenólicos (*Ilex paraguarienses*)	Nucleosil 120 C_{18} 250 x 4.6	ácido fórmico 1% (A) e ACN (B) (gradiente binário)	$[M+H]^+$íons ESI	13
Catequinas, ácido gálico, ácido quínico, ácido coumaroilquínico	-	-	$[M+H]^+$íons ESI	64
Flavonóis, TF's, hidroxinamatos e alcalóides (chá preto e chá verde)	Phenomenex RP-Max C_{12} 250 x 4.6	ACN, água e ácido fórmico (1%) (gradientes diversos)	$[M+H]^+$ e $[M+H]^-$ ESI, MS^n	17
Catequinas (chá verde e chá preto)	Zorbax Eclipse XBD C_{18} 250 x 4.6	água (0.05% TFA) (A) e ACN (0.05% TFA) (B) (gradiente linear)	$[M+H]^+$ APCI	95
Catequinas (chá verde)	Zorbax SB C_{18} 150 x 2.1	ACN/acetato de amônio (10%/2mM pH5.0) (gradiente)	$[M+H]^+$e $[M+H]^+$ EI e ESI	55

Catequinas e cafeína (chá verde e tintura de chá verde)	Altima C_{18} 250 x 4.6	água (0.1% TFA) (A) e ACN (B) (gradiente)	$[M+H]^+$ FAB, PB/GD	78
Catequinas (chá verde)	Phenomenex Luna C_{18} 250 x 4.6 250 x 3.0	água/MeOH/ácido fórmico (A) (74.7/25/0.3) ACN/ácido fórmico (B) (99.7/0.3) (gradiente linear)	$[M+H]^+$ API / ESI	61
Catequinas (chá verde)	Phenomenex Luna C_{18} 250 x 4.6	água/MeOH/ácido fórmico (A) (74.7/25/0.3) ACN/ácido fórmico (B) (99.7/0.3) (gradiente linear)	$[M+H]^+$ API / ESI	60
Chá verde, chá preto e chás do mediterrâneo	Supersphere 100 C_{18} 125 x 2.0	água/ACN (2.5%) (A) MeOH/ água (2.5% ACN) (B) (gradiente)	$[M+H]^+$ ESI	6
Teamina, catequinas, metilxantinas e ácido clorogenico (chá verde e chá preto)	Spherigel C_{18} 250 x 4.6	ácido fórmico 0.5% (A) ACN (B) (gradiente)	$[M+H]^+$ e $[M+H]^-$ ESI	97

EGCg, epigalocatequina; ECg, epicatequina galato; EGC, epigalocatequina; EC, epicatequina; TF, teaflavina; TR, tearrubina; TFA, ácido trifluoroacetico; EI, impacto eletrônico; ESI, ionização eletrospray; FAB, Bombardeamento rápido de átomos; MS, Espectrometria de massas; APCI, Ionização química à pressão ambiente; CID, detector de ionização química; TSI, ionização termospray; ACN, acetonitrila; TFA, ácido trifluoroacético.

e os compostos identificados por tempo de retenção, espectros de absorção UV-VIS e de massas.

Pelillo et al.[61] analisaram extrato de chá verde por HPLC-DAD e HPLC-MS. Foram inicialmente testadas colunas com diâmetros internos diferentes (3,0mm e 4,6mm), mostrando limites de detecção melhores para a coluna de 4,6mm. Posteriormente, as catequinas foram identificadas, utilizando a interface "eletrospray".

Neilson et al.[56], utilizaram a técnica HPLC-ESI/MS para identificação complementar aos dados obtidos por DAD. O trabalho enfatizou principalmente a rapidez nas análises (5 min para as catequinas e 10 min para as teaflavinas). Baseando-se nas fragmentações e valores de absorção máxima, os autores puderam identificar catequinas e teaflavinas em chá verde, verde descafeinado, "oolong" e chá preto.

A análise dos fenólicos principais de extratos e infusões de mate (*Ilex paraguienses* St. Hil.) por HPLC-ESI/MS foi recentemente publicada. Neste trabalho isômeros do ácido cafeoilquínico foram identificados, bem como alguns compostos glicosilados derivados de quercetina e kaempferol[13].

Considerações finais

A introdução de equipamentos modernos e sofisticados vem proporcionando uma riqueza de informações não apenas sobre a composição e as propriedades dos componentes de alimentos, com implicações marcantes no controle de qualidade dos alimentos como no seguimento dessas substâncias ao longo das vias metabólicas e a elucidação da relevância dos metabólitos na saúde. Em relação aos flavonóides de chás, a utilização de HPLC, CE e MS, inclusive HPLC-MS, vem contribuindo de maneira altamente significativa para melhor

entendimento das propriedades químicas, ocorrência, variação na composição e os fatores influentes e o comportamento destes durante processamento e estocagem. Com a continuação de desenvolvimento metodológico e refinamento da instrumentação analítica, as perspectivas de maiores avanços em análise e química de alimentos são ótimas.

PERES, R. G.; RODRIGUEZ-AMAYA, D. B. Methods for the determination of flavonoids in tea: A review. Alim. Nutr., Araraquara, v. n., p. , 2007.

ABSTRACT*:* Green and black teas (*Camellia sinensis*) are widely consumed beverages in the entire world, principally in the Orient and Europe. In the last decade, these beverages have been subject of intense investigations in relation to their possible beneficial effects on health. Catechins and theaflavins are the most important phenolics in green and black tea, respectively. Possible therapeutic and pharmacological applications have also been attributed to the infusions of "mate", a drink widely consumed in Brazil, probably due to caffeoylquinic derivatives and other phenolics. Modern analytical techniques, such as high performance liquid chromatography (HPLC), mass spectrometry (MS) and capillary electrophoreses (CE), have provided important data about the composition of phenolic compounds in the above mentioned beverages. The present review article discusses advances in instrumention and analytical methodology applied to phenolic compounds in teas.

KEYWORDS: tea, polyphenols, flavonoids, HPLC, CE, MS

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CAPÍTULO 2

Métodos por eletroforese capilar para a determinação de polifenóis e ácidos orgânicos em vinhos: Uma revisão

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Métodos por eletroforese capilar para a determinação de polifenóis e ácidos orgânicos em vinhos: Uma revisão

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Resumo

Compostos fenólicos ocorrem em vinho em abundância e tem sido objeto de inúmeras pesquisas científicas nos últimos anos, devido aos supostos benefícios à saúde. Ácidos orgânicos exercem influência direta na qualidade e propriedades sensoriais de vinhos, bem como na sua estabilidade e controle microbiológico. A determinação tanto de compostos fenólicos quanto de ácidos orgânicos em vinho é, portanto, de extrema importância. Nos últimos anos a eletroforese capilar tem sido bastante utilizada na resolução de problemas analíticos na área de ciência de alimentos, fornecendo informações sobre processamento, controle de qualidade, adulteração, composição química, etc. Vem substituindo gradativamente a cromatografia liquida de alta eficiência (HPLC) com vantagens que vão desde baixo custo operacional até baixo consumo de solventes, rapidez, minimização do preparo das amostras. Com fenólicos, a eletroforese capilar tem sido utilizada com sucesso para a determinação de isômeros do resveratrol, ácidos fenólicos e flavonóides de maneira geral. Em ácidos orgânicos sua utilização se dá nos modos de detecção direta e indireta. Este artigo de revisão tem como objetivo a discussão do uso de eletroforese capilar na análise de compostos fenólicos e ácidos orgânicos em vinhos.

Palavras-Chave: Ácidos orgânicos, Compostos fenólicos, Flavonóides, Eletroforese Capilar, Vinho

Abstract

Phenolic compounds occur in abundance in wine and have been the object of intensive research in recent years because of their potential benefits to health. Organic acids have a direct influence on the quality and sensory properties of wine, as well as in their stability and microbial control. The determination of phenolic compounds and organic acids of wine is, therefore, of paramount importance. In recent years, capillary electrophoresis (CE) has been widely used in the resolution of analytical problems in food science, furnishing information about processing, quality control, adulteration, chemical composition, etc. It is gradually substituting high performance liquid chromatography, with the advantages of low operational cost, low solvent consumption, short analysis time, minimal sample preparation. With phenolics, CE has been successfully used for the determination of resveratrol isomers, phenolic acids and flavonoids in general. With organic acids, this technique has been employed in the direct and indirect mode. This review article has the objective of discussing the use of CE in the analysis of phenolic compounds and organic acids in wine.

Key words: Organic acids, Phenolic compounds, Flavonoids, Capillary electrophoresis, Wine

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Introdução

Com o advento do novo campo da promoção da saúde, dentro da medicina preventiva, o melhor caminho para se alcançar a longevidade e o envelhecimento ameno, a humanidade passou a ter grande interesse por alimentos funcionais, definidos como sendo alimentos que proporcionam efeitos fisiológicos benéficos à saúde, além de satisfazer as necessidades nutricionais clássicos. De acordo com Roberfroid[1], um alimento funcional deve ter relevante efeito na saúde e bem estar ou resultar na redução do risco de doenças. O componente funcional pode ser um macronutriente, um micronutriente ou um componente não nutritivo.

A procura por evidências científicas que pudessem comprovar e suportar hipóteses de que alguns compostos de alimentos realmente trouxessem benefícios adicionais à saúde, nos últimos anos, contribuiu para um aumento expressivo no número de publicações científicas sobre a composição dos alimentos em termos das supostas substâncias bioativas. Entre estas publicações, estão os estudos sobre os compostos fenólicos de vinho.

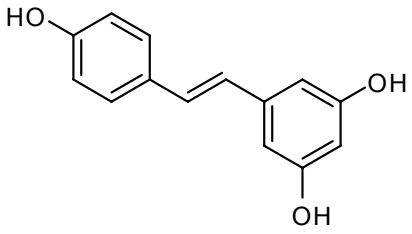
Por outro lado, existem compostos não considerados funcionais, como os ácidos orgânicos, mas que exercem influência direta e importante em vinhos, como, por exemplo, em propriedades sensoriais como sabor, cor e aroma, além de contribuírem a sua estabilidade e controle microbiológico.

O presente artigo de revisão discute métodos eletroforéticos aplicados a compostos fenólicos e ácidos orgânicos presentes em vinhos.

Compostos fenólicos em vinho

Fenólicos ocorrem em vinhos tintos em teores maiores (1000–4000mg/L) que nos vinhos brancos (200-300mg/L). Vinho tinto contém uma predominância de compostos solúveis em água que incluem ácidos fenólicos, o trihidroxistilbeno resveratrol, flavonóis, flavanóis. Também há presença de grande quantidade de procianidinas (taninos) e antocianinas (500-900mg/L)[2-5]. Vinhos tintos mais novos, geralmente, apresentam relativamente altos níveis de procianidinas e antocianinas, conferindo ao vinho a cor púrpura. A maturação dos vinhos tintos forma novos pigmentos oligoméricos e monoméricos vermelho-alaranjados com massa molecular de 2000 a 4000 Daltons. A figura 1 mostra as estruturas das principais classes de compostos presentes em vinho.

A contribuição dos fenólicos não está relacionada apenas a sua influência direta na qualidade dos vinhos em propriedades enológicas importantes, como cor em vinhos tintos, turvamento, aroma, adstringência, entre outros[6], mas também com o possível benéficio do consumo moderado de vinho na saúde.



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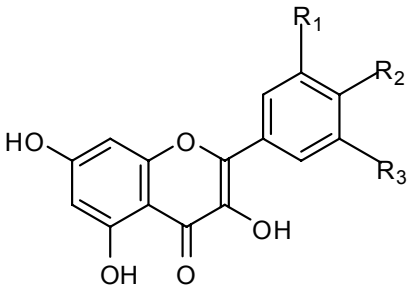

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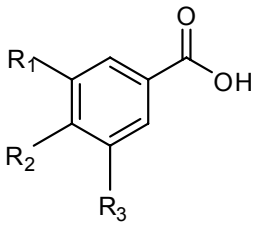

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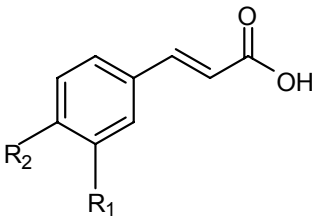

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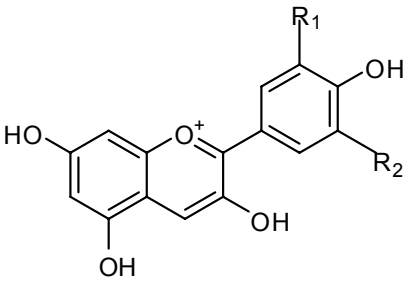

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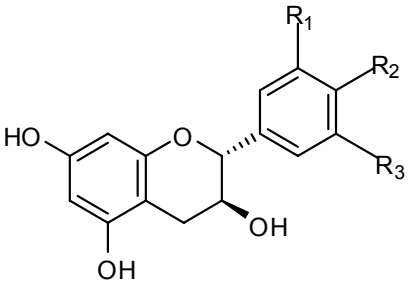

FLAVANÓIS

Figura 1. Estruturas das principais classes de compostos fenólicos em vinhos.

O paradoxo francês, relatado por Renaud & de Lorgeril[7], ganhou grande destaque, pois, os autores constataram que apesar dos franceses possuírem hábitos não recomendáveis como alta ingestão de gorduras saturadas e sedentarismo, a incidência de doenças cardiovasculares na população em geral era relativamente baixa.

Este fenômeno foi reforçado por um estudo conduzido na Dinamarca por Gronbaech et al[8] onde pessoas que tomaram 3 a 5 taças de vinho por dia tiveram, independentemente da idade ou educação, aproximadamente 50% menor risco de morte por doenças cardiovasculares durante os 12 anos da pesquisa. O consumo de vinho foi também associado com o baixo risco de morte por outras causas durante o período da pesquisa. Outros estudos apresentaram evidências científicas que levam a crer que o consumo moderado de vinhos reduz consideravelmente o risco de doenças cardiovasculares[9-17].

Outro estudo importante conduzido por Renaud et al. (2004)[18] acompanhou durante 8 anos (1978-1985) 36.583 homens franceses de meia idade, e observaram que o consumo comedido de vinho tinto (equivalente a 60g etanol/dia) pode reduzir o risco de morte por doença cardíaca em até 37% em comparação a população abstêmia, sendo o efeito mais pronunciado quanto menor for a pressão sistólica média. Uma análise de 13 estudos epidemiológicos envolvendo um total de 209.418 participantes demonstrou uma redução de 32% no risco de morte por doença aterosclerótica no grupo consumidor de vinho tinto (em comparação à população abstêmia), redução maior que a de 22% observada para o grupo consumidor de cerveja.

Estes efeitos benéficos do consumo de vinho foi atribuído aos antioxidantes polifenólicos, tais como o resveratrol e as proantocianidinas, que na dieta francesa estão em cerca de 1800-3000ug/L [19]. Além dos polifenóis, o vinho contém etanol que, se consumido

em pequenas quantidades (1 a 2 doses por dia de qualquer bebida alcoólica), pode reduzir em 20% o risco de isquemia cardíaca, além de aumentar o HDL colosterol em 12%.

Estudos *in vitro* indicam que o resveratrol pode atuar como cardioprotetor por uma série de mecanismos, como: inibição da agregação plaquetária, da proliferação de celulas musculares lisas e da oxidação do LDL-colesterol; redução da síntese de certos lipídeos e eicosanóides que podem promover inflamação e aterosclerose; e supressão de certos tipo de arritmia[20]. Outro estudo *in vitro* mostrou ainda a atividade anticarcinogênica e quimiopreventiva do resveratrol, que pode atuar nas fases de iniciação, promoção e progressão de células tumorais[21].

Análise de fenólicos em vinho por eletroforese capilar

A cromatografia liquida de alta eficiência (HPLC) é, sem dúvida, a técnica mais utilizada quando se busca informação mais completa sobre a composição de fenólicos em alimentos. Em vinho, HPLC tem sido bastante utilizada na determinação de compostos fenólicos com detecção espectofotométrica[22-24]. Mais recentemente, o acoplamento a espectrometria de massas possibilitou a confirmação da identidade dos analitos, bem como a obtenção de dados que podem ajudar na elucidação de mecanismos complexos e reações envolvidos no envelhecimento de vinhos[25-26]. A HPLC, no entanto, está sendo gradativamente substituído por electroforese capilar (CE).

A literatura nos últimos anos sugere que a eletroforese capilar tem se tornado uma ferramenta indispensável em laboratórios analíticos modernos nas análises de alimentos. Em CE, bebidas podem ser injetadas diretamente no capilar ou requerem pouco tratamento

da amostra, com apenas diluição ou filtração. Desvios padrão relativos entre 2 a 5% para área do pico e 0.5 a 1.0% para tempo de migração, tem sido obtidos na análise de bebidas.

A eletroforese é uma técnica de separação de alta eficiência baseada na migração diferencial de espécies iônicas ou ionizáveis quando submetidas a um campo elétrico[27]. Em CE, a separação e conduzida em tubos de sílica fundida de dimensões capilares de 15 a 100μm de diâmetro interno, e 50 a 100cm de comprimento, preenchidos com um eletrólito condutor, e submetidos a ação de um campo elétrico. Devido a fatores geométricos (a relação entre a área superficial interna e volume é apreciavelmente grande), um capilar possibilita a dissipação eficiente do calor gerado pela passagem da corrente elétrica (efeito Joule). Além disso, a alta resistência elétrica do capilar permite o estabelecimento de campos elétricos elevados (100 a 1000 V/cm), resultando em separações de alta eficiência (geralmente excede 10^5 pratos teóricos), excelente resolução e tempos de análise bastante curtos.

A utilização de capilares de sílica fundida introduziu à técnica uma importante peculiaridade: a geração do chamado fluxo eletrosmótico. Este fluxo é conseqüência de uma interação entre a solução e as paredes do capilar. Quimicamente, a sílica é caracterizada pela presença de vários grupos silanóis, os quais, em média apresentam um caráter ácido. Em contato com o meio aquoso, alguns destes grupos são ionizados e, com a ionização, a superfície do capilar torna-se negativamente carregada, gerando um saldo positivo de espécies carregadas positivamente na solução. Quando um campo elétrico é imposto tangencialmente à superfície, forcas elétricas causam um movimento unilateral de íons em direção ao eletrodo de carga oposta. Durante a migração, os íons transportam moléculas de água, induzindo a um fluxo de solução como um todo, na direção do cátodo, conhecido como fluxo eletrosmótico normal[27-28]. Para promover a inversão do fluxo, são

adicionados ao eletrólito condutor, surfactantes catiônicos, principalmente os derivados de sais quaternários de amônio de cadeia longa. Desta forma, uma camada de semi-micelas é adsorvida na superfície do capilar, promovendo a organização de uma camada de ânions na solução, que sob a ação do campo elétrico, migra na direção do ânodo, definindo o chamado fluxo eletrosmótico invertido[29].

O volume de injeção é critico em CE. Por exemplo, um capilar de 1m x 75μm (diâmetro interno) contem cerca de 5μL de tampão e o volume da amostra devera ser menor que 50nL (para evitar carregamento excessivo).

Os métodos permitem a análise simultânea de uma faixa ampla de moléculas. Como os polifenóis são moléculas geralmente carregadas em meio alcalino, são separados pelas técnicas eletroforéticas.

A eletroforese capilar propriamente dita não é aplicável a moléculas sem carga. Entretanto, a técnica desenvolvida por Terabe et al.[30] chamada de cromatografia eletrocinética micelar (MECK), a qual é híbrida da eletroforese e cromatografia e envolve a introdução de um surfactante na solução tampão (por exemplo, SDS), é apropriada às moléculas sem carga.

O uso de MEKC em análise de flavonóides tem recebido atenção[31] e os fatores como resolução, seletividade, voltagem aplicada, temperatura do capilar, concentração e natureza do eletrólito tem sido estudados. A introdução de solventes orgânicos modifica a interação entre as micelas e solutos, alterando a retenção e resolução[32].

A separação dos flavonóides foi melhorada com a adição de SDS a pH 8,3, sendo que o aumento do pH, por exemplo a pH 10,5, teve efeito nulo ou apenas pequena influência. A separação procede pela ionização dos grupos hidroxilas[33].

Em vinhos CE foi utilizada para a análise de flavonóides em diversas condições experimentais. A *Tabela 1* mostra as principais aplicações e características dos métodos, em sua maioria nas modalidades de MEKC e CZE.

A análise de nove flavonóides em vinho, incluindo apigenina, luteolina, naringenina, kaempferol, miricetina e quercetina, foi realizada por EC-UV[34] onde as condições experimentais do método foram: preparação de amostra por extração em fase sólida (SPE), eletrólito de 35mM de bórax a pH 8.9, com detecção a 250nm.

O uso do detector eletroquímico em sistema de eletroforese na análise de vinho foi proposto[35] e o efeito do potencial do eletrodo, pH, tempo de injeção, voltagem e concentração do tampão foram estudados. Após a otimização, foi escolhida como eletrólito 100mM tampão borato a pH 9.2, o tempo de corrida sendo vinte minutos. O método otimizado foi aplicado à determinação simultânea de *trans*-resveratrol, epicatequina e catequina em vinho tinto.

CE combinada a isotacofore foi utilizada para aumentar a capacidade de separação e sensibilidade durante a análise por CZE de 14 flavonóides e fenólicos em vinho tinto[36]. Sob condições otimizadas, o tempo de corrida foi de 45 minutos e o limite de detecção foi de 30ng/mL para ácidos fenólicos, 100ng/mL para quercetina e kaempferol e 250ng/mL para catequina.

Vinte e cinco compostos polifenólicos em bebidas foram estudados por CE, comparada a HPLC. Tampões clássicos foram utilizados (borato e fosfato) e a mistura dos

Tabela 1. Principais métodos eletroforéticos para polifenóis em vinhos.

