MARLA SGANZERLA

STUDY OF CAPSAICINOIDS AND VOLATILE COMPOUNDS IN BRAZILIAN *Capsicum chinense* PEPPERS

ESTUDO DE CAPSAICINOIDES E COMPOSTOS VOLÁTEIS EM PIMENTAS BRASILEIRAS Capsicum chinense

CAMPINAS

2015

UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Engenharia de Alimentos

MARLA SGANZERLA

STUDY OF CAPSAICINOIDS AND VOLATILE COMPOUNDS IN BRAZILIAN *Capsicum chinense* PEPPERS

ESTUDO DE CAPSAICINOIDES E COMPOSTOS VOLÁTEIS EM PIMENTAS BRASILEIRAS Capsicum chinense

Thesis presented to the Faculty of Food Engineering of the University of Campinas in partial fulfillment of the requirements for the degree of Ph.D. in Food Science.

Tese apresentada à Faculdade de Engenharia de Alimentos da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Ciência de Alimentos.

Supervisora/Orientadora: Profª. Dra. Helena Teixeira Godoy

ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL DA TESE DEFENDIDA PELA ALUNA MARLA SGANZERLA E ORIENTADA PELA PROFª. DRA. HELENA TEIXEIRA GODOY

_____
Assinatura do Orientador

CAMPINAS
2015

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca da Faculdade de Engenharia de Alimentos
Helena Joana Flipsen - CRB 8/5283

Sg15s

Sganzerla, Marla, 1986-
Study of capsaicinoids and volatile compounds in Brazilian *Capsicum chinense* peppers / Marla Sganzerla. – Campinas, SP : [s.n.], 2015.

Orientador: Helena Teixeira Godoy.
Tese (doutorado) – Universidade Estadual de Campinas, Faculdade de Engenharia de Alimentos.

1. Pimenta. 2. Capsicum. 3. Aroma. 4. Compostos orgânicos voláteis. 5. Otimização. I. Godoy, Helena Teixeira,1957-. II. Universidade Estadual de Campinas. Faculdade de Engenharia de Alimentos. III. Título.

Informações para Biblioteca Digital

Título em outro idioma: Estudo de capsaicinoides e compostos voláteis em pimentas brasileiras *Capsicum chinense*
Palavras-chave em inglês:
Peppers
Capsicum
Aromas
Volatile organic compounds
Optimization
Área de concentração: Ciência de Alimentos
Titulação: Doutora em Ciência de Alimentos
Banca examinadora:
Helena Teixeira Godoy [Orientador]
Cláudia Hoffmann Kowalski Schroder
Cristiano Augusto Ballus
Roger Wagner
Stanislau Bogusz Junior
Data de defesa: 27-02-2015
Programa de Pós-Graduação: Ciência de Alimentos

BANCA EXAMINADORA

Profª. Dra. Helena Teixeira Godoy
(Orientadora)

Dra. Cláudia Hoffmann Kowalski Schroder
(Membro Titular)
Laboratório Nacional Agropecuário - LANAGRO

Dr. Cristiano Augusto Ballus
(Membro Titular)
FEA - UNICAMP

Profº. Dr. Roger Wagner
(Membro Titular)
DTCA-UFSM

Profº. Dr. Stanislau Bogusz Junior
(Membro Titular)
ICT-UFVJM

Dra. Adriana Dillenburg Meinhart
(Membro Suplente)
FEA-UNICAMP

Dra. Daniele Rodrigues
(Membro Suplente)
IESI-Max Plank

Profª. Dra. Juliana Azevedo Lima Pallone
(Membro Suplente)
FEA-UNICAMP

ABSTRACT

The *Capsicum* peppers are largely used due their sensorial properties as color, pungency and aroma. These characteristics occur by the presence of determinate classes of compounds including carotenoids, capsaicinoids and volatiles. These secondary metabolism derivatives compounds are influenced by two factors: genetics and plant-environment interactions and are susceptible to water availability conditions. This study had a proposal of the characterization of the capsaicinoids and volatile composition of the *Capsicum chinense* pepper fruits. The analysis were carried out by using of chromatographic methods (UPLC, UHPLC-MS and GC-MS). The results obtained and the characterization of the *C. chinense* fruits regarding the mainly compounds which influenced in the quality of the peppers provide the scientific subsidies for improvement of general quality, agricultural practices, industrial applications and breeding programs and also, the knowledge about substances potentially beneficial to the human organism. Initially, was developed and validated a fast, efficient and reproducible method to analyze capsaicinoids in Brazilian *Capsicum chinense* fruits by means the use of an optimization strategy to extracted the capsaicinoids. The extracts were obtained following the condition 100% of methanol and 10 min on ultrasound assisted extraction. The analytical method developed in an ultra high performance liquid chromatographic system coupled to a mass spectrometer permits the separation of 8 capsaicinoids in 4 min of time analysis expending only 2 mL of solvent as mobile phase. The method achieved was fully validated and show the effectiveness and satisfactory performance to answer the analytical needs of this research area. Different accessions of C. chinense fruits were analyzed, the contents of capsaicin and dihydrocapsaicin were in the range of 156 - 1442 $\mu g.g^{-1}$ and 26 - 478 $\mu g.g^{-1}$ of fresh fruit, respectively. Characterization of aroma profile of Capsicum chinense peppers 'Habanero' type, using Headspace Solid-Phase Microextraction (HS-SPME), was performed after an optimization step by GC-MS. Central composite design and Derringer's desirability function strategies were used for this optimization, in order to

evaluate simultaneously the 'total sum peak areas' and 'number of extracted compounds' responses. The maximized results for both responses were obtained using PDMS/Car/DVB fiber, temperature of 40 °C and extraction time of 30 min. Eighty-two compounds were tentatively identified in the volatile fraction of the 'Habanero' pepper and the most abundant were hexyl isovalerate, cis-hexenyl isovalerate, hexyl 3-methylbutanoate, 3,3-dimethylcyclohexanol, longifolene, and 2-methyl-1-tetradecene. The compound 2,3-dimethylcyclohexanol and longifolene were reported for the first time in 'Habanero' pepper. In addition, Brazilian 'Habanero' peppers grown under different condition of water availability were evaluated regarding their capsaicinoids and volatiles composition. The higher values of capsaicin and dihydrocapsaicin were found in the mature peppers, achieving ranges from 2.85 - 3.33 $mg.g^{-1}$ for capsaicin and 1.06 - 1.71 $mg.g^{-1}$ for dihydrocapsaicin. Total volatile compounds presented higher values for the peppers in green stage of maturity with ranges from 195.47 - 298.94 $mg.kg^{-1}$, whereas in the mature samples the range was from 68.74 - 118.50 $mg.kg^{-1}$. According the results obtained with the PCA performed was possible separe the 'Habanero' peppers in function of the degree of maturity. While, PCA apllied, separately, for green and mature peppers showed a clear separation in function of the harvest date. This fact can be justified by the large influence of maturity degree and harvest date on the peppers composition. The peppers were clustered in function of the water availability treatments received, but to better understand the effects of water availability on the peppers composition, another strategy of evaluation, with more frequent monitoring would be interesting, as well as the inclusion of sensorial studies, as the olfactometry technique.

Keywords: chili pepper; 'Habanero'; water stress; pungency; aroma; HS-SPME; GC-MS; UHPLC; optimization.

RESUMO

As pimentas do gênero *Capsicum* apresentam ampla aplicação em função dos atributos sensoriais de cor, pungência e aroma, decorrentes da presença de carotenoides, capsaicinoides e voláteis. No entanto, variações no conteúdo desses metabólitos secundários dependem de fatores como sazonalidade, ritmo circadiano, desenvolvimento da planta, características edafo-climáticas, ataque de patógenos. Neste estudo foi realizada a caracterização da composição de capsaicinoides e fração volátil de frutos de *Capsicum chinense*. A caracterização foi realizada através do uso de métodos analíticos cromatográficos (UPLC, UHPLC-MS e GC-MS). Com a execução desta pesquisa, foi possível caracterizar os frutos de *C. chinense* quanto à composição de metabólitos relacionados aos seus principais parâmetros de qualidade, fornecendo subsídios científicos para o conhecimento e melhoria da qualidade em termos de produção, aplicação industrial, compreensão de rotas metabólicas e melhoramento genético, além de gerar informações a respeito de substâncias potencialmente benéficas, devido a suas ações no organismo humano. Inicialmente foram desenvolvidos e validados os métodos, usando ferramentas estatísticas. De acordo com a etapa de otimização para a extração dos capsaicinoides, 100% de metanol combinado com o uso do banho de ultrassom por 10 minutos foram empregados. O método analítico desenvolvido através do uso de cromatografia em fase líquida de ultra eficiência permitiu a separação de 8 capsaicinoides em 4 minutos de análise cromatográfica e mostrou-se eficiente de acordo com a validação realizada. 9 acessos de pimentas *C. chinense* foram analisadas. Os teores de capsaicina e dihidrocapsaicina obtidos estavam nas faixas de 156 -1442 $\mu g.g^{-1}$ e 26 - 478 $\mu g.g^{-1}$ (peso de fruto fresco), respectivamente. A caracterização do perfil de voláteis em pimentas 'Habanero', através do uso de HS-SPME (Headspace Solid-Phase Microextraction), foi realizada após etapa de otimização. Delineamento composto central e ferramenta de desejabilidade foram usadas a fim de avaliar, simultaneamente, duas respostas: soma total da área dos picos e número de compostos extraídos. O resultado maximizado para as duas

respostas usando a fibra tripla (DVB/Car/PDMS) indicou o uso das condições de temperatura de extração de 40 °C e 30 min de extração. 82 compostos foram tentativamente identificados na fração volátil da pimenta 'Habanero' e os ésteres foram os compostos mais abundantes. O 2,3-dimetilciclohexanol e longifoleno foram identificados pela primeira vez em pimentas 'Habanero'. Além disso, pimentas 'Habanero' submetidas à diferentes condições de disponibilidade de água durante seu cultivo foram avaliadas quanto a composição de capsaicinoides e voláteis. Maiores quantidades de capsaicina e dihidrocapsaicina foram encontradas na pimentas maduras, alcançando valores entre as faixas de 2,85 - 3,33 $mg.g^{-1}$ para capsaicina e 1,06 - 1,71 $mg.g^{-1}$ para dihidrocapsaicina. Os teores de compostos voláteis totais foram maiores nas pimentas verdes (195,47 - 298,94 $mg.kg^{-1}$), independente do tratamento de estresse hídrico que receberam. Os resultados obtidos com o PCA realizado possibilitaram, inicialmente, separar as pimentas 'Habanero' em função do grau de maturação. Enquanto, PCA aplicado, separadamente, nas pimentas verdes e maduras demonstrou uma separação evidente em função da data de colheita. Isso se deve ao fato de que influências do grau de maturação e época da colheita se sobressaíram no estudo. As pimentas foram agrupadas de acordo com o tratamento de irrigação recebido, mas para uma melhor compreensão dos efeitos dos tratamentos de estresse hídrico sobre a composição das amostras, seria necessário uma estratégia de estudo com monitoramento mais frequente dos frutos formados além de outras investigações sensoriais, como o uso de olfatometria.

Palavras-chave: pimentas; 'Habanero'; estresse hídrico; pungência; aroma; UHPLC; HS-SPME; GC-MS; otimização.

SUMÁRIO

"Quelli che s'innamorano di pratica senza scienza son come il nocchiere, che entra in naviglio senza timone o bussola, che mai ha certezza su dove si vada.

Leonardo da Vinci

“I was just guessing at numbers and figures

Pulling the puzzles apart

Questions of science, science and progress

Do not speak as loud as my heart

(…)

Nobody said it was easy

No one ever said it would be this hard"

Chris Martin, *The scientist*

Dedico este trabalho aos meus pais, Derli e Carmen,

ao meu irmão Marcos, ao Felipe, meu noivo,

e a todos professores que participaram e

contribuíram para a minha formação,

desde os primeiros anos da educação básica

até a conclusão de mais esta etapa da vida acadêmica.

AGRADECIMENTOS

À Faculdade de Engenharia de Alimentos e ao Departamento de Ciência de Alimentos pela oportunidade de realização do doutorado e elaboração desta tese.

À professora Dra. Helena Teixeira Godoy, orientadora deste doutorado, por me aceitar como sua aluna e por todo apoio, confiança, carinho e pela amizade que me foram dados ao longo deste período. Agradeço imensamente pela oportunidade de trabalharmos juntas, foi um período muito gratificante e enriquecedor, seus conselhos e ensinamentos me acompanharão em toda minha vida profissional e pessoal.

Aos meus pais, Derli e Carmen, e ao meu irmão Marcos, o maior incentivador da minha trajetória, por todo o amor e carinho. Obrigada por estarem ao meu lado em qualquer circunstância, apoiando minhas decisões, compreendendo as dificuldades impostas pela distância e sempre me incentivando a seguir em busca dos meus objetivos.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), pela concessão da bolsa de Doutorado e pela concessão da bolsa de estudos de Doutorado Sanduíche no Exterior (PSDE, processo nº 1262/12-2).

À Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), pela aprovação do projeto (processo nº 2011/17094-2), cujo financiamento tornou possível a aquisição do equipamento GC-MS e dos materiais e reagentes para a realização de grande dos experimentos desta tese.

Aos membros da banca examinadora, Drª. Cláudia Hoffmann Kowalski Schroder, Dr. Cristiano Augusto Ballus, Dr. Roger Wagner, Dr. Stanislau Bogusz Junior, Drª. Adriana Dillenburg Meinhart, Drª. Daniele Rodrigues e Drª. Juliana Azevedo Lima Pallone, pela sua disponibilidade em colaborar no aprimoramento deste trabalho através das suas importantes correções e sugestões.

Ao Felipe, meu companheiro, grande amor da minha vida, que está sempre ao meu lado (apesar dos nossos desencontros pelo mundo) desde quando o conheci, no início deste doutorado. Está sempre disponível a me ouvir e me ajudar

no que for preciso, oferecendo-me a sua cumplicidade, dedicação incondicional, seus ensinamentos e seu amor. Ter te conhecido foi a melhor coisa que me ocorreu, não há palavras para descrever a felicidade que este encontro me proporcionou e proporciona em todos os meus dias.

Aos meu sogros Ademir e Ana, por me acolherem como se fosse uma filha, pelo carinho, convivência e todo suporte no retorno a Campinas.

Aos colegas e amigos tão especiais, Cristiano, Sergio, Sabrina, Leonardo, Ana Paula e Juliana por todos os bons momentos que compartilhamos, bem como pelo apoio e suporte nos momentos difíceis. Sem vocês, este caminho teria sido repleto de dias mais duros.

Aos colegas do Laboratório de Análise de Alimentos, Adriana, Milene, Dani Rodrigues (Dani Bio), Paula, Francisco (Chico), Pollyane, Marcus, Danilo, Janclei, Michelly, Alane, Dani Neves, Kleidson, Elenice, Tayse, Thais, Maria Rosa, Mateus, Stefany, e Wellington pelo carinho e pelos momentos de alegria e descontração que compartilhamos. Agradeço também aos funcionários do laboratório, às técnicas Renata, Marcela e Miriam e em especial, ao Seu Dirceu, um exemplo de pessoa e profissional, pelas conversas nas longas jornadas de trabalho no laboratório e sua constante disposição em ajudar.

À professora Dra. Juliana Azevedo Lima Pallone, pela amizade, pelo carinho e atenção, além das agradáveis conversas nesses anos. Ao professor Dr. Marcelo Alexandre Prado, pela oportunidade de participar do Programa de Estágio Docente sob sua orientação na TP 513.

Aos funcionários da Secretaria de Pós-Graduação, em especial ao Cosme e ao Marcos, por todo o suporte e paciência, principalmente durante o processo de defesa. Ao pessoal da Secretaria do DCA, Marcos, Jardete e Guiomar pela disponibilidade e ajuda com diversas documentações durante o período do doutorado.

A todos os amigos que me acompanharam durante o período do doutorado, mesmo estando longe, em especial, Francine, Sá, Graci, Dai, e Beto.

Ao Thiago, meu professor de italiano, que com muita competência e habilidade me ensinou esta língua tão bela e encantadora. "*Sei um grande amico, Thiago! Ho uma grande ammirazione per te e ti voglio tanto bene, grazie per tutto!*"

Desidero ringraziare al Professore Dr. Carlo Bicchi, della Universitá degli Studi di Torino (Italia), per avermi consentito di svolgere il mio interscambio di dottorato nel suo laboratorio (Laboratory of Phytochemical Analysis), mettendo a disposizione tutta la sua competenza, suo tempo e grande conoscenza su il mondo dei composti volatili e per i preciozi consigli per la vita. Ringrazio anche ala Professoressa Patrizia Rubiolo e alle tutte ricercatrice del laboratorio, Chiara Cordero, Cecilia Cagliero, Erica Liberto e Barbara Sgorbini. Ai miei colegui e amici, Alessandra, Luca, Thais, Cristina, Rosanna, Stefano, Annalisa e Giulia per la amicizia, per i bellissimi momenti trascorsi, per l'ora del caffe e per tutti momento di pausa pranzo che abbiamo avuto insieme.

Ringrazio anche alla tutta famiglia Maiorca per l'accoglienza, principalmente alla mia grande amica Francesca: "Non c'era posto migliore per andare a vivere che non insieme a te, sono stata fortunata!".

Per tutto che mi há permesso vivere, grazie mille, Italia e a presto!

A todos que já foram meus professores durante esta longa jornada de estudos.

E agradeço também a todos que, porventura não tenham sido aqui citados, colaboraram para a realização deste trabalho e para que eu pudesse chegar até aqui.