Método	***Analitos***	***Dimensões capilar (cm x µm)***	***Eletrólito***	***Detecção***	***Voltagem (kV)***	***Temperatura (°C)***	***Tempo de corrida (min)***	***Referência***
CZE	Resveratrol	65 x 25	100 mmol/L borato (pH 9.24)	eletroquímica	30	ambiente	35	Gao et al.[41]
MEKC	resveratrol	57 x 75	20 mmol/L borato 25 mmol/L polietilino glicol 400, 25 mmol/L SDS	DAD	28	25	15	Brandolini et al [42]
CZE	9 flavonóides	45 x 75	35 mmol/L borato, (pH 8.9)	250nm	24	ambiente	20	Wang et al.[34]
CE	*trans*-resveratrol, epicatequina, catequina	70 x 25	100 mmol/L borato,(pH=9.2)	eletroquímica	12	ambiente	20	Peng at al.[35]
CZE-ITP	14 fenólicos	ITP: 9.0 x 80 CZE: 16 x 30	***Eletrólito 1***: água/MeOH (4:1), 25 mmol/L MOPSO, 50 mmol/L tris, 15 mmol/L borato, pH 8.5, 0.2% 2-HEC, 5 mmol/L β-ciclodextrina ***Eletrólito 2***: água/MeOH (4:1) 25 mmol/L TAPS, 50 mmol/L tris 40 mmol/L borato, pH 8.7 0.2% 2-HEC, 5 mmol/L β-ciclodextrina	254nm	27	25	45	Hamoudová et al.[36]
CZE	11 fenólicos	50 x 7 5	100 mmol/L borato (pH 9.5)	280nm	20	30	20	Garcia-Viguera et al. Andrade et al. [37,38]
CE	ácidos fenólicos	43 x 50	50 mmol/L carbonato (pH 8.3)	UV	15	25	15	Cartoni et al.[69]
MEKC	*cis* and *trans*-resveratrol, quercetin, catequina, ácido galico	60 x 50	10 mmol/L fosfato, 6 mmol/L borato, 50 mmol/L deoxicolato de sódio (pH 9.3)	220nm	20	40	12	Prasongsidh et al [40]

CZE	*cis*- and *trans*-resveratrol	25 x 75	40 mmol/L borato (pH 9.5)	320nm	5	25	8	Berzas Nevado[71]
CE	18 compostos	57 x 75	25 mmol/L fosfato e 10 mmol/L borato (pH 8.8)	DAD	15	20	30	Minussi et al[72]
NACE	quercetina, miricetina, catequina,epicatequina e resveratrol	50;30;8.5x50	5 mmol/L ácido malônico, 9.6 mmol/L TBAOH em MeOH (pH 13.5)	230nm	10-30	25	4-35	Demianová et al[73]

CE, eletroforese capilar; CZE, eletroforese capilar de zona; MEKC, cromatografia eletrocinética micelar; ITP, isotacoforese; DAD, detector de arranjo de diodos; NACE, eletroforese capilar em meio não aquoso; SDS, dodecil sulfato de sódio; TBAOH, hidróxido de tetradecil trimetilamônio; 2-HEC, 2-hidroxietileno celulose; TAPS, acido 3-amino propanosulfônico N-[tris(hidroximetil)metil]; MOPSO, ácido β-Hidroxi-4-morfolino propanosulfonico.

mesmos, e ensaios foram realizados com aditivos como surfactante SDS (0.1M) e etanol (5%). O tampão contendo 5% de etanol apresentou melhor separação dos padrões e com adição de surfactante pôde ser separado um composto importante, o furfural. As técnicas HPLC e CE foram complementares e puderam, em conjunto, permitir a caracterização completa[37,38].

Minussi et al.[72] utilizou um método por CZE para determinação de 18 compostos fenólicos em vinhos brasileiros, portugueses, chilenos e uma amostra argentina. Estes autores também determinaram a atividade antioxidante através do cátion ABTS, utilizando acido gálico como padrão.

Em vinhos portugueses, dez compostos fenólicos foram quantificados, comparando HPLC e CZE. Pequenas diferenças nos teores foram encontradas como no caso do ácido cafeico, com valores superiores encontrados no método por CE, e flavonóis como a miricetina, kaempferol e quercetina que foram detectados por HPLC e não por CE. O método por CE foi aplicado em vinhos do porto, estudando o envelhecimento relacionado aos compostos fenólicos[38].

Resveratrol, um dos compostos antioxidantes e cardioproteores mais importantes encontrados em vinho[39] em sua forma *cis* e *trans*, foi primeiramente determinado em uvas e, posteriormente, em vinhos por eletroforese capilar juntamente com ácido gálico, catequinas, quercetina, *cis* e outros fenólicos[40]. Foi utilizado o tampão fosfato-borato a pH 9.1, o tempo de análise sendo 11 minutos. Amostras e padrões foram injetadas sem preparação previa. Geralmente estes compostos requerem métodos de pré-concentração ou extração, pois apresentam-se em baixas concentrações. Outros autores relataram

metodologias para o resveratrol[41,42,70] por eletroforese capilar, inclusive com um artigo revisão[43].

Análise de ácidos orgânicos em vinho por eletroforese capilar

A determinação de ácidos orgânicos em vinhos fornece informações relevantes no monitoramento dos processos de fermentação, gerando informações importantes sobre a estabilidade do produto, propriedades sensoriais (sabor, cor) e controle microbiologico[44].

Freqüentemente os vinhos são produzidos em duas etapas ou duas fermentações sucessivas. A primeira, a fermentação alcoólica, é realizada com leveduras, e a segunda, a fermentação malolática, que envolve a conversão do acido málico em lático e dióxido de carbono (CO_2), por bactérias láticas.

Em vinhos, existe uma diferenciação entre os ácidos provenientes da uva (tartárico, málico, cítrico) e ácidos que originam-se no processo de fermentação (succínico, acético e lático). Pequenas quantidades de outros ácidos como galacturônico, glucorônico, citramálico, dimetilglicérico, pirúvico e cetoglutárico também podem estar presentes[44]. O controle dos ácidos orgânicos, portanto, possibilita um acompanhamento de etapas importantes na produção de vinho, como processos de fermentação alcoólica e malolática e processos ligados ao envelhecimento de vinhos. Ácido tartárico, por exemplo, é um parâmetro critico para a estabilização do vinho. Os ácidos orgânicos ainda podem ser considerados decisivos no controle de adulteração em vinhos.

Vários métodos têm sido propostos para a determinação de ácidos orgânicos em vinhos como métodos enzimáticos e não-enzimáticos (Mato et al. 2005)[46]. Dentro os não enzimáticos, foram utilizados métodos baseados em cromatografia em camada delgada

(TLC)[45], cromatografia gasosa (GC)[47], cromatografia líquida de alta eficiencia (CLAE)[48] e cromatografia de íons (IC)[49].

Métodos eletroforéticos estão tornando mais preferidos e utilizados na análise de ácidos orgânicos em vinhos devido as características como simplicidade, alta resolução, preparação mínima de amostra, baixo consumo de reagentes e solventes, rápido condicionamento do sistema de análise, entre outras. A Tabela 2 mostra um resumo dos principais métodos para análise de ácidos orgânicos em vinho por eletroforese capilar de zona (CZE).

A grande maioria dos métodos utiliza capilares de sílica fundida, geralmente de 50 a 75 um de diâmetro interno. Saavedra e Barbas (2003)[50] empregaram capilares recobertos com poliacrilamida que apresentaram excelente reprodutibilidade se comparados a capilares convencionais. Os métodos principais utilizam detecção na região do UV e podem ser divididos por modos de detecção, em detecção direta e indireta[46].

Na detecção direta[50-55], a absorção do eletrólito na região do UV é menor que a dos ácidos, portanto, os analitos ao passarem pela janela de detecção do capilar geram sinal positivo. No modo indireto [56-67], a absorção do eletrólito na região do UV é maior que a dos analitos, ou seja, ao passarem pela janela de detecção, a absorbância decresce e são obtidos picos negativos. Comprimentos de onda mais utlizados estão entre 185 e 254nm.

Tabela 2. Métodos eletroforéticos para ácidos orgânicos em vinhos.

Ácidos orgânicos	*Dim. capilar (cm x μm)*	*Tampão*	*Voltagem (kV)*	*Detecção*	*Temp. (^oC)*	*Tempo de corrida (min)*	*Referência*
tartárico, málico, cítrico, succínico, acético, lático	67 x 70	7 mmol/L MES/His; 0,5 mmol/L TTAB; 30% MeOH (pH 6)	-25	condutividade	-	12	Huang et al.[69]
tártarico, málico	70 x 75	5 mmol/L ftalato; 0.5 mM TTAB; 50 mmol/L MES (pH 5.2)	-30	indireta (205nm)	20	5	Kelly and Nelson[56]
tártarico, málico, lático	100 x 75	5 mmol/L ftalato; 0.5 mmol/L CTAB (pH 5.6)	-20	indireta (254nm)	20	20	Levi et al.[57]
málico, lático, acético, succínico, cítrico	60 x 77	5 mmol/L cromato; 0.154% (p/v) PDDPi cromato (pH 8)	-30	indireta (254nm)	-	8	Stathakis and Cassidy[58]
tartárico, málico, cítrico, succínico, acético., lático	80.5 x 75	5 mmol/L PDC; 0.5 mmol/L CTAB (pH 5.6)	-25	indireta (200nm)	20	7	Soga[59]
tartárico, málico, succínico, adípico, glutámico, acético, lático, shikimico.	48 (UV) 60 (cond.) x 50	7.5 mmol/L PAB 10.5 mmol/L BIS TRIS; 0.1 mmol/L TTAB (pH 7.0)	-30	condutividade e indireta (254nm)	-	7 (cond.). 8 (UV)	Klampfl et al.[60]
tartárico, málico, succ., acético, lático	60 x 75	3 mmol/L fosfato; 0.5 mmol/L MTAB (pH6.5)	-20	direta (185nm)	-	6	Castiñeira et al[54,55]
tartárico, málico, succínico, cítrico, acético, lático	65 x 50	3 mmol/L BTA; 15mmol/L Tris; 1.5 mmol/L TEPA (pH 8.4)	-25	indireta (240nm)		9	Sing Fung and Man Lau[61]

acético, cítrico, fumárico, lático, málico, oxálico, succínico, tartárico	50 x50 recoberto poliacrilamida	200 mmol/L fosfato (pH 7.50)	-14	direta (200nm)	20	13	Saavedra and Barbas[50]
tartárico, málico, succínico, cítrico, acético, lático	110 x 75	7.5 mmol/L PDC; 0.5 mmol/L CTAB; 0.5 mmol/L EDTA (pH 5.6)	0 a -22 kV em 0,5 min	indireta (210nm)	15	18	De Villiers et al.[62]
tartárico, málico, succínico, acético, lático	78 x 75	5 mmol/L PDC; 0.5 mmol/L CTAB (pH 5.6)	25	indireta (200nm)	18	7.5	Esteves et al.[63]
cítrico, tartárico, málico, succínico, acético, lático	100 x 75	5 mmol/L ftalato; 0.5 mmol/L OFM – anion BT (pH 7)	- 20	indireta (254 mm)	-	15	Kenney[64]
tartárico, málico, succínico, cítrico, acético, lático	44 x 75	3 mmol/L PMA; 3 mmol/L EDTA (pH 7.5)	- 20	indireta (220 nm)	30	12	Arellano et al.[65,66]
tartárico, málico, cítrico, succínico, acético, lático	80.5 x 75	5 mM PDC; 0.5 mmol/L CTAB (pH 5.6)	-25	indireta (200 nm)	18	7.5	Kandl and Kupina[67]

succínico, málico, tartárico, acético	60 x 75	10 mmol/L tetraborato; 0.5 mmol/L TTAOH; 10 ppm Ca^{2+} and Mg^{2+} (pH 9.3)	-7	direta (185 nm)	20	20	Garcia-Moreno et al.[59] Moreno et al.[51,52]
tartárico, málico, succínico, acético, cítrico, lático	60 x 75	7.5 mmol/L NaH_2PO_4; 2.5 mmol/L Na_2HPO_4; 2.5 mmol/L TTAOH; 0.24 mmol/L $CaCl_2$ (pH 6.40)	-25	direta (185 nm)	25	3.5	Mato et al.[53]

MÊS, ácido etanosulfônico 2-(*N*-morfolino); HIS, histidina; TTAB, brometo de tetradecil trimetilamônio; PDC ácido piridinodicarboxílico; CTAB, brometo de cetiltrimetilamônio; BIS-TRIS, bis(2-hidroxietil)imino-tris(hidroximetil)aminometano; MTAB, brometo de miristil trimetilamônio; BTA1,3,5-benzeno tricarboxílico; PMA, ácido piromelitico; EDTA, etilenodiamintetracetato;TTAOH, hidróxido de tetradecil trimetilamônio; PAB, ácido 4 aminobenzóic

Os métodos eletroforéticos para a análise de ácidos orgânicos em vinho, utilizam geralmente os tampões bis(2-hidroxietil)imino-tris(hidroximetil)aminometano (BIS-TRIS)[60,61], ácido bórico[59,60], 1,3,5-benzeno tricarboxílico (BTA)[61], ácido etanosulfônico 2-(*N*-morfolino) (MES)[56], cromato[58], ácido 4-aminobenzóico (PAB) [60], fosfato[50,53], ftalato[57,64], ácido piridinodicarboxílico (PDC)[62,63], ácido piromelitico (PMA)[65,66] ou tetraborato[51,52]. São também adicionados ao eletrólito, modificadores (inversores) do fluxo eletrosmótico (surfactantes catiônicos) como brometo de cetiltrimetilamônio (CTAB),[67] etilenodiamintetracetato (EDTA)[62], brometo de miristil trimetilamônio (MTAB)[54,55], brometo de tetradecil trimetilamônio (TTAB)[56,60] ou hidróxido de tetradecil trimetilamônio (TTAOH) [51,52]. Em relação ao tempo de análise, métodos eletroforéticos apresentam grande vantagem em relação aos métodos por HPLC aplicados a análise de ácidos orgânicos em vinhos. A maioria dos métodos apresenta tempo de corrida inferior a 15 minutos. O método apresentado por Mato et al.[53] por detecção direta a 185nm requereu apenas 3,5 minutos.

Como desvantagem principal apontada por alguns autores, a reprodutibilidade é considerada inferior quando comparada a métodos enzimáticos e cromatográficos. Isso, porém, pode ser contornado com a utilização de padrão interno, com o qual o tempo de migração e área dos analitos são relacionados. Padrões internos mais utilizados são ácido bútirico,[64] ácido oxálico[53], entre outros.

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CAPÍTULO 3

HPLC-DAD-ESI/MS Identification and Quantification of Phenolic Compounds in *Ilex paraguarienses* Beverages and On-line Evaluation of Individual Antioxidant Activity

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HPLC-DAD-ESI/MS Identification and Quantification of Phenolic Compounds in *Ilex paraguarienses* Beverages and On-line Evaluation of Individual Antioxidant Activity

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KEYWORDS: Flavonols; antioxidant capacity; Ilex paraguarienses; beverages; HPLC-DAD-ESI-MS; phenolics

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Abstract

"Chimarrão" and "tererê" are mate (*Ilex paraguariensis*) infusions widely consumed in South America. This paper describes the application of HPLC-DAD-ESI/MS method for the identification and quantification of caffeoylquinic acids (CQA), flavonol glycosides and purine alkaloids in these beverages. Infusions were prepared from commercial samples of *Ilex paraguarienses* from Southern Brazil. The cafeoylquinic acids, 4,5-diCQA, 3-CQA, 5-CQA, and 4-CQA were the major compounds, having 238-289, 153-242, 183-263, and 123-188 μg/mL, respectively, for chimarrão and 206-265, 122-218, 164-209, 103-169 μg/mL, respectively for tererê. Caffeine also had high amounts while glycosides of quercetin and kaempferol were found at much lower levels. The individual antioxidant activity was also determined by an on-line system that measured their $ABTS^{\cdot+}$ radical scavenging activity, showing that the antioxidant capacity was not proportional to the concentrations of the phenolic compounds. 3-CQA, quercetina-3-*O*-ramnosylglucoside, and quercetina-3-*O*-glucoside were the major contributors to the antioxidant capacity, although the quercetin glycosides had concentrations less than 10 times that of 3-CQA.

INTRODUCTION

"Mate" or "yerba mate" (*Ilex paraguarienses* St. Hilaire) is used to prepare tea-like beverages widely consumed in South America. It is estimated that 30% of the population in this region drink over 1 liter per day of mate infusions (*1*). Around 80% of the planted area is in Brazil (*2*), where it contributes very positively to the local economy with an estimated production of 650,000 tons of mate per year (*3*). Typical beverages from mate consumed in Brazil are "chimarrão" (infusion prepared from dry mate with hot water) and "tererê" (infusion prepared with cold water).

Some therapeutic properties have been attributed to mate such as antirheumatic, hypocholesterolemic, anti-thrombotic, anti-inflammatory, anti-obesity, anti-aging, hepatoprotective and diuretic activities (*1, 4-9*). Some of the pharmacological properties have been attributed to the high content of phenolic compounds, especially caffeoyl derivatives (*6, 7, 10-12*).

High performance liquid chromatography with diode array and mass detectors (HPLC-DAD-MS) has been used to identify the phenolic compounds of *Ilex paraguariensis*. Using an atmospheric pressure chemical ionization (APCI) interface in the negative-ion mode and collision-induced dissociation (CID) of precursor ions, along with UV-diode array detection, Carini et al. (*13*) analyzed mate from Argentina and identified 10 constituents: three naturally occurring caffeoylquinic acid (neo-chlorogenic, chlorogenic and crypto-chlorogenic acids), three isomeric dicaffeoylquinic acids, rutin (quercetin-3-rutinoside), a diglycosyl derivative of luteolin, and two caffeoyl-glucosides isomers. Quantification was not carried out, however. Also analyzing Argentinian mate by the UV

and MS (ESI) spectra, 28 compounds were identified by Bravo et al. (*14*), including seven of those reported by Carini et al.*(13)* Percentages of polyphenol groups were presented.

Phenolic compounds in *Ilex paraguariensis* were quantified in some articles (*7, 11, 12*), confirming the predominance of chlorogenic acid derivatives, but the data were expressed as total contents or relative percentages. The antioxidant properties have also been studied in chemical and biological systems (*13-21*).

Aqueous extracts of *Ilex paraguariensis* have been shown to scavenge superoxide anions and the 2,2,-diphenyl-1-picrylhydrazyl (DPPH) radical (*13, 21*) and inhibit oxidation induced by 2,2'-azobis-(2-aminopropane) hydrochloride (AAPH) (*9, 16, 21*). The ferric reducing antioxidant power (FRAP) and 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) (*14*) assays have also been employed. *In vivo*, water extracts of mate were evaluated with healthy volunteers and the results suggested that *Ilex paraguariensis* antioxidants reached the plasma, increased the aqueous phase antioxidant capacity of the plasma and inhibited copper-induced autoxidation of LDL (*15*).

Numerous papers assessing the antioxidant activity of extracts of foods and beverages have been published. The values obtained, however, represent the total antioxidant capacity of the food. Although often correlated or attributed to some food constituent groups (e.g., polyphenols), these are presumed rather than direct measurements and the contribution of individual compounds is not known. Recently, on-line HPLC methods for the evaluation of the individual antioxidant activity of food constituents have been introduced (*22, 23*). The HPLC-separated analytes react postcolumn with preformed radical cation $ABTS^{\cdot+}$ (*22*) or radical $DPPH^{\cdot}$ or inhibit luminol chemilumiscence (CL) (*23*). In the on-line $ABTS^{+}$ method, the eluate reacts with a stabilized solution of $ABTS^{+\cdot}$ radical and the absorbance is monitored at high wavelengths such as 720 nm or 600 nm. The

solution with the radical has a deep blue color and if a quenching reaction occurs, there will be a loss of color and a negative peak will be registered. This method was applied to green and black tea samples, using HPLC-MS for identification of the phenolic compounds (*24*).

The objectives of this work were to identify and measure the amounts of the principal phenolic compounds in the Brazilian beverages chimarrão and tererê by HPLC-ESI/MS and HPLC-DAD and evaluate their individual contribution to the total antioxidant capacity through an on-line HPLC system.

MATERIALS AND METHODS

Chemicals. Standards of the flavonoids quercetin-3-rhamnosylglucoside, quercetin-3-*O*-glucoside, kaempferol-3-*O*-glucoside and 3,5-dicaffeoylquinic acid were purchased from Extrasynthese (Genay, France); caffeine, teobromine and 5-*O*-cafeoylquinic acid were obtained from Sigma Chemical Company (St. Louis, MO, EUA). HPLC-grade methanol and acetonitrile were purchased from Tedia Company (Fairfield, OH, USA). The 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid was from ICN Biomedicals Inc. (Aurora, OH, USA) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and butyl-hydroxyanisol (BHA), from Aldrich (St. Louis, MO, EUA). Membrane filters were purchased from Millipore (Bedford, MA, USA).

Stock standard solutions were prepared at a concentration of 1 mg/mL in methanol and stored at -20^{o}C for a maximum of two months. Water was purified using a Milli-Q system from Millipore Corporation (Millipore, Bedford, MA, USA).

Sample Preparation. The mate samples (S1 to S6) were purchased from supermarkets in São Paulo, Brazil, and were produced in the states of Paraná, Santa Catarina, and Rio Grande do Sul (two sample lots from each state). Two packages of each sample lot were ground to pass a 40-mesh screen and mixed.

Chimarrão was prepared in duplicate by adding 250 mL of deionized water at 70^{o}C to 5g of the sample. The infusion was stirred for 3 min and then filtered through a 0.45 μm membrane filter (Millipore, Bedford, MA, USA) (*25*). Tererê was prepared using the same procedure, but the water temperature was 5^{o}C.

An aliquot of chimarrão or tererê was diluted 20-fold with deionized water and injected in the HPLC system.

Preparative HPLC. 3-Caffeoylquinic acid (CQA) and 4-CQA have the same MS and UV spectra and are not commercially available as standards. So it was necessary to prepare (*26*) and isolate them by preparative HPLC (*27*). For this, the isomers were separated and collected using a preparative Phenomenex C_{18} column, 15 μm, 250 x 21.2 mm (Torrance, CA, USA). The mobile phase and gradient system were those employed by Negishi et al. (*27*). These authors identified the isomers by MS and NMR. The preparative HPLC procedure was performed on a Waters Prep System (Milford, MA, USA), comprising of a HPLC Prep pump model 600E, Rheodyne injector, dual absorbance detector model 2487, a preparative flow cell, and a fraction collector (WFC) connected to the recorder model BD40 (Kipp & Zonen, Delft, The Netherlands).

HPLC analysis. The HPLC equipment used for the separation and quantification of the phenolic acids consisted of a Waters alliance model 2695 separation module with a photodiode array detector model 996, equipped with an X-Bridge C_{18} column, 5μm, 4.6 x 150 mm, a microbore detection cell and, for data collection and treatment, an Empower 2

software (Waters Corporation, Milford, MA, USA). The system was thermostated at 35°C with a Waters temperature control module and the flow rate was 1.0 mL/min. A binary convex gradient was employed with the following solutions: H_2O/formic acid (99:1 v/v) as phase A and acetonitrile/formic acid (99:1.0 v/v) as phase B. Starting with 95A/5B, the proportion was altered to 40A/70B in 5 min, then to 20A/80B in 15 min, and to 95A/5B in 30 min. The injection volume was 10 μL.

Quantification was done by external standardization, using the respective standards. However, tricaffeoylquinic acid, for which there is no commercially available standard, was estimated from the 3,5-dicaffeoylquinic acid curve.

HPLC-ESI/MS analysis. The extracts were analyzed by a Micromass ZMD quadrupole mass spectrometer, with electrospray interface and MassLysnx software version 3.5 (Micromass UK Ltd, Manchester, UK) for data acquisition.This instrument was coupled to a Waters separation module, model 2695, equipped with a photodiode detector model 996 and a microbore cell (Waters Corporation, Milford, MA, USA). The mass spectrometer parameters were set as follows: ionization mode, electrospray positive ion; capillary voltage, 4.0 kV; source block temperature, 140°C; desolvation temperature, 400°C; nebulizer nitrogen flow rate, 50 L/hr; desolvation nitrogen gas flow, 568 L/hr; LM resolution, 10; HM resolution, 12; ion energy, 1.0 V; cone voltage, 35 V; RF lens, 0.5 V; extractor lens, 5 V. Spectra were recorded by scanning the mass range from m/z 100 to 1000 with scan time of 1 s.

Preparation of $ABTS^{\cdot+}$ solution. ABTS (2 mmol/L) was dissolved in 10 mmol/L phosphate-buffered solution and adjusted to pH 7.4 with ammonium persulfate solution in ultrapure water (0.5 mmol/L final concentration) to produce the $ABTS^{\cdot+}$. To maximize the

conversion of ABTS to $ABTS^{\cdot+}$, the mixture was prepared in an amber glass volumetric flask and stored in the dark at room temperature for 16 h under constant stirring. Fresh working solutions of the appropriate concentrations were prepared daily; the final dilution was 1:40 v/v in water.

On-line $ABTS^{\cdot+}$ assay. Determination of the antioxidant activity was based on the $ABTS^{\cdot+}$ assay of Koleva et al. *(22)*. The on-line set-up utilized in the present study is shown in **Figure 1**. The HPLC equipment consisted of a Shimadzu HPLC system pump model LC-10ADvp, system controller model SCL-10Avp, an autosampler Waters model 717Plus, DAD detector model SPD-M10Avp, controlled by a Class VP software v. 5.03. Separation was carried out on a Synergi Max-RP C_{12} column, 4 μm, 4.6 x 250 mm (Phenomenex, Torrance, CA, USA) and the flow rate was 1.0 mL/min. The HPLC column and the PEEK (polyether ether ketone) tubing were maintained at 35°C, using a Waters column heater, model TCM/CHM (Waters Corporation, Milford, MA, USA). When the HPLC eluent from the DAD detector (recorded from 200 to 600 nm and monitored at 280 nm) arrived at a connection in "T" form (Altech, Deerfield, IL, USA) where the $ABTS^{\cdot+}$ was added, the compound reacted with the cation radical and decolorization was monitored by a second detector, a Waters UV/VIS tunable absorbance detector, model 486. The antioxidant on-line part of the system was controlled and monitored by Waters Empower 2 software. The $ABTS^{\cdot+}$ solution flow rate was 0.8 mL/min, delivered by a Waters 515 HPLC pump. After mixing through a 13.7 x 0.25 mm i.d. PEEK tubing (Upchurch Scientific Corporation, Oak

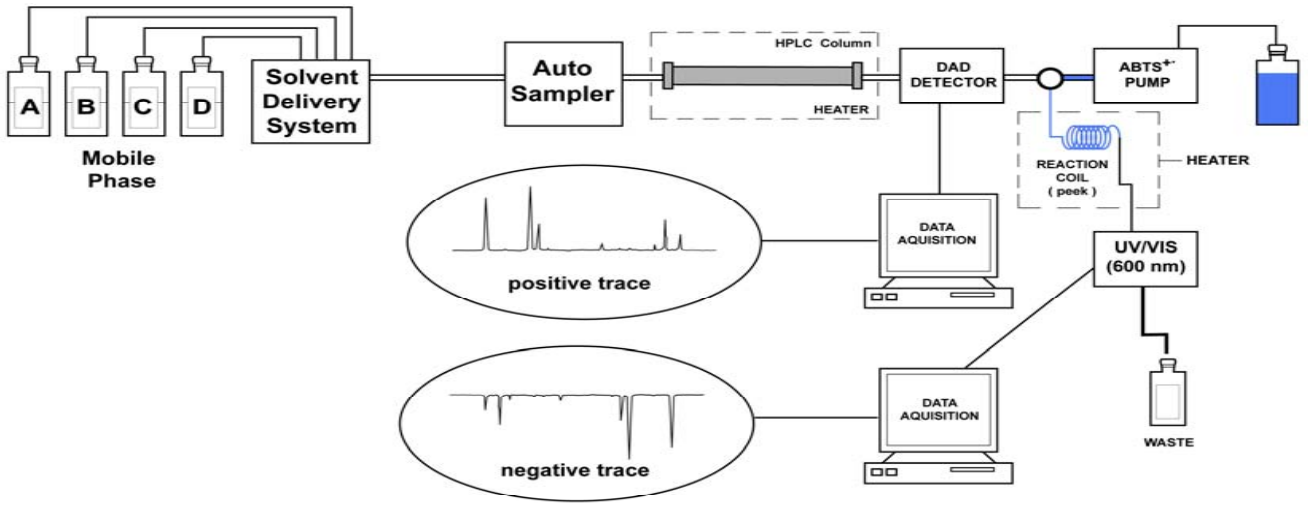


Figure 1. Set-up for the HPLC-ABTS$^{\cdot+}$ on-line system.

Harbor, WA , USA) (used as reaction coil), the absorbance was measured by a Waters tunable absorbance detector, model 486, set at 600 nm.

To calculate the antioxidant activity, Trolox was injected with the extracts at three concentrations to construct the calibration curves. The Trolox equivalent antioxidant capacity (TEAC) was calculated by dividing the slope of the compound's curve by the slope of the Trolox curve (*22*).

RESULTS AND DISCUSSION

Identification of the phenolic compounds and purine alkaloids of *Ilex paraguariensis* infusions. The typical chromatogram of the phenolics and purine alkaloids of *Ilex paraguariensis* influsions is shown in **Figure 2**. The structures of the compounds identified are in **Figures 3** and **4**. The HPLC-ESI/MS and UV data used for identification are presented in **Table 1**, which were compared with those of Bravo et al. (*14*).

Peak 1 was identified as caffeine. It had a retention time of 13.9 min, maximum absorbance at 272 nm (with typical spectra of methylxanthine), and positively charged molecular ion $[M+H]^+$ at m/z 195. It co-chromatographed with caffeine standard.

Peaks 2, 3, and 5 had similar UV spectra (λ_{max} at 326 nm, shoulder at 296 nm), typical of chlorogenic acid. Their MS spectra exhibited $[M+H]^+$ at m/z 355, indicative of isomers of chlorogenic acid ($C_{16}H_{18}O_{19}$ (M.W. 355), which had been reported as important

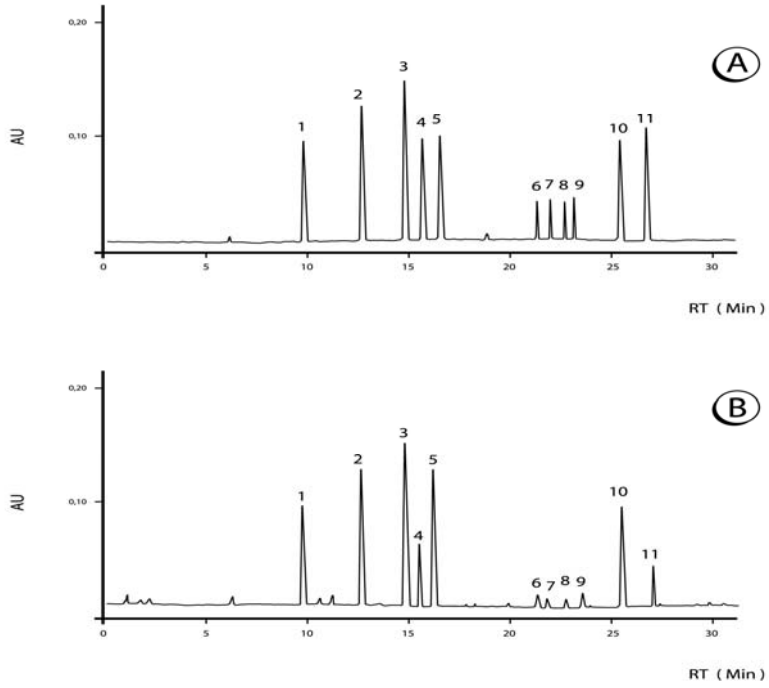
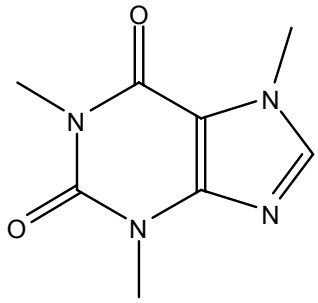
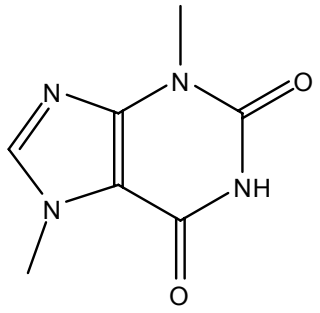


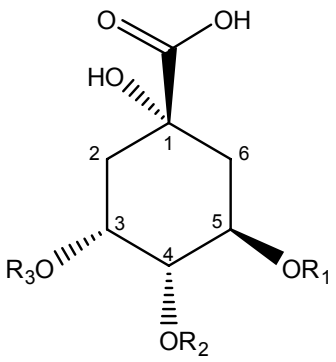
Figure 2. Chromatograms of (A) standards and (B) phenolic compounds of *Ilex paraguariensis* infusion. Peak identification: 1, caffeine; 2, 3-*O*-Caffeoylquinic acid; 3, 5-*O*-Caffeoylquinic acid; 4, Teobromine; 5, 4-*O*-Caffeoylquinic acid; 6, Quercetin-3-rhamnosylglucoside; 7, Quercetin-3-*O*-glucoside; 8, Kaempferol-3-*O*-glucoside; 9, 3,5-Dicaffeoylquinic acid; 10, 4,5-Dicaffeoylquinic acid; 11, Tricaffeoylquinic acid. Chromatographic conditions are described in the text.



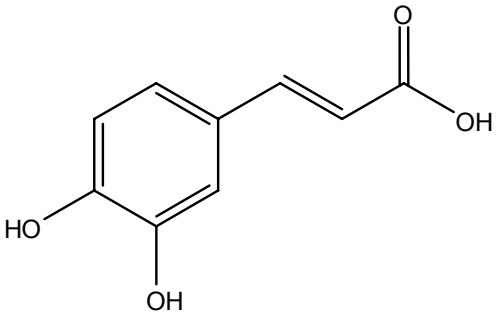
Caffeine



Teobromine



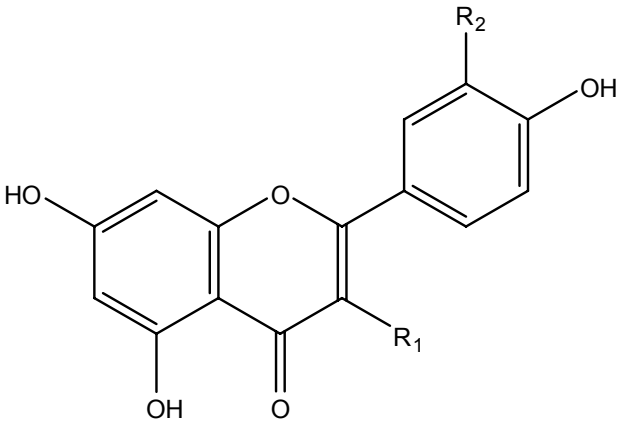
Quinic acid



Caffeic acid

Compound	R_1	R_2	R_3
3-CQA	H	H	caffeic acid
4-CQA	H	caffeic acid	H
5-CQA	caffeic acid	H	H
3,5-diCQA	caffeic acid	H	caffeic acid
4,5-diCQA	caffeic acid	caffeic acid	H
tri-CQA	caffeic acid	caffeic acid	caffeic acid

Figure 3. Structures of purine alkaloids and caffeoylquinic compounds found in *Ilex paraguariensis* infusions.



Compound	R_1	R_2
Quercetin-3-rhamnosylglucoside	*O*-rutinose	OH
Quercetin-3-*O*-glucoside	*O*-glucose	OH
Kaempferol-3-*O*-glucoside	*O*-glucose	H

Figure 4. Structures of flavonols found in *Ilex paraguariensis* infusions.

Table 1. Identifying characteristics of the phenolic compounds and purine alkaloids of infusions from *Ilex paraguariensis.*

Peak	RT (min)	λ_{max} nm	$[M+H]^+$	Fragment ions	Structure attribution
1	13.9	272	195		Caffeine
2	16.8	326,296sh	355	193,181	3-*O*-Caffeoylquinic acid
3	19.8	326,296sh	355	193,181	5-*O*-Caffeoylquinic acid
4	19.8	272	181		Teobromine
5	20.1	326,296sh	355	193,181	4-*O*-Caffeoylquinic acid
6	24.2	256,354	611		Quercetin-3-rhamnosylglucoside
7	25.0	256,344	465	303	Quercetin-3-*O*-glucoside
8	25.6	266,342	449		Kaempferol-3-*O*-glucoside
9	27.4	326,296sh	517	355,193	3,5-Dicaffeoylquinic acid
10	27.8	326,296sh	517	355,193	4,5-Dicaffeoylquinic acid
11	28.5	326,296sh	679	517,181,163	Tricaffeoylquinic acid

sh, shoulder

and major constituents of mate (*6, 26*). Peak 3 co-chromatographed with the 5-CQA standard and peaks 2 and 5 with 3-CQA and 4-CQA standards prepared as described in the experimental section. The order of elution was the same as that of Bravo et al. (*14*) and Negishi et al. (*28*).

Peak 4 was identified as teobromine by the UV (λ_{max} at 272 nm) and MS (with $[M+H]^+$ at m/z 181) spectra and co-chromatography with its standard.

Minor peaks 6, 7 and 8 were identified as flavonoids from the UV and MS spectral data. Peak 6 (λ_{max} at 256 and 354 nm, $[M+H]^+$ at m/z 611) was identified as quercetin-3-rhamnosylglucoside (quercetin-3-rutinoside) known as rutin. Peak 7 (λ_{max} at 256 and 344 nm, $[M+H]^+$ at m/z 465) was identified as quercetin-3-*O*-glucoside and peak 8 (λ_{max} at 266 and 342 nm, $[M+H]^+$ at m/z 449) as kaempferol 3-*O*-glucoside. These compounds co-chromatographed with the respective standards.

Peaks 9 e 10 both had $[M+H]^+$ at m/z 517, indicative of dicaffeoyl quinic acid isomers, which were found in mate leaves by some authors (*6,11*). The loss of one dehydrated caffeic acid molecule $[M\text{-caffeic acid-}H_2O]^+$, giving the fragment at 355 m/z, the elution profile similar to that reported in C_{18} columns by others authors (*11,28*), and the UV data (λ_{max} at 326 nm, shoulder at 296 nm) all agreed to the identification of peaks 9 and 10 as 3-5-dicaffeoylquinic and 4-5 dicaffeoylquinic acids. Additionally, peak 9 co-chromatographed with the standard of 3,5-dicaffeoylquinic acid.

Another major compound found in the mate beverages was peak 11 with UV spectra (λ_{max} at 326 nm, shoulder at 296 nm) and $[M+H]^+$ at m/z 679 of a caffeoyl quinic derivative. The fragment at m/z 517 could be attributed to tricaffeoylquinic acid with a loss of a dehydrated molecule of caffeic acid that corresponded to the fragment at m/z=163.

Tricaffeoylquinic acid was cited by Yoshimoto et al. (*28*) in sweet potato and by Bravo et al. (*14*) in mate extracts, identified by HPLC/MS.

Quantification of the phenolic compounds and purine alkaloids of *Ilex paraguariensis* infusions. Table 2 shows the concentrations of phenolic acids and purine alkaloids of the chimarrão e tererê infusions prepared from mate. The methylxanthine caffeine varied from 124 to 268 μg/mL for chimarrão and from 114 to 246 μg/mL for tererê, while teobromine varied from 64 to 126 μg/mL for chimarrão and 54 to 114 μg/mL for tererê. This agrees with the work of Clifford and Ramirez-Martinez *(12),* in which caffeine levels were approximately twice as much as those of teobromine. Bastos et al. *(29)* encountered slightly higher values (150-294 μg/mL) for caffeine in chimarrão prepared from *Ilex paraguariesis* from the state of Parana. The samples in this work came from three states.

The ranges for 3-CQA, 5-CQA, and 4-CQA in the present work were 153-242, 183-263, and 123-188 μg/mL, respectively, for chimarrão and 122-218, 164-209, 102-169 μg/mL, respectively for tererê. Values in the literature are given as chlorogenic acid or 5-CQA, without distinguishing the three isomers.

Three flavonol glycosides (quercetin-3-rhamnosylglucoside, quercetin-3-*O*-glycoside e kaempferol-3-*O*-glycoside) were detected and quantified (**Table 2**). The first glycoside varied from 8 to 18 μg/mL in chimarrão and from 7 to 12 μg/mL in tererê. The ranges for the second glycoside were 6-13 μg/mL for chimarrão and 4-12 μg/mL for tererê, and for the third glycoside trace levels to16 μg/mL for chimarrão and trace levels to 12 μg/mL for tererê. The flavonol concentrations presented in the literature are in terms of the

Table 2. Concentrations (μg/mL) of Phenolic Compounds and Purine Alkaloids of Infusions of *Ilex paraguariensis.*

Compound	S1		S2		S3		S4		S5		S6	
	Chimarrão	**Tererê**	**Chimarrão**	**Tererê**	**Chimarrão**	**Tererê**	**Chimarrão**	**Tererê**	**Chimarrão**	**Tererê**	**Chimarrão**	**Tererê**
Caffeine	146±11	124±9	265±12	234±10	190±13	168±9	124±8	114±9	268±12	246±10	197±15	175±11
3-*O*-Caffeoylquinic acid	183±6	167±4	153±7	122±5	193±8	168±8	166±4	135±7	189±5	169±6	242±9	218±4
5-*O*-Caffeoylquinic acid	263±4	203±5	212±9	199±6	183±8	164±10	213±12	209±7	219±8	193±6	210±9	185±6
Teobromine	72±3	57±3	125±9	114±8	102±6	77±5	64±7	54±4	126±8	113±1	90±4	73±3
4-*O*-Caffeoylquinic acid	153±8	125±4	188±4	169±9	139±8	114±12	153±7	133±1	130±5	104±3	123±8	102±6
Quercetin-3-rhamnosylglucoside	10±1	9±1	12±2	11±2	10±1	8±1	10±1	8±1	18±1	12±1	8±1	7±1
Quercetin-3-*O*-glucoside	12±1	10±1	13±1	12±1	13±2	7±1	8±1	6±1	10±1	11±1	6±1	4±1
Kaempferol-3-*O*-glucoside	9±1	7±1	10±1	8±1	tr	tr	tr	tr	12±1	13±1	16±1	15±1
3,5-Dicaffeoylquinic acid	112±7	103±8	138±8	112±8	167±7	123±3	145±5	123±6	156±3	126±4	165±4	145±5
4,5-Dicaffeoylquinic acid	238±8	206±7	278±6	254±7	289±6	256±8	267±7	224±8	279±5	239±6	287±5	265±8
Tricaffeoylquinic acid[a]	125±9	102±5	128±7	106±4	156±6	134±5	136±4	108±5	143±6	139±6	154±4	143±5

[a]Estimated from the 3,5-dicaffeoylquinic acid calibration curve. tr, trace levels.

aglycone, not the glycoside form. Comparison of quantification of flavonols in the glycoside and aglycone forms is the subject of another paper.

For 3,5-dicaffeoylquinic acid, the ranges were 112-167 μg/mL for chimarrão and 103-145 μg/mL for tererê; the corresponding ranges for 4,5-dicaffeoylquinic acid were 238-289 and 206-265 μg/mL. Comparison could not be made with the literature because these acids were quantified in terms of the dried leaves (*7*).

Tricaffeoylquinic acid ranged from 125 to 156 μg/mL in chimarrão and 102 to 143 μg/mL in tererê. This acid has not been previously quantified.

It is noteworthy that the concentrations of all the compounds quantified were lower in tererê than in chimarrão, which is coherent with the fact that the former was prepared with cold water and the latter with hot water, reflecting better extraction of the compounds studied in the latter infusion.

Antioxidant activity of the phenolic compounds. **Figure 4** shows the HPLC profile of the infusions (positive trace indicates the absorbance obtained with the DAD detector at 280 nm and the negative trace the absorbance obtained by the second detector at 600 nm). An indication that the on-line measurement was coherent is the fact that caffeine (peak 1), which is not an antioxidant, did not give a negative peak. In the column used for the antioxidant activity, teobromine co-eluted with 5-CQA, giving the highest positive peak and a small negative peak because teobromine is not an antioxidant.