LISTA DE ILUSTRAÇÕES

LISTA DE TABELAS

INTRODUÇÃO GERAL

Natural do continente americano, a pimenta do gênero *Capsicum* é cultivada do norte ao sul do Brasil, o qual é um importante centro de diversidade genética dessas espécies (WAGNER, 2003). Segundo o pesquisador Geovani Amaro da Embrapa Hortaliças, 17 espécies do gênero *Capsicum* são encontradas no país. Ao todo, o Brasil produz por ano cerca de 160 mil toneladas de pimenta, numa área cultivada equivalente a 6 mil hectares (O ESTADO DE SÃO PAULO, 2010). A produtividade média depende do tipo de pimenta cultivada, variando de 10 a 30 toneladas por hectare. Em 2005, o volume das exportações brasileiras de pimentões e pimentas atingiu mais de 9.200 toneladas, 8,7% maior que no ano de 2004, posicionando-se como a segunda principal hortaliça exportada pelo país, atrás apenas do melão, representando uma contribuição de 13,5% no valor total de exportações dessa categoria e com mercado estimado em 2008, em mais de R$ 100 milhões e em crescimento, tornando o agronegócio de pimentão e outras pimentas *Capsicum*, um dos mais atraentes no Brasil (RIBEIRO et al., 2008).

Acredita-se que o crescimento das exportações está relacionado à avidez do mercado por este condimento, que é frequentemente empregado em formulações alimentícias industrializadas ou caseiras nos mais diversos países (STEINHAUS; SCHIEBERLE, 2005; NISHA et al., 2009). A indústria de alimentos as emprega como agente colorante e flavorizante em molhos, sopas, carnes processadas, lanches, doces e bebidas alcoólicas (PINO et al., 2007). Além da indústria

alimentícia, indústrias farmacêuticas e cosméticas também são responsáveis pela crescente utilização desses frutos em suas formulações (SANTOS, 2009).

As pimentas são largamente utilizadas por contribuírem com sabor, cor e aroma aos alimentos (LIU et al., 2010; ORNELAS-PAZ et al., 2011), devido à presença de substâncias pungentes, pigmentos e aromas, que tem sua composição monitorada a fim de estabelecer a qualidade dos frutos de acordo com a ASTA (American Spice Trade Association), que é um importante elemento no mercado de especiarias nos Estados Unidos e, atualmente possui abrangência internacional (ASTA, 2010). Entre os componentes químicos das pimentas que determinam essas propriedades para o seu uso como condimento, estão a composição de metabólitos secundários como a capsaicina e seus análogos estruturais, os carotenoides e vários compostos voláteis, especialmente as pirazinas, além de ácidos orgânicos (RIBEIRO et al., 2008). As pimentas também são ricas em substâncias com propriedades antioxidantes, que reduzem o risco de desenvolvimento de câncer e de outras doenças crônico-degenerativas (RIBEIRO et al., 2008). Elas tem aplicação na medicina tradicional, contribuindo através das suas ações antimicrobiana, inseticida, anti-convulsiva e sedativa (OTERO et al., 2000; SOUZA et al., 2006).

Variações no conteúdo total bem como nas proporções relativas de metabólitos secundários nas plantas ocorrem em diferentes níveis. Essas variações podem ser sazonais e diárias, intraplanta, inter e intraespecíficas. Apesar da existência de um controle genético, a expressão pode sofrer modificações resultantes da interação de processos bioquímicos, fisiológicos, ecológicos e

evolutivos (HARTMANN, 1996). De fato, os metabólitos secundários representam uma interface química entre as plantas e o ambiente circundante, portanto, sua síntese é frequentemente afetada por condições ambientais (KUTCHAN, 2001). Assim, fatores como a sazonalidade, ritmo circadiano, desenvolvimento da planta, temperatura, índice pluviométrico, disponibilidade hídrica, radiação ultravioleta, nutrientes, altitude, composição atmosférica e ataque de patógenos, interferem diretamente no metabolismo secundário de plantas.

Atualmente, diante das mudanças climáticas globais, as práticas agrícolas são focadas na otimização do manejo de recursos através de um melhor controle das quantidades de água e nutrientes requeridos para melhorar os aspectos gerais da planta e sua produtividade (SERRANO et al., 2010). Contudo, deve-se considerar que fatores fisiológicos críticos, tais como fotossíntese, comportamento estomatal, mobilização de reservas, expansão foliar e crescimento, podem ser alterados por estresse hídrico e, consequentemente, levar a alterações no metabolismo secundário (TAIZ; ZIEGER, 2002).

Outros fatores que podem afetar o conteúdo final de metabólitos secundários estão relacionados às condições de colheita e estocagem, sendo que a constância de concentrações de metabólitos secundários é praticamente uma exceção (GOBBO-NETO; LOPES, 2007).

Das espécies de *Capsicum* cultivadas no Brasil, a espécie *Capsicum chinense* destaca-se por abranger pimentas suaves e doces, como a pimenta 'Biquinho', e também as recordistas em pungência, como a 'Murupi' e a 'Habanero'. As pimentas da espécie *C. chinense* são conhecidas como pimentas de cheiro

devido às suas características aromáticas pronunciadas nas inúmeras variedades de frutos que a espécie produz, os quais podem ter diferentes formatos e cores que variam entre amarelo, laranja, vermelho e preto, quando maduros. A área de maior diversidade de *C. chinense* é a bacia Amazônica, sendo que a espécie foi domesticada pelos indígenas amazônidas e, por isso, é considerada a mais brasileira dentre as espécies domesticadas (RIBEIRO et al., 2008).

A participação das pimentas do gênero *Capsicum* nas exportações de hortaliças é de grande relevância para a economia do país e a ampliação do conhecimento sobre a composição desses frutos pode valorizá-los ainda mais. Existe, no Brasil, uma grande diversidade genética de pimentas do gênero *Capsicum*, com produção e mercado em franca expansão, bem como renomados centros de pesquisa com adequada infra-estrutura voltados ao estudo de conservação das espécies. No entanto, ainda são poucos os trabalhos que se dedicam à caracterização da composição de metabólitos secundários neste tipo de amostra.

O objetivo geral deste estudo foi caracterizar a composição de capsaicinoides e fração volátil de frutos de *C. chinense* em dois estádios de maturação, verde e madura, obtidos em condições controladas de aporte hídrico, com e sem deficiência hídrica mediante o desenvolvimento e validação de métodos adequados para estas análises. A caracterização da composição de capsaicinoides e fração volátil das diferentes pimentas *C. chinense* obtidas com e sem deficiência hídrica compreende as principais características (pungência e aroma) relacionadas à qualidade dos frutos que são de interesse tecnológico e industrial, além de fornecer informações

a respeito dessas substâncias potencialmente benéficas à saúde humana devido suas ações no organismo que estão sendo apontadas por inúmeros autores. De acordo com a proposta deste estudo também poderão ser obtidos dados de grande valia para estudos de melhoramento genético e forma de cultivo da espécie, com otimização do uso de recursos hídricos atendendo a uma importante demanda ambiental.

Referências

ASTA. American Spice Trade Association. A history of ASTA. <http://www.astaspice.org/i4a/pages/index.cfm?pageid=3291> acesso em 02 de junho de 2010.

GOBBO-NETO, L.; LOPES, N. P. Plantas medicinais: fatores de influência no conteúdo de metabólitos secundários. Química Nova, 30(2):374-381, 2007.

HARTMANN, T. Diversity and variability of plant secondary metabolism: a mechanistic view. Entomologia Experimentalis et Applicata, 80:177-188, 1996.

KUTCHAN, T. M. Ecological arsenal and developmental dispatcher. The paradigm of secondary metabolism. Plant Physiology, 125:58-60, 2001.

LIU, L.; CHEN, X.; LIU, J.; DENG, X.; DUAN, W.; TAN, S. Determination of capsaicin and dihydrocapsaicin in *Capsicum anuum* and related products by capillary electrophoresis with a mixed surfactant system. Food Chemistry, 119:1228-1232, 2010.

NISHA, P.; REKHA, S. S.; PANDIT, A. B. The degradation kinetics of flavor in black pepper (*Piper nigrum* L.). Journal of Food Engineering, 92:44–49, 2009.

O ESTADO DE SÃO PAULO. Pimentas para todos os gostos. Caderno Agrícola, p. 6-7, 17 de março de 2010.

ORNELAS-PAZ, J. de J.; CIRA-CHÀVEZ, L. A.; GARDEA-BÉJAR, A. A.; GUEVARA-ARAUZA, J. C.; SEPÚLVEDA, D. R.; REYES-HERNÁNDEZ, J.; RUIZ-CRUZ, S. Effect of heat treatment on the content of some bioactive compounds and free radicalscavenging activity in pungent and non-pungent peppers. Food Research International, doi:10.1016/j.foodres.2011.01.006, 2011.

OTERO, R.; NÚÑEZ, V.; BARONA, J.; FONNEGRA, R. JIMÉNEZ, S. L.; OSORIO, R.G.; SALDARRIAGA, M; DÍAZ, A. Snakebites and ethnobotany in the northwest region of Colombia. Part III: Neutralization of the haemorrhagic effect of Bothrops atrox venom. Journal of Ethnopharmacology, 73:233-241, 2000.