Table 3 displays the mean concentrations of the phenolic compounds and the TEAC value, clearly showing that the antioxidant capacity was not proportional to the analytes' concentrations. 3-CQA, quercetin-3-*O*-glucoside, quercetin-3-rhamnosylglucoside had the highest TEAC values (about 1.00), although the quercetin glycosides had concentrations

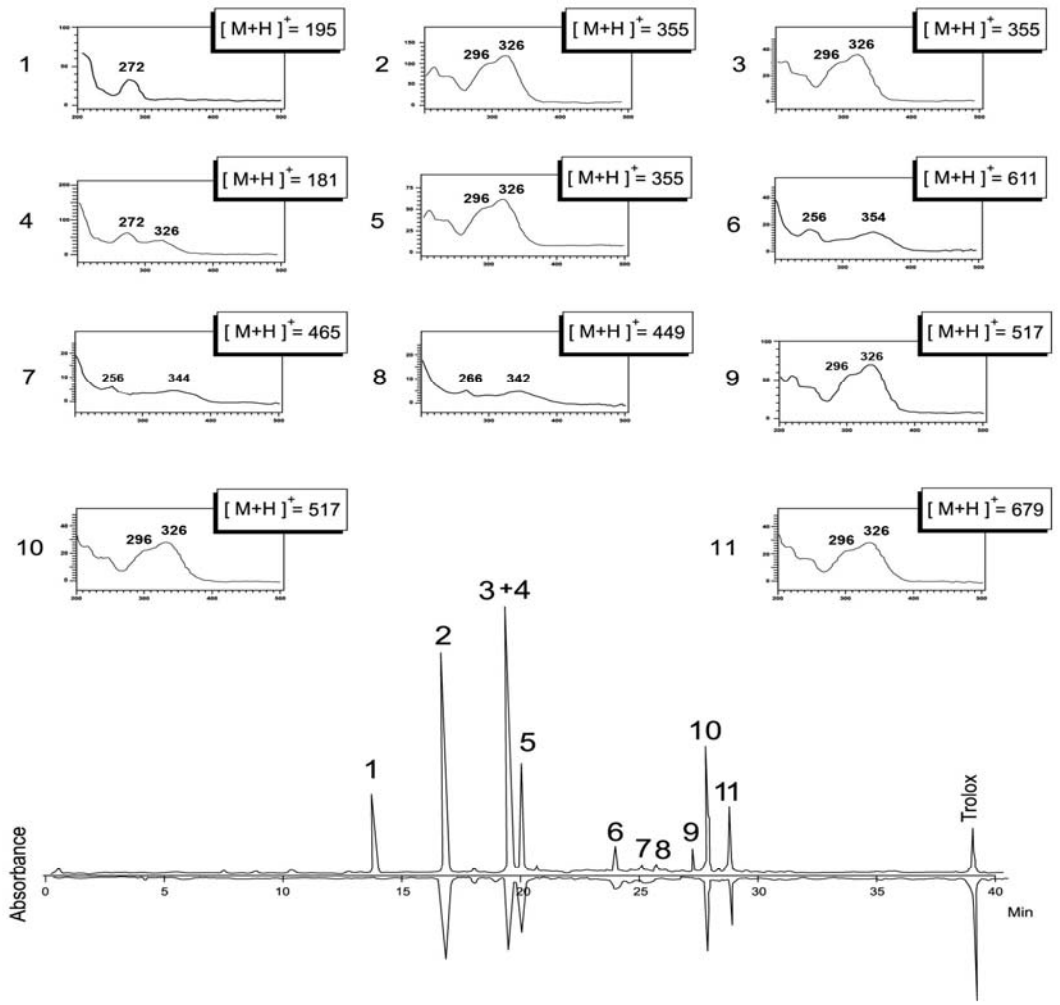


Figure 4. On-line HPLC ABTS^{+} analysis of the *Ilex paraguarienses* beverage chimarrão. Positive Trace: PDA at 280 nm and Negative Trace: UV-VIS at 600 nm. Peak identification: 1, caffeine; 2, 3-*O*-Caffeoylquinic acid; 3, 5-*O*-Caffeoylquinic acid; 4, Teobromine; 5, 4-*O*-Caffeoylquinic acid; 6, Quercetin-3-rhamnosylglucoside; 7, Quercetin-3-*O*-glucoside; 8, Kaempferol-3-*O*-glucoside; 9, 3,5-Dicaffeoylquinic acid; 10, 4,5-Dicaffeoylquinic acid; 11, Tricaffeoylquinic acid. Chromatographic conditions are described in the text.

Table 3. Individual antioxidant capacity of the phenolic compounds of chimarrão (*Ilex paraguariensis).*

Compound	Mean Concentration (µg/mL)	TEAC (a_c/a_T)
3-*O*-Caffeoylquinic acid	187.6	1.01
5-*O*-Caffeoylquinic acid	216.6	0.76
4-*O*-Caffeoylquinic acid	147.6	0.87
Quercetin-3-rhamnosylglucoside	11.3	1.00
Quercetin-3-*O*-glucoside	10.3	1.01
Kaempferol-3-*O*-glucoside	11.7	0.92
3,5-Dicaffeoylquinic acid	147.2	0.83
4,5-Dicaffeoylquinic acid	273.0	0.80
Tricaffeoylquinic acid	140.3	0.95
Trolox	-	1.00

TEAC, trolox equivalent antioxidant capacity; a_c, slope of compound calibration curve; a_T, slope of trolox calibration curve.

less than 10 times that of 3-CQA. Tri-CQA, kaempherol-3-*O*-glucoside, and 4-*O*-CQA had TEAC values of 0.95, 0.92 and 0.87, respectively, although the CQA´s had levels more than 10 times that of the kaempferol glycoside. The major phenolic compounds of the mate infusions, 4,5-diCQA and 5-CQA, had lower TEAC values.

LITERATURE CITED

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CAPITULO 4

Quantification of Phenolic Compounds and Purine Alkaloids in *Ilex paraguarienses* Infusions

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Quantification of phenolic compounds and purine alkaloids in *Ilex paraguarienses* infusions

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Abstract

Hot and cold infusions of mate (*Ilex paraguariensis* dried leaves) are widely consumed in Brazil. These infusions have been shown to have beneficial effects on health, which have been mainly attributed to their phenolic compounds. This work had a two-fold objective: (1) compare quantification of flavonols in the glycosidic and aglycone forms, and (2) compare the phenolic and purine alkaloid composition mate taken from two stages of industrial production and of hot and cold infusions. The HPLC quantitative method used for the quantification of the flavonol aglycones obtained by hydrolysis and that used for quantifying the glycosides, other phenolics and the purine alkaloids performed well. Both methods showed good linearity, limits of detection and quantification, recovery and repeatability. For all thirteen samples, analyzed as hot or cold infusion, equivalent results were obtained for quercetin and kaempherol, thus these flavonols are amenable to quantification either in the glycoside or aglycone form. Understandably, the levels of all the analytes in all the samples were lower in the cold infusion. In both infusions the phenolic and purine alkaloid concentrations tended to be higher in the samples obtained from the producers than in the commercial samples, indicating that losses occurred in the additional processing and probably storage that the latter samples underwent.

1. Introduction

The dried leaves of *Ilex paraguarienses* St. Hilaire commonly called "mate" or "yerba mate" is used to prepare tea-like beverages widely consumed in South America. It is estimated that 30% of the population in this region drink over 1 liter per day of mate infusions (Delacassa and Bandoni, 2001). Around 80% of the planted area is in Brazil with an estimated production of 650,000 tons of mate per year (Maccari, 2005). Typical beverages from mate consumed in Brazil are "chimarrão" (infusion prepared with hot water) and "tererê" (infusion prepared with cold water).

Some therapeutic properties have been attributed to mate such as antirheumatic, hypocholesterolemic, anti-thrombotic, anti-inflammatory, anti-obesity, anti-aging, hepatoprotective and diuretic activities (Gonzalez et al., 1993; Athayde et al., 2000; Delacassa and Bandoni, 2001; Filip et al., 2000, 2001; Andersen and Fogh, 2001; Gugliucci, 2002). Some of the pharmacological properties have been attributed to the antioxidant activity of the high content of phenolic compounds, especially caffeoyl derivatives (Clifford and Ramirez-Martinez, 1990; Gugliucci, 1996; Mazzafera, 1997; Filip et al., 2000; 2001).

The phenolic compounds of Argentinian mate have been identified by high performance liquid chromatography with diode array and mass detectors (HPLC-DAD-MS) (Carini et al., 1998; Bravo et al., 2007). However, quantification was not carried out in the first paper and only relative percentages were calculated in the second. Filip et al. (2000) analyzed phenolic compounds in dried powdered leaves of Argentinian *Ilex* species and Pomillo et al., (2002) the purine alkaloids in Argentinian mate infusions, but in both cases the quantitative data were also given in percentages. Clifford and Ramirez-Martinez (1990)

quantified caffeine, teobromine, total chlorogenic acids and total dichlorogenic acids, on a mg per cup basis, of infusions prepared from Brazilian mate purchased in the UK and Germany. For the mate leaves, these analytes were reported as percentages on a dry weight basis. In a Brazilian study, Bastos et al. (2006) determined caffeine, caffeic acid and 5-CGA (in μg/mL) in chimarrão. Thus, absolute quantitative data on the phenolic compounds and purine alkaloids of mate or its infusions are lacking.

In a previous paper (Chapter 3), the phenolic compounds and purine alkaloids of infusions prepared from commercial Brazilian mate were identified by HPLC-DAD-ESI/MS and the absolute concentrations determined by HPLC-DAD. On-line evaluation of the individual antioxidant activity, measured by their $ABTS^{+}$ radical scavenging activity, showed that quercetin-3-*O*-glucoside and kaempherol-3-*O*-glucoside were principal contributors to the antioxidant capacity of the infusions, along with 3-*O*-caffeoylquinic acid and tricaffeoylquinic acid, although the latter compounds had levels more than 10 times those of the flavonols.

The flavonols are found in plant foods in the glycosidic form. In flavonol analysis, hydrolysis of the glycosides is usually done to minimize interferences and simplify the subsequent chromatography since each flavonoid can occur as various glycosides. Hydrolysis is generally done simultaneously with extraction, using HCL in aqueous methanol under reflux or ambient conditions, but optimization in terms of the HCl concentration, time and temperature of hydrolysis (Hertog et al., 1993, Häkkinen et al., 1998; Nuutila et al., 2002), is necessary to insure complete hydrolysis without degrading the aglycones.

The objectives of the present study were: (1) to compare the quantification procedure of flavonols in the glycosidic with that in the aglycone form and (2) to compare

the phenolic and purine alkaloid composition at two stages of mate production and of hot and cold infusions.

2. Materials and methods

2.1. Chemicals

Standards of the flavonol aglycones, kaempferol and quercetin, and the purine alkaloids, caffeine and teobromine, were purchased from Sigma Chemical Company (St. Louis, MO, USA). Standards of quercetin-3-rhamnosylglucoside, quercetin-3-*O*-glucoside, kaempferol-3-*O*-glucoside, 3,5-dicaffeoylquinic acid were obtained from Extrasynthese (Genay, France). HPLC-grade methanol and acetonitrile were purchased from Tedia Company (Fairfield, OH, USA); analytical-grade HCl and formic acid from Merck (Darmstad, Germany); and butyl-hydroxyanisol (BHA) from Aldrich (St. Louis, MO, EUA). Membrane filters were provided by Millipore (Bedford, MA, USA).

Stock standard solutions were prepared at a concentration of 1 mg/mL in methanol and stored at -20^{o}C for a maximum of 2 months. Water was purified using a MilliQ system from Millipore Corporation (Millipore, Bedford, MA, USA).

3-Caffeoylquinic acid and 4-caffeoylquinic acid, which are not commercially available, were prepared and isolated as described in Chapter 3.

2.2. Sample preparation

The mate samples from PR1 to PR7 were yerba "cancheada", kindly donated by Dr. Agenor Maccari (Federal University of Paraná, Curitiba, PR, Brazil), which were obtained directly from producers in the state of Paraná, Brazil. The other mate samples (S1 to S6) were purchased from supermarkets in São Paulo, Brazil and were produced in the states of Paraná, Santa Catarina, and Rio Grande do Sul (two sample lots from each state). The flowsheet for yerba mate production is shown in Figure 1. "Sapeco" consists of heating the leaves over fire in a gyrating, inclined cylinder to reduce enzymatic activity, thus controlling enzymatic browning.

Two packages of each sample lot were ground to pass a 40-mesh screen and mixed and chimarrão (hot infusion) was prepared in duplicate for each lot by adding 250 mL of deionized water at 70^{o}C to 5 g of the sample. The infusion was stirred for 3 min and then filtered through a 0.45 μM membrane. Tererê (cold infusion) was prepared using the same procedure, but the water temperature was 5^{o}C. An aliquot of chimarrão or tererê was diluted 20 fold with deionized water and immediately injected in the HPLC system.

For flavonol hydrolysis (Hertog et al., 1993), to 15 mL of the beverage, 40 mL of 62.5% methanol/water (with 2 g/L BHA) were added, followed by the addition of 10 mL 6M HCl. The solution was refluxed in a water bath at 90^{o}C for 2 h, then cooled in an ice bath and its volume completed to 100 mL with methanol. Two mL of the resulting solution were

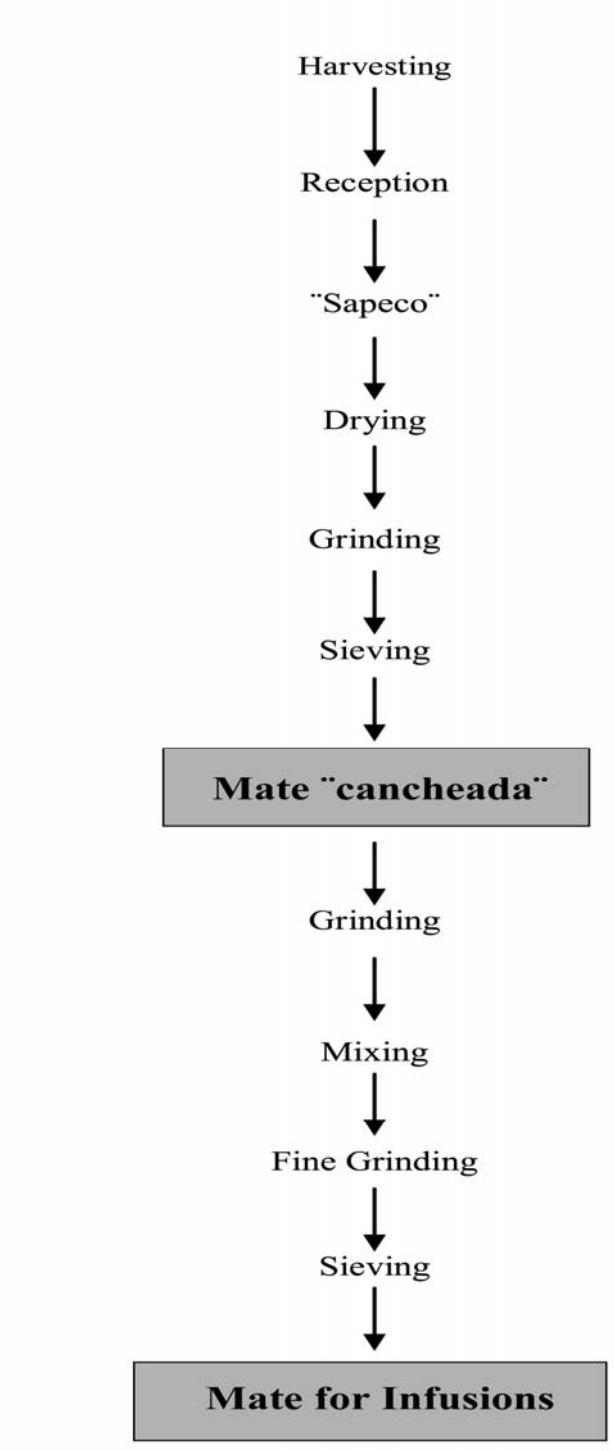

Figure 1. Industrial steps in mate production.

filtered in a 0.22 μm membrane and injected into the HPLC system. An aliquot was diluted 20 fold with deionized water and immediately injected in the HPLC and μLC systems.

2.3. μLC analysis of the aglycones

The aglycones quercetin and kaempferol were quantitated by a μLC-DAD system consisting of a Waters Alliance model 2695 separations module with a photodiode array detector model 996, equipped with a microbore detection cell. For data collection and treatment, Empower 2 Software (Waters Corporation, Milford, MA, USA) was used. Chromatographic separation was achieved using a microbore Atlantis difunctional dC_{18} column (Cat no.186001279) (3 μm,1.0 x 50 mm) (Waters Corporation, Milford, MA, USA) thermostated at 35°C with a Waters temperature control module. System flow-rate was 90μL/min. A binary convex gradient was used, the mobile phase consisting of H_2O/formic acid (99:1 v/v) as phase A and ACN/formic (99:1 v/v) as phase B. Prior to each injection, the system was equilibrated at 95/5 (A/B). This proportion was changed to 40/70 in 1.8 min, then to 20/80 in 10 min and back to 95/5 in 10 to 15 min. The chromatographic run time was 15 min and injection volume was 1.0 μL.

2.4. HPLC analysis of the phenolic compounds and the purine alkaloids

The HPLC equipment used for the separation and quantification of the phenolic compounds, including the flavonol glycosides, and purine alkaloids consisted of a Waters Alliance model 2695 separation module with a photodiode array detector model 996, equipped with an X-Bridge C_{18} column, 5μm, 4.6 x 150 mm, a microbore detection cell

and, for data collection and treatment, an Empower 2 software (Waters Corporation, Milford, MA, USA). The system was thermostated at 35°C with a Waters temperature control module and the flow rate was 1.0 mL/min. A binary convex gradient was employed with the following solutions: H_2O/formic acid (99:1 v/v) as phase A and acetonitrile/formic acid (99:1.0 v/v) as phase B. Starting with 95A/5B, the proportion was altered to 40A/70B in 5 min, then to 20A/80B in 15 min, and to 95A/5B in 30min. The chromatographic run time was 30 min and the injection volume was 10 µL.

Quantification was done by external standardization, using the respective standards. However, tricaffeoylquinic acid, for which there is no commercially available standard, was estimated from the 3,5-dicaffeoylquinic acid curve.

Both quantitative methods were validated. Linearity was evaluated by the injection of five standard solutions of each analyte at the concentration ranges expected in the samples. Limits of detection (LOD) and quantification (LOQ) were calculated as the solution concentrations that gave signal-to-noise ratios of 3 and 10, respectively. Recovery was studied at three levels, each level injected in triplicate.

2.5. ESI/MS analysis

The identity of the aglycones obtained after hydrolysis was confirmed by mass spectrometry (ESI/MS). The extracts were analyzed by a Micromass ZMD quadrupole mass spectrometer with electrospray interface, coupled to a HPLC system Waters model 2690 separations module, equipped with a photodiode detector model 996 and a microbore cell (Waters Corporation, Milford, MA, USA). Mass spectrometer parameters were set as follows: ionization mode, electrospray positive ion mode; capillary voltage, 4.0 kV; source

block temperature, 140°C; desolvation temperature, 400°C; nebulizer nitrogen flow rate, 50 L/hr; desolvation nitrogen gas flow, 568 L/hr; LM resolution, 10; HM resolution, 12; ion energy, 1.0 V; cone voltage, 35 V; RF lens, 0.5 V; extractor lens, 5 V. The spectrum was recorded by scanning the mass range from m/z 100 to 1000 with scan time of 1 s. MassLysnx Software Version 3.5 (Micromass UK Ltd, Manchester, UK) was used for data acquisition.

2.6. Statistical analysis

The quantitative results were submitted to analysis of variance ($p \leq 0.05$), the means being compared by the Tukey's test.

3. Results and discussion

3.1 Flavonol identification and performance of the quantitative method.

Typical μLC-DAD chromatogram of major flavonols in mate infusion and HPLC-DAD chromatogram of phenolic compounds and purine alkaloids are presented in Figure 2. For the aglycones, a baseline separation was achieved by a simple binary gradient for the two major compounds in less than 15 minutes and for the flavonol glycosides, purine alkaloids and other phenolics, the run time was 30 min. Preliminary assignment of peak

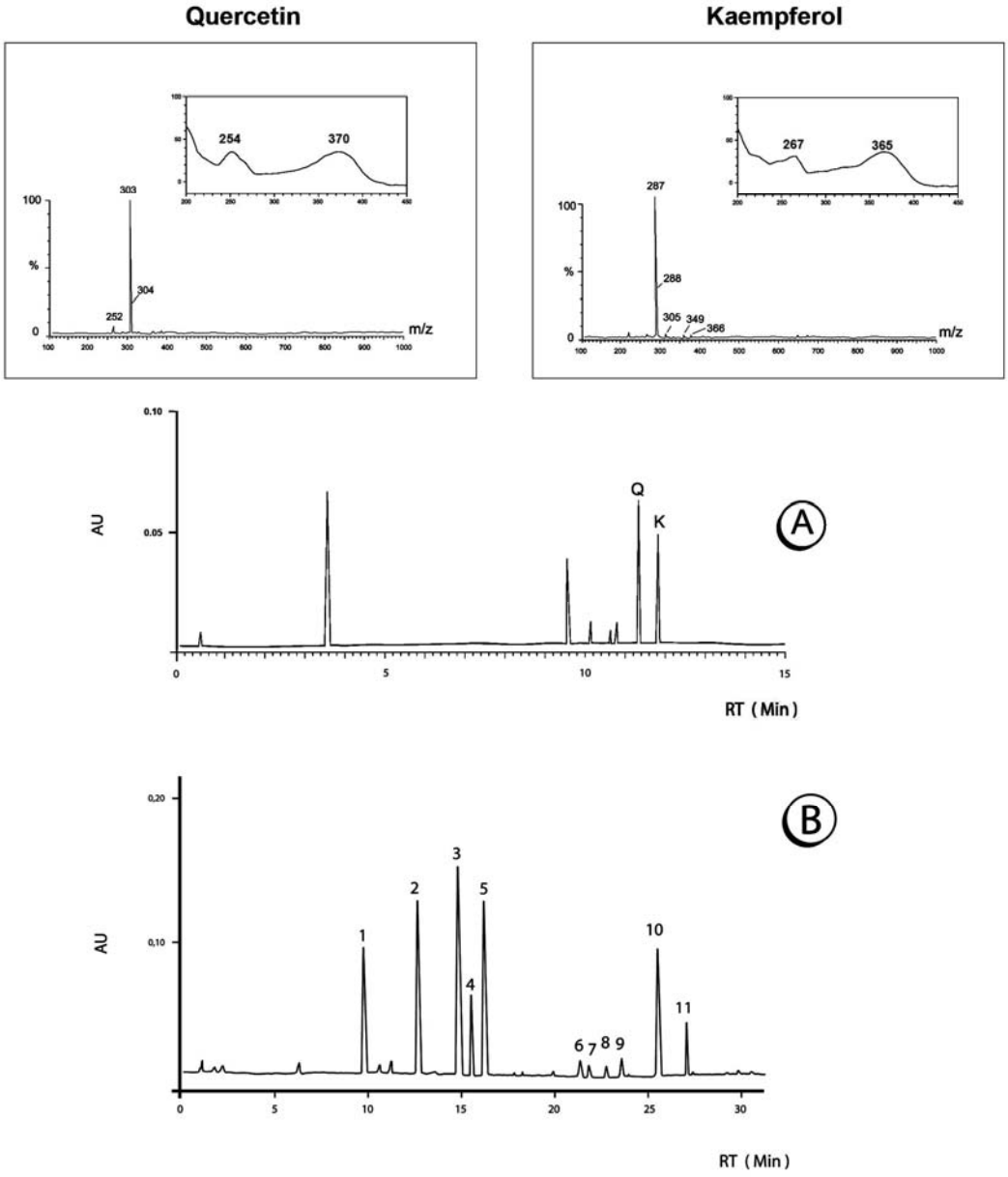

Figure 2. Absorption and mass spectra of the aglycones quercetin (Q) and kaempferol (K) and sample chromatograms: (A): chromatogram of the aglycones and (B) chromatogram of the glycosides, other phenolic compounds and purine alkaloids. Chromatographic conditions are in the text. Peak identification: Q = quercetin, K = kaempferol, 1 = caffeine, 2 = 3-*O*-caffeoylquinic acid, 3 = 5-*O*-caffeoylquinic acid, 4 = teobromine , 5 = 4-*O*-caffeoylquinic acid, 6 = quercetin-3-rhamnosylglucoside, 7 = quercetin-3-*O*-glucoside, 8 = kaempferol-3-*O*-glucoside, 9 = 3,5-dicaffeoylquinic acid, 10 = 4,5-dicaffeoylquinic acid, 11 = tricaffeoylquinic acid.

identities in the infusion samples (chimarrao and tererê) was based on comparison of peak retention time (t_R) and on-line DAD spectra with those of authentic standards, confirming two absorption bands characteristic of flavonoids, band II with maximum in the 240-285 nm range, due to the B-ring, and Band I with a maximum in the 300-550 nm range (Merken and Beecher, 2000). Identification was subsequently confirmed by μLC-ESI-MS, resulting in molecular ions of $[M+H]^+$=303 m/z ($C_{15}H_{10}O_7$) and $[M+H]^+$= 287m/z ($C_{15}H_{10}O_6$), corresponding to quercetin and kaempferol.

The performance characteristics of the quantitative methods were good (Table 1). For quercetin, R^2 was 0.9999 (concentration range of 1-80 mg/L); limit of detection, 0.6 mg/L; limit of quantification, 2.4 mg/L and % recovery, 95%. The corresponding values for kaempherol were: 0.9998 (concentration range, 1-30 mg/L), 0.4 mg/L, 1.8 mg/L and 99%. For the favonol glycosides, other phenolics and purine alkaloids, R^2 values were also generally above 0.9997, except for quercetin-3-*O*-glucoside and 4,5-dicaffeoylquinic acid, both of which had 0.9989, and kaempferol-3-*O*-glucoside, with 0.9990. Limit of detection varied from 0.4 to 0.8 mg/L, limits of quantification from 1.8 to 2.9 mg/L and recoveries from 96 to 100%. The coefficients of variation for peak area of ten consecutive injections of standard solutions were lower than 1%.

3.2. Quantitative composition

The flavonol concentrations of the hot and cold infusions of two types of mate samples are shown in Tables 2 and 3. For all thirteen samples analyzed, there was excellent agreement between the sum of the concentrations of the two quercetin glucosides and that

of the aglycone quercetin in both the hot and cold infusions. Likewise, the concentrations of the kaempherol glucoside and the aglycone kaempherol were equivalent for both influsions. Therefore, the flavonols can be quantified in either forms, provided that conditions are optimized and glycoside standards are used for the quantification in the glycoside form and the free flavonols for the hydrolyzed samples. Glycosides absorb at different wavelengths and different from those of the free flavonols. Quantification in the hydrolyzed form is attractive for its simplicity in the chromatographic step. However, the pre-chromatographic step is longer and more laborious, and drastic reagents are used. Additionally, simultaneous quantification of other phenolic and the purine alkaloids may not be recommended as degradation of these other analytes may occur, as indicated in Figure 2A. On the other hand, standards for glycosides are expensive and not easy to obtain. For mate, only two glycosides of quercetin and one glycoside for kaempferol were found. In other foods, there are more glycosides for each flavonol, making the analysis more complex

In all of the samples, the flavonol concentrations in both glycoside and aglycone forms were understandably lower in the cold infusion compared to the hot infusion. This was also true with the glycosides, other phenolic compounds and the purine alkaloids (Tables 4 and 5).

In both infusions, the phenolic and purine alkaloid concentrations tended to be higher in the samples obtained from the producers (samples PR-1 to PR-7) than in the commercial samples (S1-S6). As can be seen in Figure 1, the latter samples were subjected to additional processing steps, during which degradation and/or physical removal of the compounds investigated in this study might have occurred.

Bastos et al. (2006) found 145 to 294 mg/L in three “cancheada” samples from Paraná, values which agree with those obtained in the present study (142-248 mg/L). For 5-

CQA, the range was 357 to 447 mg/L, higher than those of the present study (145-269 mg/L). However, it is not clear if 3-CQA and 4-CQA were separated from 5-CQA in the work of Bastos et al. These compounds are not easily separated. Quantitative data for the other analytes herein quantified are not available.

4. Conclusions

The flavonols can be quantified either in the glycoside or the aglycone form provided that the conditions are optimized in each case. Each procedure has limitations and advantages to be considered in choosing the procedure to be used. Greater amounts of phenolic compounds and purine alkaloids are extracted into the infusion when hot water is used. In both hot and cold infusions, the phenolic and purine alkaloid concentrations tended to be higher in samples obtained from the producers than in the commercial samples, indicating losses during the additional processing steps or during storage of the latter samples.

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Table 1

Performance characteristics of the μLC-DAD and HPLC-DAD methods.

Phenolic compound	Retention time (min)[a]	Concentration range (mg/L)[a]	Coefficient of correlation[a]	Limit of quantification (mg/L)[a]	Limit of detection (mg/L)[a]	Recovery (%)[b]
Quercetin	11.2	1-80	0.9999	2.4	0.6	95 ± 1
Kaempferol	11.9	1-30	0.9998	1.8	0.4	99 ± 2
Caffeine	13.9	20-300	0,9997	2.9	0.8	98 ± 1
3-*O*-Caffeoylquinic acid	16.8	20-300	0,9998	2.2	0.5	96 ± 1
5-*O*-Caffeoylquinic acid	19.8	20-300	0,9999	2.3	0.5	99 ± 2
Teobromine	19.8	5-300	0.9998	2.8	0.8	100 ± 1
4-*O*-Caffeoylquinic acid	20.1	20-300	0.9999	2.3	0.5	97 ± 1
Quercetin-3-rhamnosylglucoside	24.2	1-50	0,9998	2.8	0.8	98 ± 1
Quercetin-3-*O*-glucoside	25.0	1-50	0.9989	2.6	0.7	100 ± 2
Kaempferol-3-*O*-glucoside	25.6	1-50	0.9990	2.4	0.5	99 ± 1
3,5-Dicaffeoylquinic acid	27.4	20-300	0.9997	2.3	0.4	98 ± 1
4,5-Dicaffeoylquinic acid	27.8	20-300	0.9989	2.3	0.4	99 ± 1

[a]Six concentrations, each injected in triplicate. [b]At three levels, each level injected in triplicate.

Table 2

Flavonol glycoside and aglycone contents (mg/L)[a] of hot infusions prepared from Brazilian mate (*Ilex paraguarienses*)

Sample Code[b]	Quercetin-3-rhamnosylglucoside	Quercetin-3-*O*-glucoside	Quercetin aglycone	Kaempferol-3-*O*-glucoside	Kaempferol aglycone
PR-1	15±1	19±1	35±1	12±0.1	12±0.1
PR-2	29±1	37±1	67±1	15±0.2	14±0.2
PR-3	20±2	25±2	45±2	10±0.1	9±0.1
PR-4	19±1	19±2	38±1	10±0.2	10±0.2
PR-5	15±1	18±1	35±1	21±1	22±1
PR-6	35±2	28±2	65±2	10±0.3	11±0.3
PR-7	38±2	33±2	74±2	18±0.5	18±0.5
S1	10±1	12±1	23±1	9±1	9±0.2
S2	12±2	13±1	26±1	10±1	10±0.5
S3	10±1	13±2	23±0.8	tr[c]	tr[c]
S4	10±1	8±1	18±0.5	tr[c]	tr[c]
S5	18±1	10±1	28±1	12±1	13±1
S6	8±1	6±1	15±0.3	16±1	16±0.2

[a]n = 6, each infusion prepared in duplicate, each injected in triplicate. [b]Samples PR-1 to PR-7 were obtained from the producers and samples S1 to S6 were taken from supermarkets. [c]tr = trace (LOD = 0.5 mg/L). The sums of the concentrations of the quercetin glycosides are equivalent to those of quercetin; the concentrations of the kaempferol glycoside are equivalent to those of kaempferol.

Table 3

Flavonol glycoside and aglycone contents (mg/L)[a] of cold infusions prepared from Brazilian mate (*Ilex paraguarienses*)

Sample Code	Quercetin-3-rhamnosylglucoside	Quercetin-3-*O*-glucoside	Quercetin aglycone	Kaempferol-3-*O*-glucoside	Kaempferol aglycone
PR-1	13±1	18±1	32±0.5	10±0.1	11±0.1
PR-2	26±1	35±1	62±2	13±0.2	13±0.2
PR-3	16±2	22±2	39±1	8±0.1	8±0.1
PR-4	17±1	15±2	31±0.5	7±0.2	7±0.2
PR-5	14±1	14±1	28±1	19±1	21±1
PR-6	32±2	22±2	54±2	10±0.3	10±0.3
PR-7	36±2	31±2	69±2	16±0.5	17±0.5
S1	9±1	10±1	19±1	7±1	7±0.2
S2	11±2	22±1	33±1	8±1	9±0.2
S3	8±1	7±1	16±0.7	tr	tr
S4	8±1	6±1	15±0.4	tr	tr
S5	12±1	11±1	22±1	13±1	12±1
S6	7±1	4±1	12±0.3	15±1	14±0.2

[a]n = 6, each infusion prepared in duplicate, each injected in triplicate. [b]Samples PR-1 to PR-7 were obtained from the producers and samples S1 to S6 were taken from supermarkets. [c]tr = trace (LOD = 0.5 mg/L). The sums of the concentrations of the quercetin glycosides are equivalent to those of quercetin; the concentrations of the kaempferol glycoside are equivalent to those of kaempferol.

Table 4

Other phenolic and purine alkaloid contents (mg/L) [a] of hot infusions prepared from Brazilian mate (*Ilex paraguarienses*)

Sample code[b]	Caffeine	3-CQA	5-CQA	Teobromine	4-CQA	3,5-DiCQA	4,5-DiCQA	TriCQA[c]
PR-1	184±9	202±11	244±12	98±7	145±12	144±12	184±10	125±7
PR-2	198±11	224±18	268±11	103±9	178±11	187±11	198±12	147±9
PR-3	224±8	278±12	287±15	87±8	197±10	192±10	224±8	148±8
PR-4	157±12	252±13	256±7	112±10	124±8	176±10	187±7	167±11
PR-5	224±8	219±10	245±12	67±5	187±9	134±7	197±9	134±9
PR-6	267±13	198±8	289±8	87±4	192±11	138±10	194±7	137±7
PR-7	254±15	174±8	165±8	74±6	196±13	141±9	189±9	134±9
S1	146±11	183±6	263±4	72±3	153±8	112±7	238±8	125±9
S2	265±12	153±7	212±9	125±9	188±4	138±8	278±6	128±7
S3	190±13	193±8	183±8	102±6	139±8	167±7	289±6	156±6
S4	124±8	166±4	213±12	64±7	153±7	145±5	267±7	136±4
S5	268±12	189±5	219±8	126±8	130±5	156±3	279±5	143±6
S6	197±15	242±9	210±9	90±4	123±8	165±4	287±5	154±4

[a]n = 6, each infusion prepared in duplicate, each injected in triplicate. [b]Samples PR-1 to PR-7 were obtained from the producers and samples S1 to S6 were taken from supermarkets. [c]Estimated from 3,5-dicaffeoylquinic acid calibration curve. CQA = caffeoylquinic acid. Although not statistically significant, the general means of all analytes are higher for the producers' samples, except for 4,5,-DiCQA, which is significantly higher in the commercial sample.

Table 5

Other phenolic and purine alkaloid contents (mg/L)[a] of cold infusions prepared from Brazilian mate (*Ilex paraguarienses*)

Sample code	Caffeine	3-CQA	5-CQA	Teobromine	4-CQA	3,5-DiCQA	4,5-DiCQA	TriCQA[b]
PR-1	172±9	196±12	216±10	78±9	139±9	124±8	174±8	121±9
PR-2	178±12	213±11	237±9	94±8	162±8	178±11	182±9	134±10
PR-3	212±10	256±10	269±12	83±9	179±9	185±10	208±9	132±9
PR-4	142±9	228±11	245±10	101±7	112±7	167±9	182±8	149±7
PR-5	207±9	207±10	231±9	63±4	178±8	123±8	177±9	112±8
PR-6	248±12	186±8	268±10	69±6	184±11	131±9	175±10	126±9
PR-7	239±9	163±9	145±8	71±6	190±9	133±8	179±12	130±7
S1	124±9	167±4	164±10	57±3	114±12	103±8	206±7	102±5
S2	234±10	122±5	199±6	114±8	169±9	112±8	254±7	106±4
S3	168±9	168±8	203±5	77±5	125±4	123±3	256±8	134±5
S4	114±9	135±7	209±7	54±4	133±1	123±6	224±8	108±5
S5	246±10	169±6	193±6	113±1	104±1	126±4	239±6	139±6
S6	175±11	218±4	185±6	73±3	102±6	145±5	265±8	143±5

[a]n = 6, each infusion prepared in duplicate, each injected in triplicate. [b]Samples PR-1 to PR-7 were obtained from the producers and samples S1 to S6 were taken from supermarkets. [c]Estimated from 3,5-dicaffeoylquinic acid calibration curve. CQA = caffeoylquinic acid. The general means of all analytes are higher for the producers' samples (statistically significant for 3-CQA, 5-CQA, 4-CQA and 3,5-CQA), except for 4,5,-DiCQA, which is significantly higher in the commercial sample.

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CAPÍTULO 5

Determination of catechins in green tea infusions by reduced flow micellar electrokinetic chromatography

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Determination of catechins in green tea infusions by reduced flow micellar electrokinetic chromatography

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Keywords: Capillary electrophoresis; RF-MEKC; Catechins, Tea; *Camellia sinensis*; Flavan-3-ols.

Abstract

A fast RF-MEKC method modified by sulfated-β-cyclodextrin was developed and validated for the determination of catechins in green tea. The optimal electrolyte consisted of 0.2% triethylamine, 50 mmol/L SDS and 0.8% s-ß-CD (pH=2.9), allowing baseline separation of five catechins in four min. The samples and standards were injected at 0.6 psi for 5 s under constant voltage of -30 kV. Sample preparation simply involved extraction of 2 g of tea with 200 mL water at 95^{o}C under constant stirring for 5 min. The method demonstrated excellent performance, with LOD and LOQ of 0.02 to 0.1 and 0.1 to 0.5 µg/mL, respectively, and recovery percentages of 94 to 101%. The method was applied to six samples of Brazilian green tea infusions. EGCg (23.4-112.4 µg/mL) was the major component, followed by EGC (18.4-78.9 µg/mL), ECg (5.6-29.6 µg/mL), EC (4.6-14.5 µg/mL) and C (3.2-8.2 µg/mL).

1. Introduction

Tea is a widely consumed beverage worldwide. It has been promoted as a functional food with cancer preventive action (Katiyar & Mukhtar, 1997; Yang, Lambert, Ju, Lu, & Sang, 2006; Zaveri, 2006; Béliveau & Gringas, 2004). Various other properties, such as antibacterial, antiviral, antiallergic, antiteratogenic activity (Jankun, Selman, Swiercz, & Skrzypczak-Jankun, 1997; Yang, 1997; Toschi, Bordoni, Hrelia, Bendini, Lercker, Biagi, 2000) were observed in *in vivo* and *in vitro* experiments. Using different varieties of *Camellia sinensis,* green tea is prepared from dried and unfermented leaves. It is an important source of polyphenols, especially flavan-3-ols, also called green tea catechins, a class of strong antioxidants, which might explain their protective effects (Takeo, 1992). The major catechins in green tea are: epigallocatechin (EGC), catechin (C), epigalocatechin gallate (EGCG), epicatechin (EC) and epicatechin gallate (ECG).

Catechins have been mostly determined by reverse phase high performance liquid chromatography (HPLC) (Dalluge, Nelson, Thomas, & Sander, 1998; Bronner & Beecher, 1998; Arts, Van de Putte, & Hollman, 2000; Lin, Tsai, Tsay, & Lin, 2003, Matsubara & Rodriguez-Amaya, 2006). HPLC-MS has also been employed (Pelillo, Bonoli, Biguzzi, Bendini, Toschi, &. Lercker, 2004). Capillary electrophoresis (CE) has been used in more recent years. Neutral and ionizable groups in flavonoids make it possible for these molecules to be separated in both capillary zone electrophoresis (CZE) (Horie, Mukai, & Kohata,1997) or micellar electrokinetic chromatography (MEKC) modes. Several methods have been described for catechin separation and quantification using MEKC (Bonoli,

Pelillo, Toschi, &Lercker, 2003; Horie & Kohata, 1998, Aucamp, Hara, & Apostolides, 2000).

Reduced flow-MEKC has been used in cases where it is necessary to reduce the pH to prevent analyte degradation. As a consequence, the electroosmotic flow (EOF) is significantly suppressed, a fast anodic migration occurs as the analytes partition into the SDS negatively charged micelles (Janini, Issaq and Muschik, 1997). RF-MEKC has become the method of choice of some authors in flavonoid study (Tonin, Jager, Micke, Farah, & Tavares, 2005) and phenolic acids (Risso, Peres, & Amaya-Farfan, 2007).

Cyclodextrins (CD´s) are widely used for chiral separations in CE. The anionic CD´s, like sulfated-β-cyclodextrin (s-β-CD) is negatively charged throughout the entire pH range and are used for chiral separations of neutral and acidic compounds. The neutral and charged groups in cyclodextrin allow hydrophobic and polar/electrostatic interactions and can offer a diversity of options for CE separation (Vigh, Sokolowski, 1997; Eeckhaut and Michotte, 2006). In catechin separation the use of CD´s is not usual, but it was employed by Kodama et al. (2004) for enantioseparation of catechin and epicatechin with 6-O-α-D-glucosyl-β-cyclodextrin and by Nelson et al. (1998) to improve the selectivity of the method using β-cylodextrin.

In this work, a RF-MEKC method based on a SDS and s-β-CD system was developed and optimized for the determination of five catechins in green tea in less than 5 min. RF-MEKC was chosen because catechins are stable at low pH and since at that pH condition they are neutral the only possible separation strategy is to provide a secondary charged phase in which they partition, i.e. an EKC phase.

2. Experimental

2.1. CE instrumentation and capillary conditioning

Experiments were conducted in a capillary electrophoresis system model P/ACE MDQ (Beckman Instruments, Fullerton, CA, USA) equipped with a DAD detector, a temperature control device maintained at 25°C, and a software (32 Karat® Software Version 8.0) for data acquisition.

CE separations were carried out on an uncoated fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) with 40.0 cm effective length, 50.2 cm total length, 50 μm id and 375 μm od.

At the beginning of each working day, the capillary was conditioned by running 5 min of deionized water, 30 min of 1mmol/L NaOH, followed by 10 min of the electrolyte solution. In between runs, the capillary was reconditioned by flushing with the electrolyte solution for 2 min. At the end of the day, the capillary was rinsed with deionized water (5 min), followed by 1mmol/L NaOH (10 min) and again deionized water (5 min).

2.2. Reagents and solutions

All reagents were of analytical grade and water was purified by deionization (18mΩ.cm) (Milli-Q system, Millipore, Bedford, MA, USA). The following catechin standards were obtained from Sigma (St. Louis, MO, USA): EC, C, EGCg, EGC and ECg. β-Cyclodextrin sulfated sodium salt was purchased from Fluka Biochemika (Bucks, Switzerland), sodium dodecylsulfate from Riedel-de Haen (Sweelze, Germany),

triethylamine and phosphoric acid (both HPLC grade) from Tedia company (Fairfield, OH, USA).

2.3. Sample preparation

Six samples of Brazilian green tea of different brands were purchased from a supermarket in Sao Paulo, Brazil. Sample characteristics are listed in Table 3. The infusions were prepared using an aqueous extraction simulating a normal brewing for a cup of tea. For each sample, the contents of two bags were mixed, 2 g were weighed and placed in a 250 ml conical flask and 200 ml of boiling water was added. Heating on a hot plate at 90^{o}C for 5 min was then done with magnetic stirring. The extract was filtered through a 0.45 μm pore Millex HV filter (PVDF) from Millipore (Bedfrod, MA,USA), diluted with purified water at a proportion of 1:5 and injected immediately in the CE system. The samples were prepared in duplicate, each duplicate being injected three times.

2.4. Analytical conditions

The standard solutions and sample extracts used in this study were injected hydrodynamically at 0.6 psi during 5s and monitored at 210 nm. The electrophoresis system was operated under constant voltage condition of -30 kV.

Stock standard solutions of each 5 catechin were prepared at 1000 mg/L. Working solutions were prepared by mixing appropriate volumes of the stock solutions and purified

water to give solutions containing 100, 75, 50, 25, 5 and 2.5 mg/L of each phenolic compound. Quantification was carried out by external standardization.

2.5. Method development

The initial experiments involved testing a range of concentrations (10-100 mmol/L) SDS in RF-MEKC. The SDS concentration that gave the best resolution of the compounds of interest was 50 mmol/L.

With the objective of achieving better resolution and the shortest analysis time as possible, three types of neutral or unsubstituted cyclodextrins (α-CD, γ-CD and β-CD) were examined as buffer modifiers. α-CD and γ-CD were individually incorporated in the running buffer, but no significant effect on the separation was observed. On the other hand, β-CD had a positive effect, but it was not enough to achieve complete separation of all catechins under investigation.

Figure 1 shows the electropherograms obtained with increasing s-β-CD concentration from 0 to 1.0%. Although the electropherogram without addition of s-β-CD appeared to have adequate resolution, the spectrum obtained by DAD for EC_g peak in the real sample showed the presence of an interfering substance. With the addition of s-β-CD, the spectrum was that of EC_g and the relative peak area was smaller.

It can be seen that the peaks of catechin and epigallocatechin overlapped at 0.2% s-β-CD. A baseline separation was achieved with 0.4% s-β-CD. However, 0.8% was chosen for method validation because a better separation with less tailing and better peak shape and more evenly spaced were obtained under this condition.

Because catechin interacts more strongly with s-β-CD, which migrates more rapidly than the SDS micelles, the migration time of catechin decreases with increasing concentration of s-β-CD.

The optimized electrolyte consisted of 0.2% TEA, 50 mmol/L SDS and 0.8% w/v s-β-CD (pH=2.9) adjusted with phosphoric acid. The electrolyte solution was prepared daily and filtered through a 0.45 μm membrane.

Linearity was evaluated by the injection of five standard solutions of each catechin at concentrations varying from 1.25 to 100 μg/mL. Limits of detection (LOD) and quantification (LOQ) were calculated as the solution concentrations that gave signal-to-noise ratios of 3 and 10, respectively. Peak and migration time repeatability was evaluated by 10 consecutive injections of the standard solutions. Recovery was studied at three levels, each level injected in triplicate.