PINO, J.; GONZALEZ, M.; CEBALLOS, L.; CENTURION-YAH, A. R.; TRUJILLO-AGUIRRE, J.; LATOURNERIE-MORENO, L.; SAURI-DUCH, E. Characterization of total capsaicinoids, colour and volatile compounds of Habanero chilli pepper (*Capsicum chinense* Jack.) cultivars grown in Yucatan. Food Chemistry, 104(4):1682-1686, 2007.

RIBEIRO, C. S. C.; LOPES, C. A.; CARVALHO, S. I. C. de; HENZ, G. P..; REIFSCHNEIDER, F. J. B. Pimentas *Capsicum*. Brasília: Embrapa Hortaliças, 2008. 200p.

SERRANO, M.; ZAPATA, P. J.; CASTILHO, S.; GUILLÉN, F.; MARTÍNEZ-ROMERO, D.; VALERO, D. Antioxidant and nutritive constituents during sweet pepper development and ripening are enhanced by nitrophenolate treatments. Food Chemistry, 118:497–503, 2010.

SOUSA, E. T.; RODRIGUES, F. M.; MARTINS, C. C.; OLIVEIRA, F. S. de; PEREIRA, P. A. de P.; ANDRADE, J. B. de. Multivariate optimization and HS-SPME/GC-MS analysis of VOCs in red, yellow and purple varieties of *Capsicum chinense* sp. Peppers. Microchemical Journal, 82:142-149, 2006.

STEINHAUS, M.; SCHIEBERLE, P. Characterization of odorants causing an atypical aroma in white pepper powder (*Piper nigrum* L.) based on quantitative measurements and orthonasal breakthrough thresholds. Journal of Agricultural and Food Chemistry, 53:6049-6055, 2005.

TAIZ, L.; ZEIGER, E. Plant physiology. 3. ed. Sunderland: Sinauer Associates, 2002. 690p.

WAGNER, C.M. Variabilidade e base genética da pungência e de caracteres do fruto: implicações no melhoramento de uma população de *Capsicum*. Tese de Doutorado: Escola Superior de Agricultura Luiz de Queiroz, 2003, 104p.

CAPÍTULO I

REVISÃO BIBLIOGRÁFICA

Características e composição de frutos Capsicum chinense

CAPÍTULO I

REVISÃO BIBLIOGRÁFICA

Características e composição de frutos *Capsicum chinense*

Marla Sganzerla, Helena Teixeira Godoy

Departamento de Ciência de Alimentos, Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas (UNICAMP), Rua Monteiro Lobato, 80, Cidade Universitária Zeferino Vaz, 13083-862, Campinas, SP, Brasil

1.1. Pimentas *Capsicum*

As pimentas estão entre as hortaliças mais populares no mundo, com ampla aplicação devido aos seus atributos sensoriais de cor, pungência e aroma. Os frutos da pimenteira são bastante importantes comercialmente, quantidades expressivas de diversas variedades do gênero *Capsicum* são produzidas e comercializadas. A China está entre os países que, como o México, cultivam grande quantidade de pimentas, que são muito utilizadas na sua culinária tradicional (LIU et al., 2009). No Brasil, consiste na segunda hortaliça mais exportada, respondendo por 13,5% do valor de exportações dessa categoria no ano de 2005 e com mercado estimado para 2008 em mais de R$ 100 milhões com tendência de crescimento, tornando o agronegócio de pimentão e outras pimentas *Capsicum*, um dos mais atraentes no Brasil (RIBEIRO et al., 2008).

A produção de pimenta (*Capsicum* spp.) para uso como condimento em produtos alimentícios industrializados vem crescendo e, atualmente, é uma atividade olerícola bastante rentável, inclusive para pequenos produtores e pequenas indústrias de conservas. Cinco espécies são comumente cultivadas no Brasil, principalmente no centro-sul, entre elas: *C. annuum*, *C. frutescens*, *C. chinense*, *C. baccatum*, *C. pubscens* (RIBEIRO et al., 2008).

As pimentas constituem um grupo de espécies botânicas com características próprias, que produzem frutos geralmente com sabor picante, embora também existam pimentas doces. A planta é arbustiva, atingindo 120 cm de altura, com ampla formação de ramificações laterais e possibilidade de tornar-se perene. Normalmente, ela é auto-polinizada, todavia a polinização cruzada pode ocorrer, o

que explica a grande variabilidade dos frutos do gênero *Capsicum* quanto ao tamanho, formato e cores quando maduros (GUZMAN et al., 2010).

A espécie *C. chinense* foi originalmente encontrada na bacia amazônica, mas atualmente, é cultivada do sul ao norte do Brasil devido a sua fácil adaptação em diferentes tipos de solo e clima, e pelas suas características aromáticas peculiares que despertam o interesse do consumidor (LANNES et al., 2007). Estas pimentas possuem diferentes características relacionadas à cor e pungência (PINO et al., 2006) que, de modo geral, são resultantes da formação e acúmulo de metabólitos secundários. No entanto, fatores relativos à planta e ao ambiente, como a sazonalidade, ritmo circadiano, desenvolvimento da planta, temperatura, índice pluviométrico, disponibilidade hídrica, radiação ultravioleta, nutrientes, altitude, composição atmosférica e ataque de patógenos interferem diretamente no conteúdo total desses compostos (KUTCHAN, 2001). Além disso, as plantas estão frequentemente expostas a estresses ambientais que podem resultar em deficiência hídrica, desequilíbrio nutricional e foto-inibição, entre outros.

O estresse hídrico tem forte influência sobre o metabolismo da planta, tendendo a aumentar a concentração de metabólitos secundários mediada pela alteração da expressão gênica e reduzir o crescimento vegetativo (BRUCE et al., 2002; JIANG; ZHANG, 2002; TAIZ; ZIEGER, 2002; SHARP et al., 2004). A insuficiência de água reduz tanto a produtividade quanto a qualidade dos frutos. Nessas circunstâncias as plantas desenvolvem mecanismos de adaptação à falta de água, como o fechamento dos estomas, ajuste osmótico e da parede celular, produção de folhas menores, redução da área foliar, aumento na densidade e

profundidade de raízes, além de mecanismos que provocam queda na absorção de CO_2 e interceptação de luz (MATTOS et al., 1999).

Em condições de deficiência hídrica, também ocorre o aumento da produção de radicais livres de oxigênio nas plantas. O acúmulo de solutos compatíveis pode ter algum efeito protetor contra danos oxidativos nas proteínas (ITURBE-ORMAETXE et al., 1998).

Vários trabalhos têm mostrado que existe um aumento nos teores de aminoácidos em várias situações de estresse vegetal (COOLEY; FOY, 1992; DROGE, 2002). Possíveis explicações sobre os aumentos observados incluem a inibição da síntese de proteínas, aumento da hidrólise de proteínas, decréscimo do uso de aminoácidos como fonte de carbono respiratório e/ou aumento da biossíntese de aminoácidos (COOLEY; FOY, 1992). A biossíntese de aminoácidos tem uma relação direta com o metabolismo secundário e, portanto, mudanças que afetem o teor de aminoácidos podem orientar o metabolismo para uma resposta de defesa (BUCHANAN et al., 2000).

No estudo realizado por Estrada et al. (1999), os pesquisadores constataram que o estresse hídrico afetou alguns parâmetros biológicos e os níveis de pungência durante todo o processo de maturação dos frutos do gênero *Capsicum* avaliados; os autores sugeriram que esse aumento no acúmulo de capsaicinóides nos frutos de plantas hidricamente estressadas está correlacionado com a redução do conteúdo de compostos fenólicos e de lignina.

1.2. Capsaicinoides

Dentre as características sensoriais da pimenta, a pungência é uma das que mais se destaca (MAILLARD et al., 1997), sendo o principal motivo de aceitação ou rejeição de alimentos condimentados com essa especiaria. A pungência é produzida por um conjunto de alcaloides denominados capsaicinoides, que ocorrem apenas no gênero *Capsicum* (WAGNER, 2003). Os principais capsaicinoides encontrados são a capsaicina, dihidrocapsaicina e nordihidrocapsaicina, as diferenças entre esses compostos estão relacionados com a presença de diferentes cadeias carbônicas, insaturações ou ramificações nos ácidos graxos que entram nas suas rotas de biossíntese. A capsaicina e a dihidrocapsaicina (Figura 1.1) são responsáveis por mais de 90% da pungência das pimentas (GARCÉS-CLAVER et al., 2006; SANTOS, 2009; BOGUSZ JUNIOR, 2010).