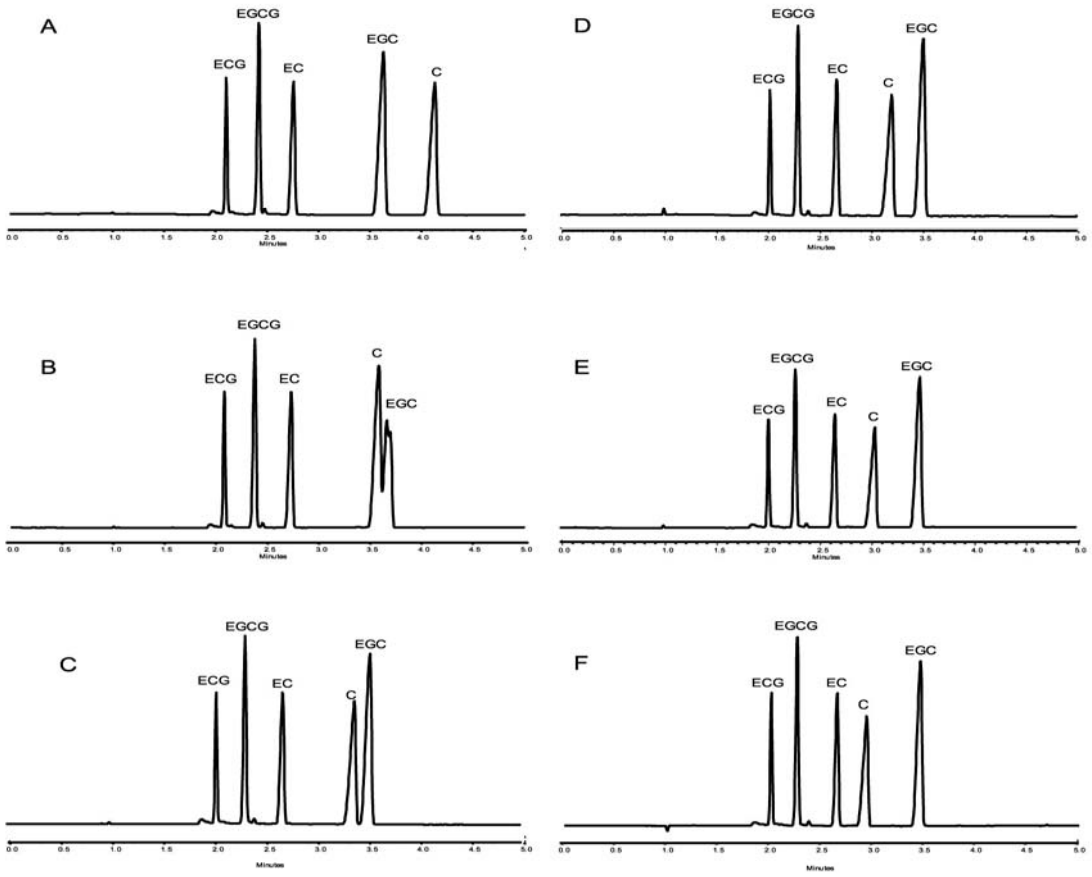

Fig. 1. Electropherograms of tea catechins obtained with the RF-MEKC method, using 0.2% TEA and 50mM SDS with 0.0% (A), 0.2% (B), 0.4% (C), 0.6% (D) 0.8% (E) and 1.0% (F) of s-β -CD (sulfated cyclodextrin salt). Conditions are described in the text.

3. Results and discussion

3.1. Method performance

Performance characteristics of the method developed are listed in Table 1. Correlation coefficients for the curves varied from 0.99983 for EC to 0.99998 for C. Limits of detection of 0.02 to 0.1, considering the signal-to-noise ratio of 3:1, and limits of quantification of 0.1 to 0.5, using the signal-to-noise ratio of 10:1, were obtained. Repeatability for migration time and peak area was excellent, the coefficients of variation (CV %) for 10 consecutive injections ranging from 0.008 to 0.019 and from 0.02 to 0.03, respectively. The theoretical plates and peak symmetry are also shown in Table1.

For comparative purposes, published methods for tea catechins, with some evaluation data (8 out of 17 methods presented in Chapter 1) are described in Table 2 and the performance characteristics are shown in Table 3. All methods had good linearity with R greater than 0.99. LOQ, determined for only three methods, ranged from 0.01 to 18 mg/l. LOD was determined for all methods and it varied from 0.013 to 6.07. Recovery was done for only one method and it ranged from 93.5 to 100.5%. The peak area repeatability, determined for 5 methods, varied from 0.88 to 6.9%. Thus, the method herein developed compares very favorably with other developed methods.

3.2. Catechins of Brazilian tea

The electropherogram (Figure 2) shows excellent separation of the catechins from a Brazilian green tea infusion. The use of negatively charged s-β-CD) permitted the separation of an interfering substance from ECg and anticipated the elution of catechin. Table 4 presents the catechin concentrations for 6 Brazilian green tea analyzed by the optimized method. The catechins, identified by co-chromatography and comparison of their absorption spectra with those of standards, were EGCg (23.4-112.4 μg/ml) as the major component, followed by EGC (18.4-78.9 μg/ml), ECg (5.6-29.6 μg/ml), EC (4.6-14.5 μg/mL) and C (3.2-8.2 μg/ml). In green tea of two brands commercialized in Campinas, Brazil, the corresponding values determined by HPLC were 41-80, 37-72, 7.3-12, 7.0-14 and 1.3-4.5 μg/ml (Matsubara and Rodriguez-Amaya, 2006), thus in excellent agreement with the present data especially considering that different samples collected in different years were analyzed. The HPLC method of Matsubara and Rodriguez-Amaya, however, took 30 min.

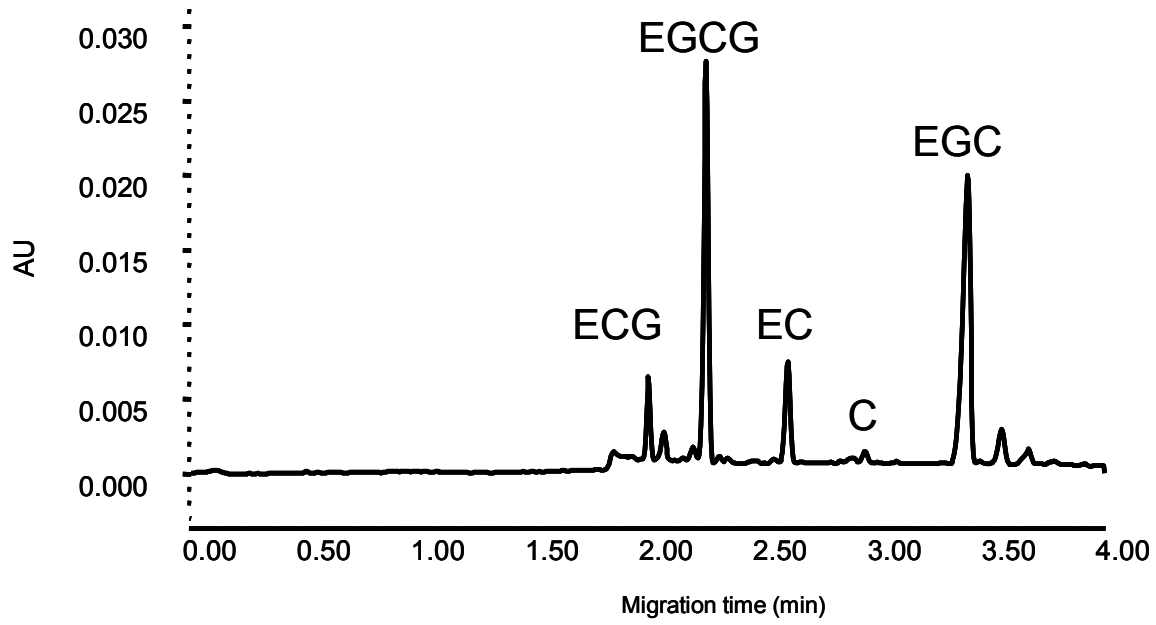

Fig. 2. Typical electropherogram of the catechins of Brazilian green tea. Conditions are described in the text. Peak Identification: ECg, epicatechin gallate; EGCg, epigallocatechin gallate; EC, epicatechin; C, catechin; EGC, epigallocatechin.

Table 1. Performance characteristics of the RF-MEKC method developed.

Analyte	Concentration range[a] (µg/mL)	Coefficient of correlation[a]	Limit of quantification (µg/mL)[a]	Limit of detection (µg/mL)[a]	Recovery (%)[b]	Peak symmetry[c]	Plates/m	Migration Time[c] (min) (%CV)	Peak area repeatability[c] (%CV)
ECg	1.25-100	0.99994	0.1	0.05	99±2	0.99	77422	2.005 (0.008)	0.02
EGCg	1.25-100	0.99987	0.1	0.02	94±3	0.87	69698	2.269 (0.008)	0.02
EC	1.25-100	0,99983	0.1	0.03	97±2	0.75	51854	2.629 (0.011)	0.03
C	1.25-100	0.99985	0.5	0.1	100±3	0.70	32614	2.997 (0.019)	0.02
EGC	1.25-100	0.99998	0.5	0.1	101±4	0.69	43630	3.422 (0.015)	0.03

[a]Seven concentrations, each injected in triplicate.[b]At three levels, each level in triplicate.[c]Ten consecutive injections.

Table 2. Description of the methods for catechins in tea for which evaluation of performance was carried out

Method	*Analytes*	*Capillary Dimension (cm x µm)*	*Electrolyte*	*Detection (nm)*	*Voltage (kV)*	*Temperature (°C)*	*Run time (min)*	*Reference*
CZE	EGCg, ECg, EGC EC, C, GA and Adenine	77 x 57	150mmol/L borate (pH 8,5)	210	20	20	20	Arce et al. (1998)
MEKC	EGCg, Ecg, EGC, EC, C, GCg and Caffeine	50 x 85	20mmol/L borate-phosfate 25 mmol/L SDS (pH 7,0)	200	30	21	20	Barroso et al. (1999)
MEKC	C,EC,EGC, ECg, EGCg, Tiamine and GA	58 x 50	25mmol/L phosfate (pH=7.) 100mmol/LSDS e 6% MeOH	200	14	25	13	Aucamp et al. (2000)
MEKC	GC, C, EGC, EGCg, EC, ECg, GCg, TP, TB, GA, Caffeine	40 x 50	20mmol/L phosfate, 50mmol/L tetraborate 200mmol/L SDS (pH 7.0)	200	20	29	5	Bonoli et al. (2003)
MEKC & MEEKC	SA, *p*-Coumaric acid, VA, CA, GA, 3,4-Dihidroxibenzoic acid, 4-hidroxibenzoic, C, EGC, ECg, , EC, GC, Caffeine and TP	48,5 x 50	MEKC: 2.89% SDS, 2% MeOH, e 95.5% phosfate 25mmol/L pH 2.0 MEEKC: 2.89% SDS. 1.36% heptane, 7.66% cyclohexanol, 2% acetonitrile e 86.1% Phosfate (25mmol/L, pH 2.0)	200	-20 -27	35	14	Huang et al. (2005), (2007)
MEKC	(+)-C,(-)-C,(+)-EC,(-)-,Cg, (-)-EGC,(-)-EGCg, (-)-ECg, (-)-EGCg,(-)-ECg and Caffeine	64,5 x 50	200 mmol/L borate-20mmol/L phosfate (pH 6.4) 240 mmol/L and 25mmol/L 6G-β-CD	210	25	20	35	Kodama et al. (2004)
MEEKC	ECg, EGCg, EC, C, EGC, GC, Caffeine and TP	24 x 50	50mmol/L phosfate (pH 2.5) SDS (2.31% a 2.89% e n-heptane (1.36%) and nine alcohols as co-surfactants: (1-butanol or tert-butanol or 1-pentanol or 2-pentanol or 3-pentanol or cyclopentanol or 1-hexanol or 2-hexanol and cyclohexanol)	230	-10	40	10-18	Pomponio et al. (2003)

CZE= Capillary zone electrophoresis; MEKC= Micellar electrokinetic chromatography; MEEKC= Microemulsion electrokinetic capillary chromatography; EGCg= Epigallocatechin gallate; ECg= Epicatechin; EGC= Epigallocatechin; EC= Epicatechin; GCg= Gallocatechin Gallate; GC= Gallocatechin; Cg= Catecnhin gallate; C= Catechin; TB= Teobromine; TP= Teophiline; CA= Caffeic acid; GA= Gallic acid; SA= Syringic acid; VA= Vanilic acid.

Table 3. Summary of the validation parameters of published CE methods for catechins in tea

CATECHIN	R	LOQ (mg/L)	LOD (mg/L)	Recovery (%)	Peak area % CV	Reference
EC	0.996	0.14	0.04		3.38	Arce et al. (1998)
C	0.996	0.15	0.04		4.18	
EGC	0.993	0.21	0.06		4.50	
EGCg	0.998	0.10	0.03		3.70	
ECg	0.997	0.14	0.04		4.30	
EC	0.9998		3.0		3.0	Barroso et al. (1999)
C	0.9999		5.0		2.41	
EGC	0.9999		2.0		2.68	
EGCg	0.9998		5.0			
ECg	0.9997		4.0		2.95	
EC	0.996	2.0	1.0			Aucamp et al. (2000)
C	0.999	2.0	1.0			
EGC	0.997	4.0	2.0			
EGCg	0.994	11.0	6.0			
ECg	0.994	10.0	3.0			
EC	0.999		2.3		2.70	Bonoli et al. (2003)
C	0.992		1.2		2.38	
EGC	0.999		1.7		2.06	
EGCg	0.999		1.8		1.47	
ECg	0.999		2.3		2.77	
EC	1.0000		0.064		2.19	Huang et al. (2007)
C	0.9836		0.069		0.80	
EGC	0.9981		0.294		0.88	
EGCg	0.9944		0.031		2.77	
ECg	0.9905		0.013		3.73	
EC	0.9995		0.14			Huang et al. (2005)
C	0.9991		0.45			
EGC	0.9995		0.15			
EGCg	0.9996		0.13			
ECg	0.9998		0.10			
EC	0.9994		0.46			Huang et al. (2005)
C	0.9990		1.13			
EGC	0.9993		0.40			
EGCg	0.9996		0.62			
ECg	0.9995		0.35			
(+)-EC			0.5	93.5	1.0	Kodama et al. (2004)
(-)-EC			0.5	99.2	1.1	
(-)-C			0.2	98.4	1.5	
(+)-C			0.2	100.5	1.6	
(-)-EGC			0.5	98.2	1.8	
(-)-EGCg			0.5	99.8	6.9	
(-)-ECC			0.2	99.7	6.0	
EC	0.9999	3.80	1.27			Pomponio et al. (2003)
C	0.9999	0.90	0.30			
EGC	0.9999	1.50	0.48			
EGCg	0.9998	18.18	6.07			
ECg	0.9999	2.64	0.88			

EGCg= Epigallocatechin gallate; ECg= Epicatechin; EGC= Epigallocatechin; EC= Epicatechin; C= Catechin.

Table 4. Catechin contents of six Brazilian green tea samples.

Sample*	***Sample***	***Catechin concentration (µg/ml)***				
code	***characteristics***	***ECg***	***EGCg***	***EC***	***C***	***EGC***
GT1	Lemon flavoured,	12.3	84.5	12.9	4.5	74.6
	Leaves and buds	(0.2)	(6.5)	(0.2)	(0.1)	(3.4)
GT2**	Leaves and buds	29.6	80.4	7.8	8.2	39.4
		(1.0)	(8.9)	(0.1)	(0.1)	(2.5)
GT3	Leaves	13.3	96.7	14.5	4.0	78.9
		(0.7)	(5.1)	(0.2)	(0.1)	(4.6)
GT4	Apple flavoured	9.6	112.4	9.8	5.4	68.9
	Leaves and buds	(0.2)	(7.2)	(0.1)	(0.1)	(5.6)
GT5	Leaves and buds	13.4	70.4	13.9	6.8	72.4
		(0.3)	(3.4)	(0.2)	(0.1)	(3.9)
GT6	Leaves and buds,	5.6	23.4	4.6	3.2	18.4
	minimum 3 years old.	(0.1)	(1.1)	(0.1)	(0.1)	(1.4)

ECg= Epicatechin; EGCg= Epigallocatechin gallate; EC= Epicatechin; C= Catechin EGC= Epigallocatechin;*Two lots of each sample were analyzed;** Imported material from Germany, packed in Brazil; GT=Green tea.

4. Conclusions

A RF-MEKC method, which utilizes 0.2% TEA, 50 mmol/L SDS and w/v 0.8% β-CD sulfated salt (pH=2.9) adjusted with phosphoric acid, was developed and evaluated. With direct injection of green tea infusions, the analysis time was less than 4 min and excellent selectivity and resolution were obtained. Applied to green tea samples, the method provides fast quantitative routine analyses of catechins of green tea.

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CAPÍTULO 6

Determination of polyphenols and phenolic acids in Brazilian wine by capillary electrophoresis

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Determination of polyphenols and phenolic acids in Brazilian wine by capillary electrophoresis

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Abstract

In this work, a method for simultaneously determining the stilbene resveratrol, four phenolic acids and five flavonoids in wine by capillary electrophoresis (CE) was developed and evaluated. The CE electrolyte composition and instrumental conditions were optimized using 2^{7-3} factorial design and response surface analysis, showing TBS (tetraborate)/MeOH as the most influential variables. The optimal electrophoretic conditions, minimizing the chromatographic resolution statistic (CRS) values, consisted of 17 mmol/L TBS with 20% methanol as electrolyte, constant voltage of 25 kV, hydrodynamic injection at 50 mbar for 3s and temperature of 25^{o}C. The R^2 values for linearity were equal to or higher than 0.99; limits of detection and quantification were 0.01 to 0.03 and 0.04 to 0.08, respectively. Coefficients of variation for migration time and peak area obtained from ten consecutive injections were less than 2% and recoveries varied from 97 to 102%. The method was applied to 23 different samples of Brazilian wines.

1. Introduction

Moderate and regular consumption of wine may have some health benefits (Renaud and De Lorgeril, 1992; de Lorgeril and Salen, 1999). Lower incidence of coronary heart disease (CHD) (Frankel et al.,1995; Orralo at al., 2002), through an increase of the blood antioxidant capacity and reduction of the level of oxidized LDL-cholesterol, have been attributed to the phenolic compounds of wine (Shahidi and Janita, 1992; Jovanovic et al., 1994; Kanner et a., 1994; Nigidikar et al., 1998; Tadesco, et al., 2000). Phenolic compounds, such as phenolic acids, catechins and other flavonoids, also have an important role in wine quality, contributing to flavor and color. (Lea et al, 1979).

High performance liquid chromatography (HPLC) has been the method of choice for the analysis of phenolic compounds in wine (Revilla, et al., 2000; Lopez et. al., 2001; Rodriguez-Delgado et al., 2001; Fang et al, 2007), but capillary electrophoresis (CE) (Garcia-Vigueira and Bridle, 1995; Kulomaa, et. al, 1997; Pazourek et al., 2000; Minussi et al., 2003; Saenz-Lopez et al, 2004), micellar electrokinetic chromatography (MEKC) (Watanabe, et al., 1998) and nonaqueous capillary electrophoresis (NACE) (Demianová et al. 2003) have been increasingly used for this purpose. A diode array detector (DAD) has been generally used, but electrochemical (Gao et al., 2002; Peng et al., 2004) and fluorimetric detection (Rodriguez-Delgado et al., 2001) have also been employed. Comparison between HPLC and CE methods has been carried out, showing agreement of the results (Garcia-Vigueira and Bridle, 1995; Andrade et al., 2001; Wang and Huang, 2004). CE has the advantages of high speed, high resolution, low operational cost, low consumption of chemicals and convenience. Some CE studies in wine have focused solely

on the determination of phytoalexin resveratrol (Prasongsidh and Skurray, 1998; Lima et al., 1999; Dobiášová et al., 2002; Spanilá et al., 2005).

In Brazil the production of wine is concentrated in the southern region (state of Rio Grande do Sul). A couple of articles has been published on the phenolic compounds of Brazilian wines (Souto et al., 2001; Minussi et al., 2003), but the data available is still very limited.

The objective of this study was to develop and evaluate a CE method for the simultaneous determination of the most important phenolic compounds in wine and apply it to a variety of Brazilian wines.

2. Materials and methods

2.1. Instrumentation

Method development and evaluation, as well as sample analyses, were conducted in a capillary electrophoresis system model HP^{3D}CE (Agilent Technologies, Palo Alto, CA, USA), equipped with a photodiode array detector (DAD) set at 280 nm for quantification. The temperature was controlled and stabilized at 25 °C. For data acquisition, a Chemstation Software (Rev A.06.01) (Agilent Technologies, Palo Alto, CA, USA) installed in a personal computer supplied by the manufacturer, was used. Samples and standard solutions were injected hydrodynamically (50 mbar for 3 sec) and constant voltage of 25 kV was employed. The column was a fused-silica capillary (Polymicro Technologies,

Phoenix, AZ, U.S.A.) with dimensions of 48.5 cm total length, 40.0 cm effective length, 75 μm i.d. and 375 μm o.d.

2.2. Reagents and solvents

All reagents were of analytical grade and solvents of chromatographic grade, used without previous purification. Methanol was purchased from Tedia Company (Fairfield, OH, USA), hydrochloric acid from Merck KGaA (Darmstad, Germany), sodium tetraborate (TBS) and sodium dodecyl sulfate (SDS) from Riedel-de Haen (Sweelze, Germany) and Brij35 from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA). Water was purified by deionization (18mΩ) (Milli-Q system, Millipore, Bedford, MA, USA). The standards resveratrol (R5010), gallic acid (G7384), *p*-coumaric acid (C9008), quercetin (Q0125), rutin (R2303), myricetin (M6760), caffeic acid (C0625) and catechin (C0567) were purchased from Aldrich (St. Louis, MO, USA); syringic acid (86230) and kaempferol (60010) were from Fluka (St. Louis, MO, USA). Stock standard solutions of each of the 10 phenolic compounds were prepared at 1000 mg/L in 60:40 v/v water/ethanol. Working solutions were prepared by mixing appropriate volumes of the stock solutions with 60:40 v/v water/ethanol.

2.3. Sample preparation

Twenty-three samples of different types (red, white and blended) and brands of wine were purchased from a supermarket in São Paulo, Brazil. Year of production and

types of wine are listed in Table 5. These wines were chosen to get a good representation of wines produced in the region of Serra Gaucha, which are sold at reasonable prices, thus reaching a greater part of the population.

All wines were stored in the dark at 4°C until analysis. The contents of two freshly opened bottles were mixed and 1 mL was extracted with ethyl ether at a solvent/wine proportion of 8:5 mL, followed by hydrolysis with 100μL of hydrochloric acid for 15 min with magnetic stirring. The organic phase was separated from the aqueous phase, dried under nitrogen and dissolved with 2.5 mL of ethanol:water 60:40 v/v. The samples were filtered through a 0.45 μm membrane (Millex LCR PTFE) (Millipore, Sao Paulo, Brazil).

The analytes were identified by comparison of the migration times with those of standards and with wine spiked with standards under identical conditions, along with the spectra of the migrated solutes obtained with the PDA detector.

2.4. Capillary electrophoresis procedure

On each day of analysis, the capillary was conditioned by flushing with 1 mol/L NaOH for 5 min, followed by purified water (MilliQ System, Millipore, Bedford, MA, USA) for 5 min and the electrolyte solution for 30 min. In between runs, the capillary was flushed with the electrolyte solution (1 min). The electrolyte solution was prepared daily and filtered through a 0.45 μm membrane. All standards and samples were injected in triplicate.