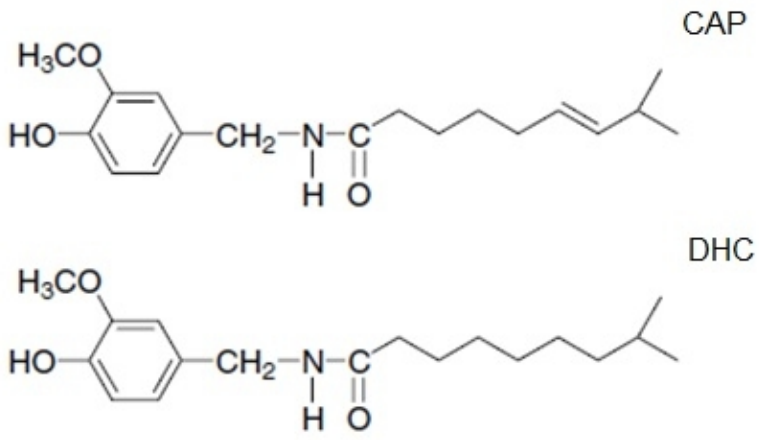

Figura 1.1. Estruturas químicas da capsaicina (CAP) e dihidrocapsaicina (DHC). FONTE: CISNEROS-PINEDA et al., 2007.

Além da sensação de ardência e queimação, os capsaicinoides possuem diversas ações no corpo humano como atividade antimutagênica, antitumoral, antioxidante, analgésica, e podem ser usados em armas como o *spray* de pimenta (HENDERSON; SLICKMAN, 1999; REILLY et al., 2001; ROSA et al., 2002; BOGUSZ JUNIOR, 2010). Além disso, investigações recentes apontam para uma interessante propriedade dos capsaicinoides no controle da obesidade (KOVACS; MELA, 2006; KANG et al., 2007).

Diante de todas essas informações, a quantificação da pungência das pimentas passa a ser de interesse tanto do consumidor como do produtor que fornece os frutos para atender às exigências das indústrias alimentícia e farmacêutica.

A medição do teor de pungência primeiramente era realizada pelo teste de Scoville, o qual deu origem a uma tabela baseada em unidades de calor, designada genericamente como Tabela de Scoville. Esse teste baseia-se na extração dos capsaicinoides em solução alcoólica, da qual são feitas diluições aquosas e este extrato é submetido à avaliação por julgadores treinados (SCOVILLE, 1912).

Atualmente, diferentes técnicas analíticas são empregadas com o objetivo de quantificar esses compostos, destacando-se técnicas clássicas de cromatografia como a cromatografia líquida de alta eficiência (HPLC) (MUELLER-SEITZ et al., 2008; SANATOMBI; SHARMA, 2008) e cromatografia gasosa (GC) (CISNEROS-PINEDA et al., 2007; PEÑA-ALVAREZ et al., 2009), a eletroforese capilar (CE) (LIU et al., 2010) e mais recentemente a cromatografia líquida de ultra eficiência (UHPLC) (HA et al., 2010).

1.3. Carotenoides

Frutos do gênero *Capsicum* apresentam uma enorme variabilidade em termos de tamanho, formato, e nas diferentes intensidades de cor variando geralmente entre o amarelo, laranja e vermelho quando maduros. Essa característica de coloração é resultante da composição em carotenoides desses frutos (GUZMAN et al., 2010).

Atualmente, cresce a demanda pelo uso de corantes naturais na indústria de alimentos. Os carotenoides compreendem um grande grupo de pigmentos naturais, cerca de 650 compostos, distribuídos na natureza entre frutas, hortaliças, animais, fungos, algas e bactérias. Esses compostos são, na sua maioria, tetraterpenoides (C40) com um extenso sistema de ligações duplas conjugadas. Tradicionalmente, eles são classificados em dois grandes grupos estruturais: os carotenos, que são basicamente hidrocarbonetos (β-caroteno, licopeno, entre outros), e xantofilas, que incluem diferentes funções oxigenadas nas moléculas (luteína, zeaxantina, β-criptoxantina, etc.). Dependendo da extensão do sistema de ligações duplas conjugadas e dos vários grupos funcionais contidos na molécula, o cromóforo, bem como as características espectrais do composto são diferentes (BRITTON, 1983).

Na natureza, os carotenoides têm funções diferentes, sendo uma das mais notáveis, o seu papel na absorção da energia luminosa em comprimentos de onda pouco acessíveis à clorofila, para que essa energia seja transferida, posteriormente, para utilização no processo fotossintético. Eles também têm funções de proteção em relação ao aparato fotossintético, dissipando o excesso de energia e inativando

espécies altamente reativas como o oxigênio singlete e a molécula de clorofila no estado excitado (FRANK; COGDELL, 1993).

Os carotenoides são razoavelmente estáveis nas matrizes em que se encontram, no entanto, eles podem se tornar muito lábeis quando são isolados ou dissolvidos em solventes orgânicos. Frequentemente, os carotenoides são encontrados esterificados a cadeias de ácidos graxos e isso contribui para sua estabilidade. Devido à insaturação dos carotenoides, eles devem ser manipulados cuidadosamente e mantidos sob a ausência de luz e oxigênio e a baixas temperaturas (KANNER; MENDEL, 1976).

Em se tratando de alimentos, esses compostos despertam o interesse de muitos pesquisadores, devido principalmente a sua atividade como pró-vitamina A, que é convertida, durante a digestão, em retinol, que é a forma ativa da vitamina (SMIDT; BURKE, 2004). De acordo com a Organização Mundial da Saúde, a deficiência de vitamina A é um problema de saúde pública e estima-se que essa deficiência possa levar à cegueira entre 250.000 e 500.000 crianças por ano (WHO, 2010). As concentrações de compostos como β-caroteno e β-cripotoxantina são de grande importância visto que apresentam atividade pró-vitamínica (HOWARD et al., 1994; MINGUÉZ-MOSQUERA; HORNERO-MÉNDEZ, 1994).

Atualmente sabe-se que os carotenoides, até mesmo os sem atividade pró-vitamínica, podem exercer algumas funções como prevenção de determinados tipos de câncer, aumento da resposta imunológica e alguns tipos de infecção e também proteção da mucosa contra gastrites (GODOY; RODRIGUEZ-AMAYA, 1994). O efeito nutracêutico dos carotenoides em animais e seres humanos se deve ao seu

elevado poder antioxidante permitindo a proteção da função celular pela eliminação de radicais livres, e consequentemente, reduzindo o risco de câncer (HORNERO-MÉNDEZ et al., 2000; MAOKA et al., 2001).

Em pimentas, pelo menos 34 carotenoides já foram extraídos e separados usando a técnica de HPLC, incluindo β-caroteno, β-criptoxantina, zeaxantina, capsantina, capsorubina, luteína, violaxantina (DELI et al., 2001; AZEVEDO-MELEIRO; RODRIGUEZ-AMAYA, 2009). O elevado teor de carotenoides em frutos de *Capsicum* faz deles uma importante fonte desses nutrientes na dieta (MARÍN et al., 2004).

A cromatografia líquida de alta eficiência (HPLC) apresenta limitações na separação de alguns carotenoides, que para ser efetiva demanda um longo tempo de análise. A fim de caracterizar com alta seletividade a composição de carotenoides em pimentas do gênero *Capsicum*, Guzman et al. (2010) desenvolveram um novo método por cromatografia líquida de ultra eficiência (UHPLC) capaz de separar suficientemente seis padrões de carotenoides (β-caroteno, capsantina, capsorubina, zeaxantina, β-cripotoxantina e anteraxantina) em tempo reduzido de análise.

1.4. Compostos voláteis

Os principais parâmetros de qualidade para pimentas *Capsicum* são a cor e a pungência (PINO et al., 2006). No entanto, atualmente as pesquisas estão direcionadas também ao estudo do aroma como um importante parâmetro para a qualidade de frutas e hortaliças (LUNING et al., 1994; GUADAYOL et al., 1997;

CREMER; EICHNER, 2000). De modo geral, os alimentos, embora nutritivos, não são apreciados sem apresentarem sabor e aroma agradáveis. O aroma é uma das características mais importantes que determina a aceitabilidade de qualquer produto alimentício (NISHA et al., 2009).

A análise de compostos voláteis tem sido um desafio para muitos pesquisadores por mais de 40 anos (STEFFEN; PAWLISZYN, 1996). Nas últimas décadas principalmente, os pesquisadores da área de compostos voláteis foram beneficiados pelos avanços na química analítica instrumental e também, pela evolução das técnicas de extração e preparo de amostra. Atualmente, o número de compostos voláteis conhecidos tem aumentado para mais de 7000 compostos, sendo identificados cerca de 300 compostos em morango e mais de 1000 no aroma de café (ZELLNER et al., 2008).

Esses compostos normalmente ocorrem em baixas concentrações nas matrizes alimentares, que são bastante complexas e consistem de diferentes compostos orgânicos com propriedades de polaridade e reatividade distintas. Entretanto, os compostos responsáveis pelo aroma são voláteis e com base nessa propriedade, procedimentos para isolá-los da amostra foram estabelecidos (KATAOKA et al., 2000).

Dessa forma, uma técnica de preparo de amostra ideal para avaliação dos compostos voláteis em pimentas deve ser simples, rápida, de baixo custo e compatível com instrumentação analítica. A microextração em fase sólida no *headspace* (SPME) é a técnica que cumpre a maioria dos requisitos das etapas de preparo de amostra para analisar compostos voláteis em pimentas (ZHANG;

PAWLISZYN, 1993; PARREIRA; CARDEAL, 2005), devido a sua simplicidade, ausência de solventes, pouca manipulação da amostra, elevada sensibilidade, curto tempo operacional e fácil automação.