2.5. Method development and evaluation of performance

During method development and optimization in the present study, the chromatographic resolution statistic equation (CRS) was used. This is a mathematical function developed originally for chromatographic separations (Schlabach and Excoffier, 1988); the CRS tends to a minimum value for chromatograms having well resolved and uniformly spaced peaks. It has been used in the CE area for optimizing fatty acids (Oliveira et al. 2001) and food dyes (Jager et al. 2005) separations.

TBS based buffers, MeOH as organic modifier, SDS or Brij35 as surfactants, time/pressure of injection, voltage and temperature were tested. Since it was not possible to select adequate electrolyte components by studying their effects by one-at-a-time approach, a factorial design was employed .

TBS as the electrolyte was assessed from 10 to 30 mmol/L. The lower level was established considering the need to maintain the pH in all of the analyses. Very low levels of TBS do not provide good buffering capacity. The upper level was limited by the electrical current. High amounts of TBS combined with high levels of SDS may result in a high current, and temperature gradients can occur in the capillary due to the Joule heating.

MeOH as organic modifier was evaluated from 0 to 10%. One of the first effects is a decrease in the electrical current, reducing the Joule heating and peak band broadening. The second effect is increasing the migration time and the resolution of the peaks. Modification of the pK_a of the analytes can occur with MeOH, thereby increasing solubilization.

The third variable was SDS, evaluated from 0 to 10 mmol/L. The absence and presence of micelles was the main objective in selecting this variable. The presence of micelles can increase the selectivity because each compound can partition to the micelle

core. Brij35 from 0 to 5 mmol/L was also included because the presence of both SDS and Brij in the electrolyte results in a mixed micelle system constituted by anionic monomers (SDS) and neutral monomers (Brij35).

Time of injection from 1 to 3s at 10 mbar, operation voltage from 10 to 25 V and temperature from 25 ^{o}C to 35^{o}C were also evaluated.

Optimization of the CE method was initially carried out, using a factorial design, for the selection of the factors with direct influence on the separation. After selection of these factors, they were optimized by a response surface methodology.

Table1 shows the studied variables for the factorial design as well as the low (-1) and high (+1) levels.

Table 1. 2^{7-3} Factorial design variable levels

Coded Levels	TBS mmol/L	MeOH % v/v	SDS mmol/L	[Brij 35] mmol/L	Injection time s/mbar	Voltage kV	Temp ^{o}C
-1	10	0	0	0	1 / 10	15	25
+1	30	10	10	5	3 / 10	25	35

TBS, sodium tetraborate; SDS, sodium dodecylsulfate.

Linearity was evaluated by the injection of five standard solutions of each compound at concentrations varying from 2.5 to 220 mg/L. Limits of detection (LOD) and quantification (LOQ) were calculated as the solution concentrations that gave signal-to-noise ratios of 3 and 10, respectively. Peak and migration time precision was evaluated by

10 consecutive injections of the standard solutions. Recovery was studied at three levels, each level injected in triplicate.

3. Results and discussion

3.1. Optimum electrolyte composition and CE conditions

The effects of the 2^{7-3} fractionary factorial design are shown in Table 2. A close look at the results values reveals that the effects of the Brij35 and SDS levels were not significant, thus the lower levels for both were adopted. The time of injection did not also have any significant effect during the studied interval and the high level was chosen in order to obtain better sensitivity. The temperature was kept at a low level to avoid bubbles that could be formed by the evaporation of the organic solvent. The most significant effects came from the variables TBS, MeOH and their interaction. These variables were therefore studied further through the response surface methodology.

According to Table 3, the best condition (lowest CRS value) was achieved with 20% MeOH and 10 mmol/L. However, these conditions did not give good separation of the phenolic compounds of wine. Thus, response surface analysis, using the reciprocal of the CRS value, was used to obtain the optimum separation condition: 20% MeOH, 17 mM TBS, 25 kV, 3 s inj. 10 mBar at 25^{o}C (Figure 1).

Table 2. Principal effects obtained from the factorial design 2^{7-3}

Variable	% Contribution
Sodium tetraborate	26.1
MeOH	12.9
Sodium dodecylsulfate	3.5
Brij35	1.6
Injection time	8.3
Voltage	0.9
Temperature	3.8
Sodium tetraborate:MeOH	18.1
Sodium tetraborate: Sodium dodecylsulfate	4.4
Sodium tetraborate:Brij	1.1
Sodium tetraborate:injection time	9.6
Sodium tetraborate:voltage	2.6
Sodium tetraborate:temperatue	6.9

Table 3. Response function results of the response surface analysis

Experiment	% MeOH v/v	TBS mmol/L	CRS
1	0	20	9.6
2	0	10	43.8
3	20	20	3.9
4	10	10	6.3
5	0	30	135.5
6	20	10	2.6
7	20	10	2.8
8	20	30	1000
9	10	20	4
10	10	30	5.1
11	20	13	3.5
12	30	10	3.8
13	30	5	7.9
14	40	3	1000
15	20	5	8.2
16	10	2	1000
17	40	25	1000

TBS, sodium tetraborate; CRS, chromatographic resolution statistic.

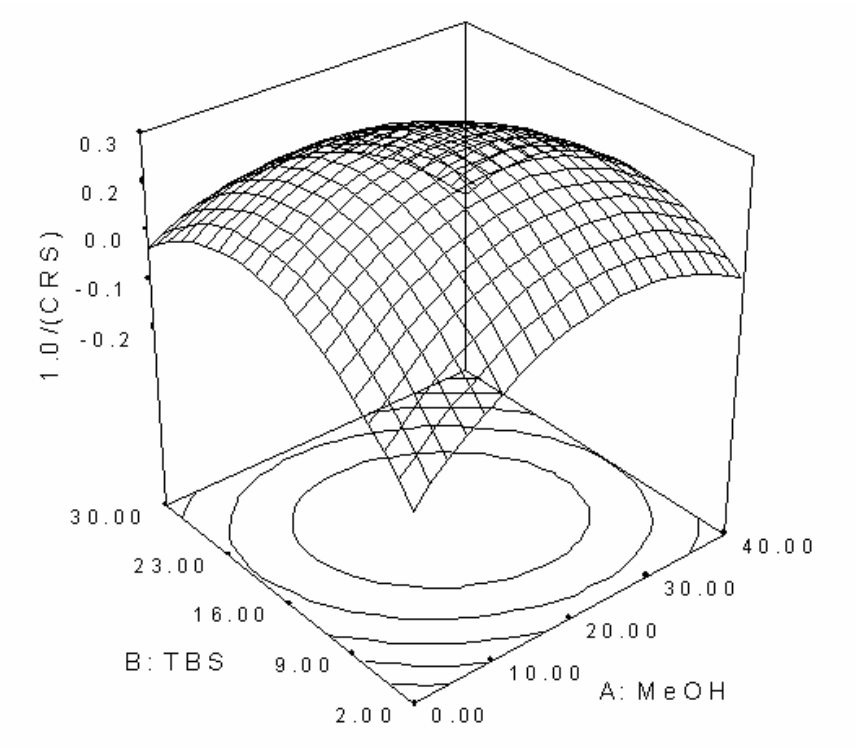

Figure 1. Response surface for electrolyte composition and CE conditions. Optimum conditions: 20% MeOH, 17mM tetraborate, 25 kV, 3 s injection 10 mBar, 25°C. TBS, sodium tetraborate; CRS, chromatographic resolution statistic

3.2. Method performance

Table 4 shows the performance characteristics of the proposed method. At the concentration range tested (2.5 to 220 mg/L), the method presented good R^2 values (from 0.9997 to 0.9936). The limit of quantification varied from 0.04 to 0.08 mg/L and the limit of detection from 0.01 to 0.03 mg/L. The coefficients of variation (CV) of ten consecutive injections of standard solutions were lower than 2% for both peak area and

Table 4. Performance characteristics of the CE method developed

Compound	Concentration range (mg/L)[a]	Coefficient of correlation[a]	Limit of quantification[a] (mg/L)	Limit of detection[a] (mg/L)	Recovery (%)[b]	Repeatability (%CV)[c]	
						Peak area	Migration time (min)
Resveratrol	2.5-80	0.9975	0.08	0.03	98±1	1.2	1.1
Catechin	2.5-80	0.9985	0.04	0.01	101±1	1.6	1.1
Rutin	2.5-80	0.9963	0.05	0.02	102±1	1.2	1.0
Syringic acid	5.0-60	0.9985	0.07	0.03	99±1	1.7	1.3
Kaempferol	5.5-60	0.9989	0.05	0.02	101±1	2.0	1.4
Coumaric acid	18-220	0.9963	0.05	0.02	98±1	0.9	1.5
Myricetin	5.0-90	0.9974	0.08	0.02	97±1	1.3	1.4
Quercetin	4.0-60	0.9976	0.05	0.01	97±1	1.4	1.4
Caffeic acid	4.0-60	0.9936	0.07	0.02	101±1	1.3	1.6
Gallic Acid	4.0-60	0.9944	0.05	0.01	99±1	1.7	1.7

[a]Seven concentrations, each injected in triplicate. [b]At three levels, each level in triplicate. [c]Ten consecutive injections.

migration time. Recoveries varied from 97% (myricetin and quercetin) to 102% (rutin). The method is considered therefore suitable for the analysis of phenolic compounds in wine.

3.3. Polyphenols of Brazilian wines

Figure 2 presents typical electropherograms of white and red wines and Table 5 the concentrations of the 10 phenolic compounds determined in 23 Brazilian wines.

Gallic (15.0-69.9 mg/L for red wine; 1.6-2.9 mg/L for white wine) and caffeic (11.7-17.9 mg/mL for red wine; 1.8-4.4 mg/L for white wine) acids were the only phenolic compounds found in all the wines. German and Walzem (2000) put together published data on phenolic compounds and the ranges were 26-320 mg/L gallic acid and 3-18 mg/L caffeic acid for red wines. The Brazilian wines, therefore, are in the lower end and caffeic in the upper end of these ranges.

Myricetin was found in all red wines (11.1-12.8 mg/L) and one white wine (1.6 mg/L). Quercetin was also encountered in all red wines (1.0-15.9 mg/L) and in one white wine (1.7mg/L). According to German and Walzem, myricetin (2-45 mg/L) and quercetin (5-53) were found only in red wines, the Brazilian values falling within these ranges.

Syringic acid was detected in seven (4.4-4.9 mg/L) out of 11 red wines and in one white wine. The range reported by German and Walzen (2000) was 4.2-5.9mg/L, in agreement with the data of the present work.

Coumaric acid was greater in blended wines (14.4-19.9), the range in red wine being 10.0-13.6 mg/L. These ranges agree with that of German and Walzen (7.5-22mg/L).

Table 5. Phenolic compound concentrations (mg/L)[a] in Brazilian wines obtained by the CE method developed.

Sample	resveratrol	syringic acid	coumaric acid	myricetin	quercetin	caffeic acid	gallic acid
Cabernet Sauvignon red 2000	3.1 ± 0.1	4.9 ± 0.1	nd	12.3 ± 0.1	11.7 ± 0.1	11.7 ± 0.1	29.1 ± 0.1
Cabernet Sauvignon red 2001	3.5 ± 0.3	4.4 ± 0.1	nd	12.8 ± 0.1	1.0[b]	17.1 ± 2.0	31 ± 3
Cabernet Sauvignon red	1.4 ± 0.2	4.5 ± 0.3	12.7 ± 0.1	12.3 ± 0.1	14.2 ± 0.1	15.9 ± 0.3	15 ± 1
Cabernet Sauvignon red	3.1 ± 0.1	4.9 ± 0.1	nd	12.3 ± 0.1	11.7 ± 0.1	11.7 ± 0.1	29.1 ± 0.1
Cabernet Franc red 2000	2.3 ± 0.1	4.5 ± 0.1	13.6 ± 0.1	12.1 ± 0.2	15.6 ± 0.4	17.9 ± 0.3	30,8 ± 1
Cabernet Franc red 2001	3.4 ± 0.1	4.4 ± 0.1	12.9 ±0 .1	11.9 ± 0.1	14.8 ± 0.3	16.4 ± 0.7	37.6 ± 0.1
Gammay red 2002	3.7 ± 0.1	nd	nd	11.1 ± 0.2	10.6 ± 0.3[b]	14.0 ± 0.4	45 ± 0.4
Gammay red 2003	1.0 ± 0.1[b]	4.5 ± 0.3	12.5 ± 0.2	11.7 ± 0.3	15.1 ± 0.5	15.2 ± 1.6	56.4 ± 2.1
Merlot red 2001	2.0 ± 0.1	nd	nd	11.2 ± 0.3	11.5 ± 0.2	17.7 ± 0.1	68.7 ± 0.2
Merlot red	1.5 ± 0.2	nd	nd	11.7 ± 0.2	13.5 ± 0.1	17.7 ± 0.1	69.9 ± 0.1
Shiraz red 2002	1.4 ± 0.2	nd	10 ± 1	12.0 ± 0.1	15.9 ± 0.2	17 ± 0.1	57.7 ± 0.1
Blended red	0.01[b]	nd	nd	14.6 ± 0.1	nd	14.8 ± 0.2	53.1±0.1
Blended	1.4 ± 0.1	4.1 ± 0.1	19.9 ± 0.3	12.7 ± 0.1	12.9 ± 0.3	14.7 ± 0.3	36.3 ± 0.2
Blended	0.4 ± 0.1 [b]	nd	14.6 ± 0.3	11.9 ± 0.1	12.1 ± 0.2	13.0 ± 0.2	35.3 ± 0,4
Blended	1.2 ± 0.2[b]	4.9 ± 0.1	16.5 ± 0.5	12.0 ± 0.3	14.6 ± 0.1	19.2 ± 0.6	28,5 ± 1
Chardonnay White 2002	nd	nd	nd	1.6 ± 0.1	1.7 ± 0.4[b]	1.8 ± 0.3	1.6 ± 0.4
Riesling white 2000	nd	nd	nd	nd	nd	2.2 ± 0.1	2.2 ± 0.2
Riesling white 2001	0.9 ± 0.2[b]	1.3 ± 0.1	2.1 ± 0.3	nd	nd	4.4 ± 1.5	2.5 ± 1.2
Riesling white 2002	nd	nd	nd	nd	nd	3.4 ± 0.3	2.5± 0.5
Riesling white	nd	nd	nd	nd	nd	3.3 ± 0.3	2.4 ± 0.2
Sauvignon Blanc white 2002	nd	nd	nd	nd	nd	2.9 ± 0.2	2.9 ± 0.2
Ugni Blanc white 2002	nd	nd	nd	nd	nd	2.2 ± 0.4	2.8 ± 0.2

[a]Means and standard deviations of triplicate analyses.
[b]Estimated by extrapolation of the calibration curve.
nd. not detected (<LOD).

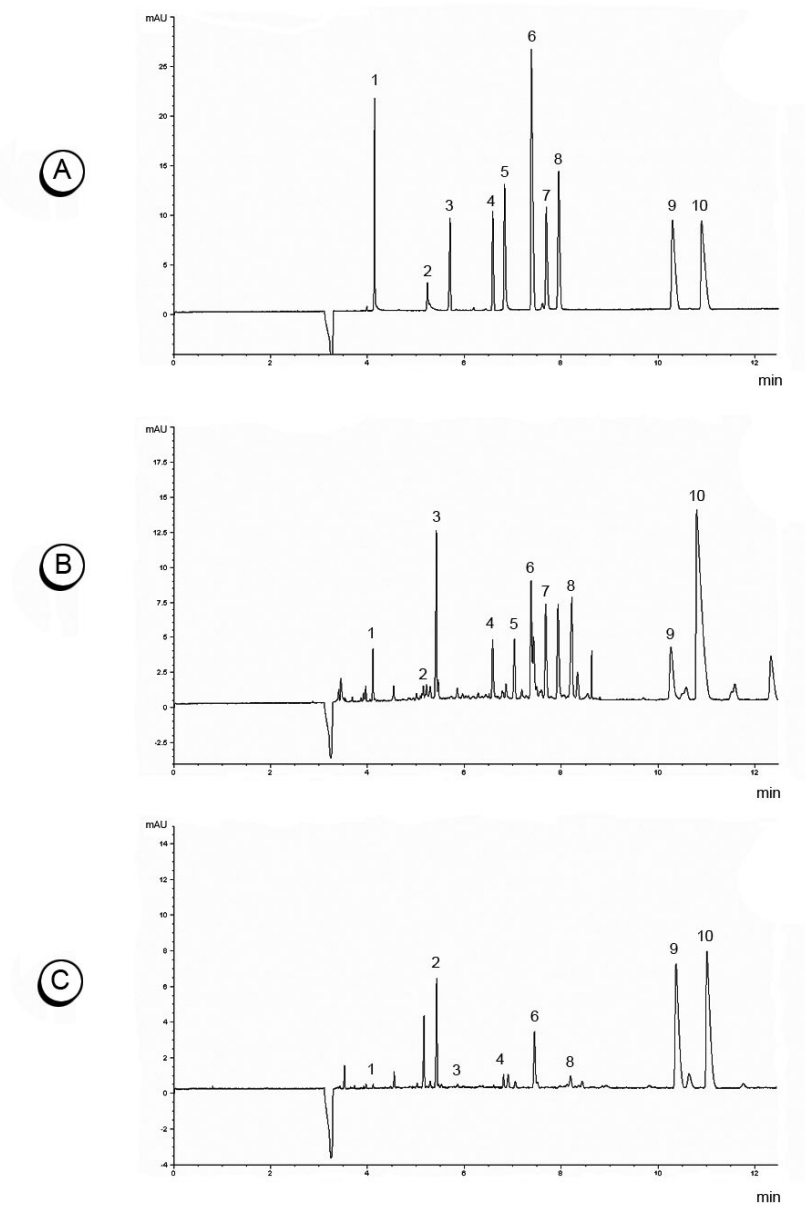

Figure 2. Electropherograms of the phenolic compounds in (a) mixture of standards, (b) red wine and (c) white wine. Peak identification: 1. resveratrol, 2. catechin, 3.rutin, 4.syringic acid, 5.kaempferol, 6.*p*-coumaric acid, 7.myricetin, 8.quercetin, 9.caffeic acid, 10. gallic acid. CE conditions are described in the text.

The concentration of the one of the most important phenolic compounds in wine, resveratrol, ranged from 1.0 to 3.7 mg/L in red wine. The range in blended wine was 0.01-1.4 mg/L; in white wine, only one had resveratrol at a low concentration (0.9 mg/L). Souto et al. (2001) found 0.82 to 5.75 mg/L in 8 types of Brazilian red wines, in agreement with the values obtained in the present work, slightly higher than the range of German and Walzen (0.1-2.3 mg/L).

The wide variation in the phenolic concentrations obtained in this and other work has two important practical implications. First, the inconsistencies in the findings of epidemiological studies, leading authors to contend that the scientific evidence is questionable or not conclusive for the beneficial effects of resveratrol and other polyphenols in wine, can be due, at least in part, to analytical and natural variability of the data on the levels of these compounds. In studies correlating these bioactive compounds with the incidence of diseases, the intake is obtained from the estimated consumption of wine, from which the amount of the bioactive substance is approximated. Second, there is now wide recognition that the levels of bioactive compounds in the diet can be maximized through agriculture, food technology and nutrition. For such a strategy, the compositional variation in the food chain has to be known. Thus, the determination of composition and investigation of the factors affecting composition of bioactive substances, using reliable methods, should remain a priority.

4. Conclusion

A simple, versatile and low-cost CE method, which utilizes methanol, sodium tetraborate and silica capillaries, was optimized by factorial design and response surface methodologies. Optimization of the electrolyte composition and CE conditions resulted in excellent selectivity. The method provided good limits of detection and quantification, as well as linearity, peak area and migration time repeatability and good recovery in the concentration levels studied. Applied to the Brazilian wines, good precision was confirmed.

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CAPÍTULO 7

Rapid quality control method for organic acids in wine by capillary electrophoresis with indirect UV detection

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Artigo a ser submetido à Revista Food Control

Rapid quality control method for organic acids in wine by capillary electrophoresis with indirect UV detection

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Abstract

A capillary electrophoresis method for six organic acids in wine was developed and validated. The optimal electrolyte consisted of 10 mmol/L 3,5-dinitrobenzoic acid (DNB) at pH 3.6 with 0.2 mmol/L cetyltrimethylammonium bromide as flow reverser. DNB was chosen because it had effective mobility similar to the organic acids, good buffering capacity at pH 3.6 and good chromophoric characteristics for indirect detection. Sample preparation involved dilution and filtration. The method showed good performance characteristics: linearity at 6 to 285 mg/L ($r > 0.99$); detection and quantification limits of 0.64 to 1.55 and 2.12 to 5.15 mg/L, respectively; separation time of less than 5.5 min. Coefficients of variation for ten injections was less than 5% and recoveries varied from 95 to 102%. Application to 23 samples of Brazilian wine confirmed good repeatability and demonstrated wide variation in the organic acid concentrations.

Keywords: Organic acids; Wine, Capillary electrophoresis, Indirect UV detection

1. Introduction

Monitoring of organic acids in wine is of paramount importance for quality and process control in the wine industry. Organic acids have a major role in the microbiological and physicochemical stability and sensory properties of wines (Ribereau-Gayon, Gloris, Maujean, & Duburdieu, 2000). These acids come from the grapes (tartaric and malic acids and, to a much lesser extent, citric acid) used as raw materials or are produced during fermentation (lactic, acetic and succinic acids) (Zoecklein, Fugelsang, Gump, & Nury, 1999). Bacterial involvement may also produce significant amounts of lactic and acetic acids.

Many analytical methods have been developed for determining organic acids. The most widely employed technique has been high performance liquid chromatography (HPLC) in various modes. Although the overall performance of HPLC methods have been found to be adequate, reversed phase HPLC with UV detection suffers from poor sensitivity and selectivity, and often requires derivatisation, increasing the time of analysis (de Villiers, Lynen, Crouch, & Sandra, 2003). Better separation is obtained with ion exchange chromatography, but expensive columns, harsh operating conditions, as well as the need for sample clean-up for complex samples as red wine, limits its use. capillary electrophoresis (CE) has therefore been increasingly promoted with the advantages of speed, simple sample preparation, efficiency of separation, low column costs, and reduced sample/solvent consumption.