Parâmetros importantes que interferem no desenvolvimento de um método de extração por SPME estão relacionados ao tipo de fibra empregada, ao tempo de exposição da fibra, à temperatura de extração, à quantidade de amostra e às condições de tempo e temperatura durante a dessorção dos analitos da fibra (KATAOKA et al., 2000). O procedimento empregado para a otimização das condições pode ser obtido através do planejamento multivariado (ARAUJO HERNANDEZ; BRERETON, 1996; BOGUSZ JUNIOR et al., 2010). Esse procedimento permite variações simultâneas dos fatores avaliados, tornando possível distinguir interações entre eles que não seriam percebidas no planejamento experimental univariado, possibilitando assim, a redução no número de experimentos necessários, sem perda de informação (MAZIDA et al., 2005; SOUSA et al., 2006).

A quantificação de compostos voláteis de diferentes variedades de pimentas é uma tarefa difícil, visto que um grande número de compostos voláteis, pertencentes a várias classes químicas como alcanos e alcenos, alcoóis, aldeídos, cetonas, ácidos carboxílicos, aromáticos, ésteres e terpenos, são encontrados nesse tipo de amostra (LIU et al., 2009).

Atualmente, tem crescido a demanda por dados quantitativos no campo das análises de aroma (BICCHI et al., 2008). Para essa quantificação através do uso de curvas de calibração é necessário que elas sejam construídas com padrões

cromatográficos sob as mesmas condições da análise da amostra (OUYANG; PAWLISZYN, 2006; OUYANG; PAWLISZYN, 2008). No entanto, a construção de curva de calibração para cada composto volátil presente na amostra torna-se inviável devido ao grande número de compostos, mas já se sabe que apenas uma pequena fração dos compostos voláteis presentes na matriz contribuem para o aroma percebido (GROSCH, 1994; VAN RUTH, 2001).

Diante desta realidade, muitos autores usam a técnica de adição de padrão interno à amostra, obtendo uma semi-quantificação. Esta técnica baseia-se na correlação entre a área do pico obtida pelo padrão de referência e área do pico do analito de interesse. Neste caso é importante observar que o padrão de referência não esteja presente na composição da amostra, que o seu pico não co-elua com demais compostos da amostra durante a corrida cromatográfica e, em técnicas de equilíbrio, como a SPME, que adição do padrão interno seja realizada de modo que não altere o perfil de extração dos voláteis da amostra. Atualmente, muitos autores publicam seus resultados com base na semi-quantificação, incluindo pesquisas recentes com frutos *Capsicum* (PINO et al., 2006; PINO et al., 2007; RODRÍGUEZ-BORRUEZO et al., 2010).

Com menor número de relatos existe também a técnica de quantificação baseada na extração múltipla (MHE) que foi introduzida por Kolb et al (1981). Esta técnica é adequada para evitar erros decorrentes do efeito-matriz em análises quantitativas envolvendo amostras complexas. O princípio da MHE aplicada à análise de voláteis é baseada em extrações consecutivas dos compostos presentes no headspace da amostra em intervalos de tempo regulares. Após a primeira

extração, o equilíbrio do analito entre a amostra e o headspace é perturbado e, assim é necessário que a situação de equilíbrio seja restaurada. A quantidade absoluta de analito após a extração é reduzido tanto na amostra sólida quanto na fase gasosa, mas a relação entre as concentrações no interior da duas fases continua a ser constante. Depois de uma segunda extração obtém-se um pico mais baixo durante a análise cromatográfica e o equilíbrio dentro do sistema amostra-headspace deve ser novamente restaurado. Prosseguindo com esse processo de extrações sequenciais é possível obter uma extração exaustiva, determinando a área total do analito que pode ser correlacionada diretamente por calibração com a sua concentração na amostra. Na prática não se repetem extrações até alcançar a exaustão do analito na amostra. De fato, são realizadas um total de 3-4 extrações para poder calcular a área total na análise cromatográfica para um determinado composto. Aplicando-se função logarítmica sobre as áreas obtidas é possível construir uma reta cuja equação permite estimar a área total para o analito na amostra.

Recentemente a técnica de MHE vem sendo aplicada em métodos que usam extração em *headspace* através da microextração em fase sólida (HS-SPME). Ezquerro et al. (2003) aplicaram a MHE adaptada para uso com HS-SPME na determinação de voláteis provenientes de embalagens. Sequencialmente, a técnica que passou a ser chamada de MSPME ou MHS-SPME (TENA; CARRILLO, 2007; BICCHI et al, 2011) dependendo dos autores, teve relatos de aplicações na análise de voláteis em alimentos e bebidas incluindo vinhos, tomates e café (PIZARRO et al., 2007; SERRANO et al., 2009; BICCHI et al., 2011).

Cresce também o interesse geral dos pesquisadores direcionado à determinação da contribuição dos compostos para o aroma de um produto, através de análises olfatométricas. Em geral, a importância sensorial de um composto depende da sua concentração na matriz e do seu limite de detecção no nariz humano (ZELLNER et al., 2008), sabe-se que esses compostos não contribuem igualmente para o aroma da amostra, assim, uma grande quantidade de determinado composto não corresponde necessariamente à alta intensidade do odor.

Na cromatografia gasosa acoplada à olfatometria (GC-O) os compostos eluídos são detectados simultaneamente por dois detectores, sendo um instrumental e outro correspondente ao sistema olfativo humano. Dessa forma, a GC-O fornece não somente uma análise instrumental, mas também uma análise sensorial.

Entre os métodos olfatométricos, existem os de detecção de frequência, os que medem tempo-intensidade da percepção (OSME) e os que utilizam diluições dos compostos até alcançar o limiar de percepção (AEDA). A análise por OSME em alimentos, monitorando tempo e intensidade de percepção de um odor fornece dados sobre a contribuição de cada composto para o aroma. A informação é demonstrada na forma de um osmegrama, no qual um pico mais alto e com maior área sugere maior importância (QIAN; REINECCIUS, 2003; LÓPEZ-FERIA et al., 2008; ZELLNER et al., 2008).

No caso de pimentas frescas e processadas, diversos trabalhos revelaram uma composição química complexa (VAN RUTH et al., 1995a; VAN RUTH et al.,

1995b; VAN RUTH et al., 2003; CARDEAL et al., 2006; SOUZA et al., 2006; PINO et al., 2006; PINO et al., 2007), com mais de uma centena de compostos voláteis identificados através de técnicas de hidrodestilação, extração líquido-líquido, amostragem de *headspace* estático, amostragem de *headspace* dinâmico e microextração em fase sólida.

A biossíntese de compostos voláteis é fortemente afetada pelas mudanças bioquímicas e fisiológicas que ocorrem durante o amadurecimento do fruto, tais como amaciamento da polpa, desenvolvimento de pigmentos, incremento na taxa de respiração e produção de etileno e alterações no metabolismo de carboidratos e lipídios (CANUTO et al., 2009). Liu et al. (2009) avaliaram a composição qualitativa e quantitativamente de voláteis em frutos de *Capsicum* e constataram variações significativas nos diferentes estádios de maturação.

No trabalho realizado por Rodríguez-Burruezo et al. (2010) os constituintes voláteis de frutos maduros de 16 variedades de Capsicum das espécies C. annuum, C. chinense e C. frutescens foram isolados por microextração em fase sólida de headspace (HS-SPME) e analisados por cromatografia gasosa-olfatometria-espectrometria de massas. Mais de 300 compostos foram identificados e a análise olfatométrica revelou que a diversidade de compostos odoríferos que contribuem para o aroma encontrada entre as cultivares estudadas foi devido a diferenças qualitativas e quantitativas de, no mínimo, 23 compostos voláteis.

Zimmermann e Schieberle (2000) e Nunes et al. (2008) reportaram que a β-ionona exerceu grande contribuição para o aroma das pimentas em diferentes estádios de crescimento. Rodríguez-Burruezo et al. (2010) também confirmaram a

elevada contribuição de muitos ésteres e iononas para os aromas frutado/exótico, além de uma baixa ou inexistente contribuição de voláteis para aroma verde/vegetal nas variedades das espécies *C. chinense* e *C. frutescens*.

1.5. Conclusões

Em relação à composição de metabólitos secundários responsáveis pela qualidade das pimentas brasileiras *Capsicum* existem poucos estudos desenvolvidos recentemente envolvendo alguns exemplares das espécies pertencentes a este gênero. Sobretudo, nota-se que o número de estudos sobre este assunto ainda é insuficiente quando se considera a grande diversidade de pimentas existentes no país, até mesmo dentro de uma espécie, como exemplo, a *Capsicum chinense*, amplamente difundida em território nacional, que compreende diferentes tipos de pimentas com propriedades sensoriais variadas.

Nesse contexto, abrem-se muitas possibilidades para estudos que permitam o conhecimento da composição destes metabólitos secundários nas diferentes pimentas, bem como a compreensão de mecanismos de síntese e acúmulos destes metabólitos nos frutos em função das condições de cultivo.

1.6. Referências

ARAUJO HERNANDEZ, P. W.; BRERETON, R. G. Experimental design 2. Optimisation. Trends in Analytical Chemistry, 15, 63-70. 10.1016/0165-9936(96)80762X, 1996.

AZEVEDO-MELEIRO, C. H. de; RODRIGUEZ-AMAYA, D. B. Qualitative and quantitative differences in the carotenoid composition of yellow and red peppers determined by HPLC-DAD-MS. Journal of Separation Science, 32(21):3652-3658, 2009.

BICCHI, C.; LIBERTO, E.; MATTEODO, M.; SGORBINI, B.; MONDELLO, L.; ZELLNER, B.; COSTA, R.; RUBIOLO, P. Quantitative analysis of essential oils: a complex task. Flavour and Fragrance Journal. 23, 382-391, 2008.

BICCHI, C.; RUOSI, M. R.; CAGLIERO, C.; CORDERO, C.; LIBERTO, E.; RUBIOLO, P.; SGORBINI, B. Quantitative analysis of volatiles from matrices of vegetable origin by high concentration capacity headspace techniques: determination of furan in roasted coffe. Journal of Chromatography A. 1218:753-762, 2011.

BOGUSZ JUNIOR, S. Caracterização química e estudo de compostos bioativos em pimentas *Capsicum*. Tese de doutorado. Faculdade de Engenharia de Alimentos, UNICAMP, 2010, 140p.

BOGUSZ JUNIOR, S.; MELO, A. M. T.; ZINI, C. A.; GODOY, H. T. Optimization of the extraction conditions of the volatile compounds from chili peppers by

headspace solid phase micro-extraction, Journal of Chromatography A, doi:10.1016/j.chroma.2010.12.060, 2010.

BRITTON, G. The biochemistry of natural pigments; Cambridge University Press: Cambridge, 1983.

BRUCE, W. B., EDMEADES, G. O., BARKER, T. C. Molecular and physiological approaches to maize improvement for drought tolerance. Journal of Experimental Botany, 53(366):13-25, 2002.

BUCHANAN, B. B., GRUISSEM, W., JONES, R. L. Biochemistry and molecular biology of plants. 1ª Edição. American Society of Plant Physiologist, Rockville. 2000, 1367p.

CANUTO, K. M.; SOUZA NETO, M. A.; GARRUTI, D. S..Composição química volátil, em diferentes estádios de maturação, de manga 'tommy atkins' produzida no vale do São Francisco. Química Nova, 32(9):2377-2381, 2009.

CARDEAL, Z. L.; GOMES DA SILVA, M. D. R.; MARRIOTT, P. J. Comprehensive two-dimensional gas chromatography/mass spectrometric analysis of pepper volatiles. Rapid Communications in Mass Spectrometry, 20:2823-2836, 2006.

CISNEROS-PINEDA, O.; TORRES-TAPIA, L. W.; GUTIERREZ-PACHECO, L. C.; CONTRERAS-MARTIN, F.; GONZALES-ESTRADA, T.; PERAZA-SANCHEZ, S. R. Capsaicinoids quantification in chili peppers cultivated in the state of Yucatan, Mexico. Food Chemistry, 104:1755–1760, 2007.

COOLEY, W. E.; FOY, C. L. Effects of SC-0224 and glyphosate on free amino acids, soluble protein, and protein synthesis in inflated duckweed (Lemna gibba). Weed Science, 40(3):345-350, 1992.

CREMER, D. R.; EICHNER, K. Formation of volatile compounds during heating of spice paprika (*Capsicum annuum*) powder. Journal of Agricultural and Food Chemistry, 48:2454-2460, 2000.

DELI, J.; MOLNAR, P.; MATUS, Z.; TOTH, G. Carotenoid composition in the fruits of red paprika (*Capsicum annuum* var. lycopersiciforme rubrum) during ripening; biosynthesis of carotenoids in red parika, Journal of Agricultural and Food Chemistry, 49:1517–1523, 2001.

DROGE, W., 2002. Free radicals in the physiological control of cell function. Physiological Reviews, 82(1):47-95, 2002.

ESTRADA, B.; POMAR, F.; DÕAAZ, J.; MERINO, F.; BERNAL, M. A. Pungency level in fruits of the Padrón pepper with different water supply. Scientia Horticulturae, 81:385-396, 1999.

EZQUERRO, O.; BEGOÑA, P.; TENA, M. T. Multiple headspace solid-phase microextraction for the quantitative determination of volatile organic compounds in multilayer packagings. Journal of Chromatogrphy A, 999:155-164, 2003.

FRANK, H.; CODGELL, R. J. Photochemistry and function of carotenoids in photosynthesis. In Carotenoids in photosynthesis; YOUNG, A., BRITTON, G., Eds.; Chapman and Hall: London, 1993.

GARCÉS-CLAVER, A.; ARNEDO-ANDREÁS, M. S.; ABADÍA, J.; GIL-ORTEGA, R.; ÁLVARES FERNÁNDES, A. Determination of Capsaicin and Dihydrocapsaicin in *Capsicum* Fruits by Liquid

Chromatography−Electrospray/Time-of-Flight Mass Spectrometry. Journal of Agricultural and Food Chemistry, 54:9303-9311, 2006.

GODOY, H. T.; RODRIGUEZ-AMAYA, D. B. Occurrence of cis-isomers of provitamin A in Brazilian fruits. Journal of Agricultural and Food Chemistry, 42:1306-1313, 1994.

GROSCH, W. Determination of potents odorants in food by aroma extract dilution analysis (AEDA) and calculation of odour activity values (OAVs). Flavour Fragrance Journal, 9:147-158, 1994.

GUADAYOL, J. M.; CAIXACH, J., RIBÉ, J.; CABAÑAS, J.; RIVERA, J. Extraction, separation and identification of volatile organic compounds from paprika oleoresin (Spanish type). Journal of Agricultural and Food Chemistry, 45:1868-1872, 1997.

GUZMAN, I.; HAMBY, S.; ROMERO, J.; BOSLAND, P. W.; O'CONNELL, M. A. Variability of carotenoid biosynthesis in orange colored *Capsicum* spp. Plant Science, 179 (2010) 49–59.

HA, J.; SEO, H. Y.; SHIM, Y. S; SEO, D. W.; SEOG, H.; ITO, M.; NAKAGAWA, H. Determination of capsaicinoids in foods using Ultra High Performance Liquid Chromatography. Food Science and Biotechnology, 19(4): 1005-1009, 2010.

HENDERSON, D. E., SLICKMAN, M. Quantitative HPLC determination of the antioxidant activity of capsaicin on the formation of lipid hydroperoxides of linoleic acid: a comparative study against BHT and melatonin. Journal of Agricultural and Food Chemistry, 47:2563-2570, 1999.

HORNERO-MÉNDEZ, D.; GOMÉZ-LADRÓN-de-GUEVARA, R.; MÍNGUEZ-MOSQUERA, M. I. Carotenoid biosynthesis changes in five red pepper (*Capsicum annuum* L.) cultivars during ripening. Cultivar selection for breeding. Journal of Agricultural and Food Chemistry, 48:3857-3864, 2000.

HOWARD, L. R.; SMITH, A. B.; WAGNER, B., VILLALON, B.; BURNS, E. E. Provitamin A and ascorbic acid content of fresh pepper cultivars (*Capsicum annuum*) and processed Jalapeños. Journal of Food Science, 59:362-365, 1994.

ITURBE-ORMAETXE, I.; ESCUDERO, P.; ARRESE-IGOR, C.; BECANA, M. Oxidative damage in peã plants exposed to water deficit or paraquat. Plant Physiology, 116:173-181, 1998.

JIANG, M., ZHANG, J. Water stress-induced abscisic acid accumulation triggers the increased generation of reactive oxygen species and up-regulates the activities of antioxidant enzymes in maize leaves. Journal of Experimental Botany, 53(379):2401-2410, 2002.

KANG, J. H.; KIM, C. S.; HAN, I. S.; KAWADA, T.; YU, R. Capsaicin, a spicy component of hot peppers, modulates adipokine gene expression and protein release from obese-mouse adipose tissues and isolated adipocytes, and suppresses the inflammatory responses of adipose tissue macrophages. FEBS Letters, 581:4389-4396, 2007.

KANNER, J.; MENDEL, H. Carotene oxidizing factors in red pepper fruits (*Capsicum annuum* L.). Ascorbic acid. Journal of Food Science, 41:183-185, 1976.

KATAOKA, H.; LORD, H. L.; PAWLISZYN, J. Applications of solid-phase microextraction in food analysis. Journal of Chromatography A, 880:35-62, 2000.

KOLB, B.; POSPISIL, P.; AUER, M. Quantitative analysis of residual solvents in food packaging printed films by capillary gas chromatography with multiple headspace extraction. Journal of Chromatography, 204, 371-376, 1981.