CE methods for organic acids encompass direct detection (Castiñeira, Peña, Herrero, García-Martín, 2000, 2002; Garcia Moreno, Jurado Campoy, & Garcia Barroso, 2001; Mato, Suárez-Luque, S., & Huidobro, 2007; Moreno, Jurado, & Barroso, 2003;

Saavedra & Barbas, 2003) and indirect detection (Arellano, Andrianary, Dedieu, Couderc, & Puig, 1997; Arellano, Couderc, & Puig, 1997; Bianchi, Careri, & Corradini, 2005; de Villiers, et al., 2003; Esteves, Lima, Lima, & Duarte, 2004; Kandl & Kupina, 1999; Kelly & Nelson,1993; Kenney, 1991; Klampfl, Katzmayr, & Buchberger, 1998; Levi, Wehr, Talmadge, & Zhu, 1993; Sing Fung & Man Lau, 2003; Stathakis & Cassidy, 1995). Although methods based on low UV-absorbance direct detection are more sensitive, not all commercially available equipments possess detection capability at 200 nm or below. For indirect detection the choice of the background electrolyte is crucial. Different electrolyte systems have been proposed, generally comprising of quaternary ammonium salt as electroosmotic flow (EOF) reverser and either phthalate (Kelly & Nelson, 1993; Kenney, 1991; Levi et al., 1993) or chromate (de Villiers et al., 2003; Stathakis & Cassidy, 1995) as chromophoric agent. The use of chromate as chromophore is limited to high pH electrolytes because together with EOF reverser it causes precipitation below pH 7.0. Phthalate is a medium mobility chromophore and for peak symmetry considerations, it is better suited for moderately low mobility anions. Since the anions originating from the organic acids under investigation in the present work have moderate to low mobilities, a low mobility chromophoric anion was pursued.

In this work, therefore, a capillary electrophoresis (CE) method with indirect UV detection based on 3,5-dinitrobenzoic acid (DNB)/cetyltrimethylammonium bromide (CTAB) system was developed and optimized for the determination of six organic acids of enological importance in 23 Brazilian wines of popular consumption. This was the first time that DNB was used as running buffer in combination with CTAB for the analysis of organic acids in wine. CTAB was used by De Villiers et al. (2003), Esteves et al. (2004) and Kandl and Kupina (1999), but with PDC (pyridinedicarboxylic acid).

2. Materials and methods

2.1. Instrumentation

The experiments were carried out in a Beckman P/ACE capillary electrophoresis system model 5010 (Beckman Coulter Inc., Fullerton, CA, USA), equipped with a UV detector set at 254 nm for indirect detection. The temperature was controlled and stabilized at 25^{o}C. For data acquisition and treatment, a P/ACE System Gold software equipped with a personal computer was used. Electrophoretic separation was carried out in a fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) with dimensions of 57 cm (50 cm to detector) x 75 μm i.d. x 375 μm o.d.

2.2. Chemicals

All of the reagents were analytical grade, used without previous purification. DL-tartaric (>99%), citric (>99%), malic (>99%), lactic (>98%) and succinic (>99%) acids and CTAB were obtained from Aldrich (Milwaukee, WI, USA). Acetic acid (100%) and DNB (>99%) were from Merck (Darmstadt, Germany). Standard stock solutions were prepared with purified water (18.2mΩ) (Milli-Q, Millipore, Bedford, MA, USA) at 1000 mg/L and stored in a freezer for future dilutions. Stock solutions of DNB at 20 mmol/L and CTAB at 10 mmol/L were also prepared. The electrolyte solution was prepared daily and filtered through a 0.45 μm membrane.

2.3. Sample preparation

Twenty-three samples of different types (red, white and blended) and brands of Brazilian wine were purchased from local markets in Sao Paulo, Brazil. Year of production and types of wine are listed in Table 2. The contents of two freshly opened bottles were mixed, diluted 1:5 with distilled water, filtered through a 0.45 μm membrane (Millipore) and immediately analyzed.

2.4. Capillary conditioning

At each day of analysis, the capillary was conditioned by pressure flushes of 1 mol/L NaOH (5 min), deionized water (5 min) and electrolyte solution (10 min), followed by an electrokinetic flush with electrolyte solution (-25 kV during 10 min). In between runs, the capillary was reconditioned with a pressurized wash with the electrolyte solution (2 min).

2.5. Development and evaluation of method performance

Ionic mobility and pK_a data of the six acids under consideration (acetic, citric, tartaric, malic, lactic, and succinic acids) were obtained from the literature (Hirokawa, Nishino, Aoki, & Kiso, 1983) and used to calculate effective mobilities. The separation pH was first optimized by screening effective mobility versus pH curves for the largest mobility differences. Once the pH region was selected, a suitable chromophoric anion was

pursued. Electrolyte concentration was then varied from 20 to 5 mmol/L and a proper value was selected taking into consideration analysis time. Considering that the effective mobilities of the organic acids were small, a flow reverser was chosen to speed up separation. The optimized electrolyte consisted of 10 mmol/L DNB and 0.2 mmol/L CTAB, the pH being adjusted to 3.6 with HCl.

Linearity was evaluated by the injection of five standard solutions of each organic acid at concentrations varying from 6 to 285 mg/L. Limits of detection (LOD) and quantification (LOQ) were calculated as the solution concentrations that gave signal-to-noise ratios of 3 and 10, respectively. Instrumental precision was evaluated by 10 consecutive injections of the standard solutions. Recovery was studied using wine samples spiked with 20 mg/L of organic acids.

The standard solutions and the samples were hydrodynamically injected at a 0.5 psi pressure in 3 s. The electrophoretic system was operated with inverted polarity and constant voltage of -25 kV. Detection was in the indirect mode at 254 nm. All standards and samples were injected in triplicate.

3. Results and discussion

3.1. Optimization of the electrolyte composition

The absence of chromophores in the organic acids under study indicated, as a possible CZE methodological alternative, the use of indirect detection. Selection of suitable conditions such as the electrolyte and its concentration, pH, as well as the electrolyte co-ion became critical in the method development due to resolution and symmetry considerations.

As can be observed from Fig. 1, above pH 7, separation is not possible because co-elution of succinic and tartaric acids occurs. At intermediate pH (4<pH<7), separation of all acids is possible but at a very narrow pH window. At the low pH region (3<pH<4), separation is more likely to be achieved. Therefore pH 3.6 was selected as the optimal pH condition.

As can also be seen in Fig.1, DNB appeared to be a suitable chromophoric anion because its effective mobility is bracketted by the solute mobilities and it has good buffering capacity at pH 3.6.

At the optimal pH, the effective mobilities of the organic acids are relatively low. Thus, a flow reverser was needed to speed up the analysis. CTAB was chosen as the flow reverser and the analysis was conducted under inverted polarity.

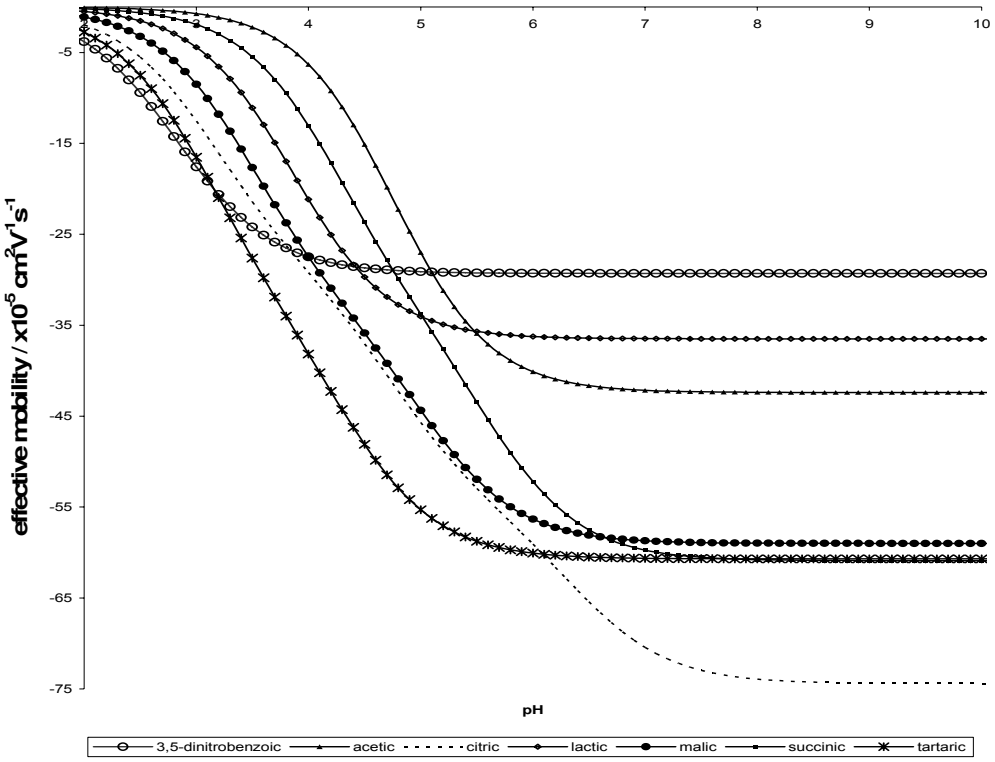

Figure 1. Curves of effective mobility values versus pH.

Fig. 2 presents the separation of standards of the organic acids under investigation at the optimal conditions. Baseline separation was achieved for all organic acids in less than 5.5 min. The proposed method was thus faster than those presented in the literature, the separation time of which varied from 6 min (Castiñeira, et al., 2000) to 20 min (Garcia Moreno et al., 2001; Levi et al., 1993), except for the methods of Mato et al. (2007) (less than 3 min), Bianchi et al. (2005) (3.5 min) and Kelly and Nelson (1993) (5 min).

3.2. Method performance

Table 1 presents the performance characteristics of the method. At the concentration ranges tested (6 to 285 mg/L), the method presented good r^2 values (from 0.9901 to 0.9988). The LOD varied from 0.64 to 1.55 mg/L and the LOQ from 2.12 to 5.15 mg/L, being lowest for malic acid and highest for citric acid in both case. As summarized by Mato et al. (2007), reported LOD varied from 0.006 to 46.4 mg/L and LOQ from 0.02 to 122 mg/L. The coefficients of variation of ten consecutive injections of standard solutions were lower than 5%. Recoveries varied from 95% (succinic acid) to the 102% (citric acid). For the few papers that reported recovery results, also summarized by Mato et al. (2007), the percentage recovery varied from 90 to 107%. When compared to values in the literature, therefore, the method developed in this study has good performance.

3.3. Organic acids of Brazilian wines

Figure 2 presents the electropherogram of the standards and Figure 3 the typical electropherogram of a Brazilian white wine, run at the optimized conditions. Table 2 shows the organic acid concentrations of 23 Brazilian wines analyzed by the proposed method.

Taking out the values which were only estimates (below the limit of quantification), the coefficients of variation of triplicate analyses varied from 0.1 to 3.9 (mean of 1.4% for 89 measurements), except for one value for acetic acid which had a CV of 7.4%. Thus, the developed method as applied to real samples has good repeatability.

Table 1. Performance characteristics of the CZE method developed.

Order of Elution	Organic Acid	Concentration range (mg/L)	Coefficient of correlation[a]	Limit of quantification (mg/L)[b]	Limit of detection (mg/L)[b]	Recovery (%)[b]
1	Tartaric	13-145	0.9983	3.42	1.02	98 ± 1
2	Malic	9-145	0.9966	2.12	0.64	99 ± 1
3	Latic	6-145	0.9988	2.35	0.70	97 ± 1
4	Succinic	7-145	0.9983	2.87	0.86	95 ± 1
5	Acetic	7-145	0.9983	3.13	0.93	101 ± 0
6	Citric	65-285	0.9901	5.15	1.55	102 ± 1

[a]Five concentrations, each injected in triplicate. [b]Injected in triplicate.

Citric acid at a concentration of 2418 mg/L was found in only one sample, a popular inexpensive Brazilian wine. Because this acid was not detected in the other wines and it is present at high level in this single wine sample, it could have been added to adjust the acidity. Citric acid as acidulant has the advantage of not forming insoluble precipitates with calcium and potassium in alcoholic solution, compared to tartaric acid (Zoecklein et al., 1999).

Tartaric (1023-2212 mg/L) lactic (35-7306 mg/L), succinic (83-700 mg/L) and acetic acid (118-1003 mg/L) were found in all the wine samples at highly variable levels. Malic acid, which is transformed to lactic acid in malolactic fermentation, was found in one sample of blended wine (2627 mg/L), two samples of red wine (2153-2243 mg/L) and three samples of white wine (1117-2112 mg/L).

Table 2. Organic acid concentrations (mg/L) in Brazilian wines obtained by the capillary electrophoresis proposed method.

Sample	Organic acids					
	Tartaric	Malic	Lactic	Succinic	Acetic	Citric
Cabernet Sauvignon red 2000	1404 ± 4		3815 ± 44	598 ± 9	1003 ± 22	
Cabernet Sauvignon red 2001	1628 ± 37		2683 ± 20	699 ± 8	752 ± 10	
Cabernet Sauvignon red	1801 ± 21		2187 ± 31	682 ± 1	620 ± 6	
Cabernet Sauvignon red	1023 ± 41		751 ± 6	142 ± 3	404 ± 14	2418 ± 14
Cabernet Franc red 2000	1603 ± 21	2153 ± 31	84±8	66 ± 6	118 ± 12	
Cabernet Franc red 2001	1642 ± 51		2566 ± 3	590 ± 18	512 ± 14	
Gammay red 2002	1498 ± 2		1981 ± 4	616 ± 14	917 ± 6	
Gammay red 2003	1459 ± 9		2504 ± 26	700 ± 9	703 ± 11	
Merlot red 2001	1536 ± 35		7306 ± 35	520 ± 2	671 ± 13	
Merlot red	1495 ± 6	2243 ± 23	35 ± 4	66 ± 3	269 ± 20	
Shiraz red red 2002	2212 ± 25		2084 ± 34	478 ± 3	814 ± 3	
Blended red	1204 ± 7		2325 ± 36	589 ± 9	775 ± 23	
Blended	1517 ± 14		2504 ± 2	631 ± 14	822 ± 5	
Blended	1555 ± 4		3392 ± 27	609 ± 1	924 ± 13	
Blended	1614 ± 33	2627 ± 21	183 ± 10	361 ± 1	224 ± 7	
Chardonnay white 2002	1273 ± 28	2112 ± 22	536 ± 2	278 ± 4	276 ± 9	
Pinot Blanc white 2001	1569 ± 38	1996 ± 18	61 ± 0.3	83 ± 3	245 ± 2	
Riesling white 2000	1521 ± 24		2761 ± 44	566 ± 10	734 ± 29	
Riesling white 2001	1345 ± 14		2269 ± 7	546 ± 8	669 ± 5	
Riesling white 2002	1451 ± 42		2381 ± 6	615 ± 5	679 ± 4	
Riesling white	1048 ± 6		2500 ± 17	338 ± 9	445 ± 7	
Sauvignon Blanc white 2002	1623 ± 11		3933 ± 18	468 ± 7	650 ± 23	
Ugni Blanc white 2002	1208 ± 25	1117 ± 8	1296 ± 6	384 ± 10	308 ± 7	

Values are the means and standard deviations of triplicate analyses.

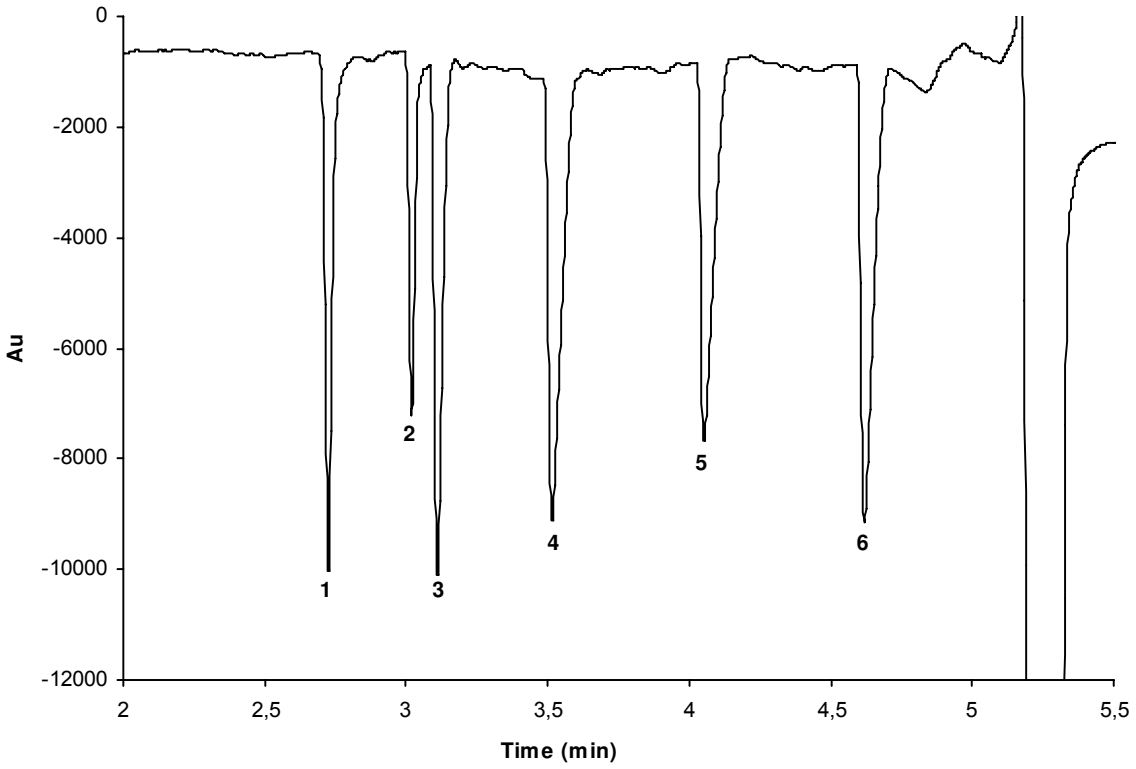

Figure 2. Eletropherogram of organic acid standards: 1-tartaric acid, 2-malic acid, 3-lactic acid, 4 - succinic acid, 5 - acetic acid, 6-citric acid. CE conditions are described in the text.

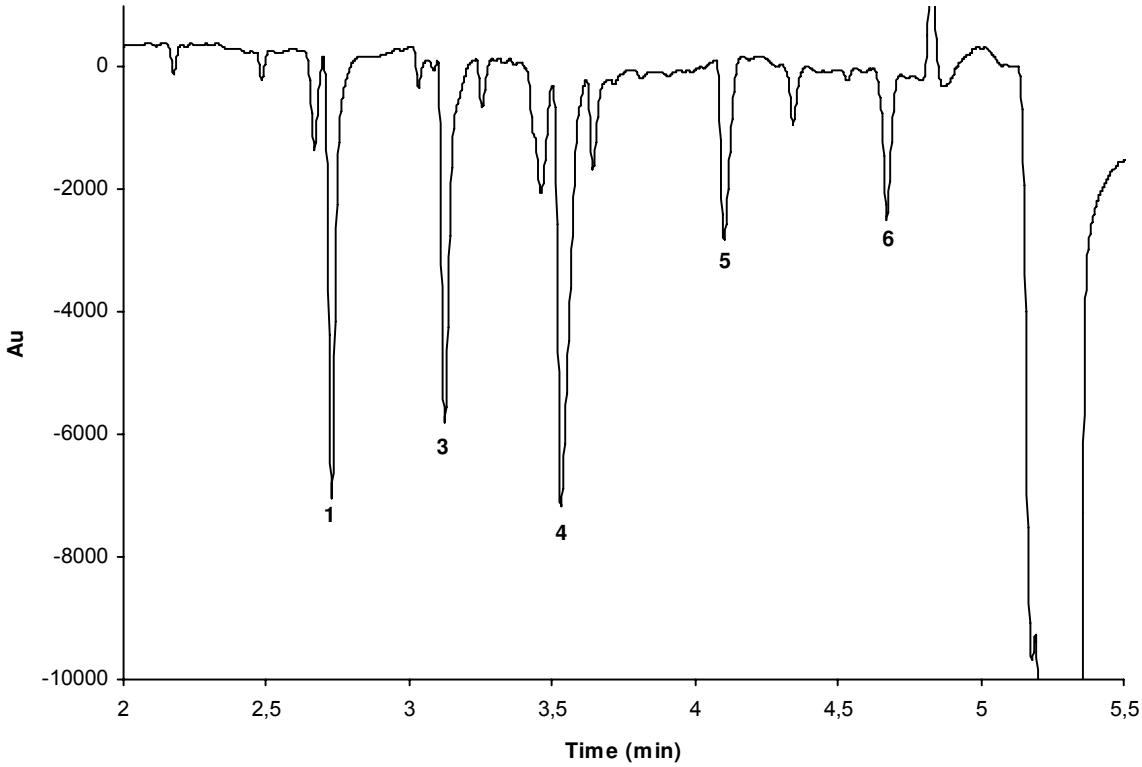

Figure 3. Typical eletropherogram of the organic acids in a sample of white wine: 1- tartaric acid, 3- lactic acid, 4 - succinic acid, 5 - acetic acid, 6-citric acid. CE conditions are described in the text.

According to Mato et al. (2007), when acetic acid is found in quantities greater than 1 g/L and lactic acid greater than various g/L, alteration of the wine could have occurred. Of the 23 Brazilian wines analyzed, one Cabernet Sauvignon red wine had 1 g/L of acetic acid, all the other wines having lower levels. The highest lactic acid content (7.3 g/L) was that of a Merlot red wine.

Recent years have seen marked improvement in the quality of Brazilian wines. The substantial variation in the organic acid composition shown in the present study indicates ample room for further improvement. The simple, rapid and reliable method herein developed can serve as a valuable tool for such an endeavor. It can also be used for quality evaluation and control in other countries.

4. Conclusion

The method developed is versatile, fast, simple and low-cost. The electrolyte/pH optimization made it possible to obtain excellent selectivity. Indirect UV detection provided good limits of detection and quantification, as well as linearity and good recovery in the concentration levels studied. Applied to Brazilian wines, good repeatability was confirmed. The organic acid concentrations of the Brazilian wines varied considerably.

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CONCLUSÕES GERAIS

1. As técnicas de eletroforese capilar e cromatografia liquida de alta eficiência acopladas a detectores de arranjo de diodos e espectrometria de massas proporcionam aplicações valiosas em alimentos, fornecendo informações sobre compostos importantes tanto na qualidade quanto nas propriedades benéficas à saúde.

2. Foram desenvolvidos métodos confiáveis por eletroforese capilar para a determinação de catequinas em chá verde, compostos fenólicos e ácidos orgânicos em vinhos.

3. Foram desenvolvidos métodos por HPLC-DAD-ESI/MS para a deterrminação simultânea de compostos fenólicos e metilxantinas em infusões de mate (*Ilex paraguariensis*).

4. Há uma marcante variação na composição dos analitos investigados em chá verde, infusões de mate e vinho, indicando a necessidade de maior controle.

5. Os flavonois quercetina e kaempferol podem ser determinados tanto na forma glicosídica como na forma de agliconas;

6. A atividade individual de compostos antioxidantes pode ser medida on-line, demonstrando que esta atividade não é proporcional a concentração;

6. Chimarrão (infusões quentes) extrai maior quantidade de compostos benéficos à saúde.

7. A comercialização de mate acarreta perdas nos compostos fenólicos e metilxantinas.