KOVACS, E. M. R.; MELA, D. J. Metabolically active functional food ingredients for weight control. Obesity Reviews, 7:59-78, 2006.

KUTCHAN, T. M. Ecological arsenal and developmental dispatcher. The paradigm of secondary metabolism. Plant Physiology, 125:58-60, 2001.

LANNES, S. D.; FINGER, F. L.; SCHUELTER, A. R.; CASALI, V. W. D. Growth and quality of Brazilian accessions of *Capsicum chinense* fruits. Scientia Horticulturae, 112: 266-270, 2007.

LIU, L.; CHEN, X.; LIU, J.; DENG, X.; DUAN, W.; TAN, S. Determination of capsaicin and dihydrocapsaicin in *Capsicum anuum* and related products by capillary electrophoresis with a mixed surfactant system. Food Chemistry, 119:1228-1232, 2010.

LIU, R.; XIONG, K.; CHAO-LUO, Y.; ZE-DAI, X.; MIN-LIU, Z.; TONG-XUE, W. Changes in volatile compounds of a native Chinese chilli pepper (*Capsicum frutescens* var) during ripening. International Journal of Food Science and Technology, 44:2470-2475, 2009.

LÓPEZ-FERIA, S.; CÁRDENAS, S.; VALCÁRCEL, M. Simplifying chromatographic analysis of the volatile fraction of foods. Trends in Analytical Chemistry, 27(9):794-803, 2008.

LUNING, P. A.; RIJK, T. D.; HARRY, J.; WICHERS, H.J.; ROOZEN, J. P. Gas chromatography, mass spectrometry, and sniffing port analyses of volatile compounds of fresh bell peppers (*Capsicum annuum*) at different ripening stages. Journal of Agricultural and Food Chemistry, 42:977-983, 1994.

MAILLARD, M. N.; GIAMPAOLI, P.; RICHARD, M. J. Analysis of eleven capsaicinoids by reverse-phase high performance liquid chromatography. Flavour Fragrance Journal, 12:409-413, 1997.

MAOKA, T; MOCHIDA, K.; KOZUKA, M., ITO, Y.; FUJIWARA, Y.; HASHIMOTO, K.; ENJO, F.; OGATA, M.; NOBOKUNI, Y.; TOKUDA, H.; NISHINO, H. Cancer chemopreventive activity of carotenoids in the fruits of red paprika *Capsicum annuum* L. Cancer Letters, 172:103-109, 2001.

MARÍN, A.; FERRERES, F.; TOMÁS-BERBERÁN, F. A.; GIL, M. I. Characterization and quantification of antioxidant constituents of sweet pepper (*Capsicum annuum* L.). Journal of Agricultural and Food Chemistry, 52:3861-3869, 2004.

MATTOS, E. A.; HERZOG, B.; LUTTGE, U. Chlorophyll fluorescence during CAM-phases in *Clusia minor* L. under drought stress. Journal of Experimental Botany, 50(331):253-261, 1999.

MAZIDA, M. M.; SALLEH, M. M. and OSMAN, H. Analysis of volatile aroma compounds of fresh chilli (*Capsicum annuum*) during stages of maturity using

solid phase microextraction (SPME). Journal of Food Composition and Analysis, 18(5):427-437, 2005.

MINGUÉZ-MOSQUERA, M. I.; HORNERO-MÉNDEZ, D. Comparative study of the effect of paprika processing on the carotenoids in peppers (*Capsicum annuum*) of the bola and agridulce varieties. Journal of Agricultural Food Chemistry, 42:1555-1560, 1994.

MUELLER-SEITZ, E.; HIEPLER, C.; PETZ, M. Chili Pepper Fruits: Content and Pattern of Capsaicinoids in Single Fruits of Different Ages. Journal of Agricultural and Food Chemistry, 56:12114–12121, 2008.

NISHA, P.; REKHA, S. S.; PANDIT, A. B. The degradation kinetics of flavor in black pepper (*Piper nigrum* L.). Journal of Food Engineering, 92:44-49, 2009.

NUNES, C., COIMBRA, M. A., SARAIVA, J.; ROCHA, S. M. Study of the volatile components of a candied plum and estimation of their contribution to the aroma. Food Chemistry, 111: 897-905, 2008.

OUYANG, G.; PAWLISZYN, J. A critical review in calibration methods for solid-phase microextraction. Analytica Chimica Acta, 627:184-197, 2008.

OUYANG, G.; PAWLISZYN, J. Recent developments in SPME for on-site analysis and monitoring. Trends in Analytical Chemistry, 25(7):692-703, 2006.

PARREIRA, F. V.; CARDEAL, Z. L. Amostragem de compostos orgânicos voláteis no ar utilizando a técnica de microextração em fase sólida. Química Nova, 28(4):646-654, 2005.

PEÑA-ALVAREZ, A.; RAMÍREZ-MAYA, E.; ALVARADO-SUÁREZ, L. Á. Analysis of capsaicin and dihydrocapsaicin in peppers and pepper sauces by solid phase

microextraction-gas chromatography-mass spectrometry. Journal of Chromatography A, 1216:2843-2847, 2009.

PINO, J.; GONZALEZ, M.; CEBALLOS, L.; CENTURION-YAH, A. R.; TRUJILLO-AGUIRRE, J.; LATOURNERIE-MORENO, L.; SAURI-DUCH, E. Characterization of total capsaicinoids, colour and volatile compounds of Habanero chilli pepper (*Capsicum chinense* Jack.) cultivars grown in Yucatan. Food Chemistry, 104(4):1682-1686, 2007.

PINO, J.; SAURI-DUCH, E.; MARBOT, R. Changes in volatile compounds of Habanero chile pepper (*Capsicum chinense* Jack. cv. Habanero) at two ripening stages. Food Chemistry, 94(3):394-398, 2006.

PIZARRO, C.; PEREZ-DEL-NOTARO, N.; GONZÀLEZ-SÀIZ, J. M. Multiple headspace solid-phase microextraction for eliminating matrix effect in the simultaneous determination of haloanisoles and volatile phenols in wines.Journal of Chromatography A. 1166(1-2):1-8, 2007.

QIAN, M.; REINECCIUS, G. Potent aroma compounds in Parmigiano Reggiano cheese studied using a dynamic headspace (purge-trap) method. Flavour Fragrance Journal, 18:252-259, 2003.

REILLY, C. A.; CROUCH, D. J.; YOST, G. S.; FATAH, A. A. Determination of capsaicin, dihydrocapsaicin, and nonivamide in self-defense weapons by liquid chromatography-mass spectrometry and liquid chromatography-tandem mass spectrometry. Journal of Chromatography A, 912:259-267, 2001.

RIBEIRO, C. S. C.; LOPES, C. A.; CARVALHO, S. I. C. de; HENZ, G. P.; REIFSCHNEIDER, F. J. B.. Pimentas *Capsicum*. Brasília: Embrapa Hortaliças, 2008. 200p.

RODRIGUEZ-AMAYA, D. B. A guide to carotenoid analysis in foods. Washington: ILSI Press, 1999. 64p.

RODRÍGUEZ-BURRUEZO, A.; KOLLMANNSBERGER, H.; GONZÁLEZ-MAS, C.; NITZ, S.; NUEZ, F. HS-SPME Comparative analysis of genotypic diversity in the volatile fraction and aroma-contributing compounds of *Capsicum* fruits from the *annuum-chinense-frutescens* complex. Journal of Agricultural and Food Chemistry, 58:4388-4400, 2010.

ROSA, A.; DEIANA, M.; CASU, V.; PACCAGNINI, S.; APPENDINO, G.; BALLERO, M.; DESSI, M. A. Antioxidant activity of capsinoids. Journal of Agricultural and Food Chemistry, 50:7396–7401, 2002.

SANATOMBI, K.; SHARMA, G. J. Capsaicin content and pungency of different *Capsicum* spp. cultivars. Notulae Botanicae Horti Agrobotanic Cluj-Napoca, 36 (2):89-90, 2008.

SANTOS, V. S. F. Caracterização morfológica e determinação da pungência em pimentas picantes. Dissertação de Mestrado. Universidade Técnica de Lisboa. Lisboa, 2009, 114p.

SCOVILLE, Wilbur L. Note on *Capsicums*. Journal of the American Pharmaceutical Association, 1:453-454, 1912.

SERRANO, E.; BELTRÁN, J.; HERNÁNDEZ, J. Application of multiple headspace-solid-phase microextraction followed by gas chromatography-mass

spectrometry to quantitative analysis oftomato aroma components. Journal of Chromatography A, 1216, 127-133, 2009.

SHARP, R. E.; POROYKO, V.; HEJLEK, L. G.; SPOLLEN, W. G., SPRINGER, G. K.; BOHNERT, H. J.; NGUYEN, H. T. Root growth maintenance during water deficits: physiology to functional genomics. Journal of Experimental Botany, 55(407):2343-2351, 2004.

SMIDT, C. R.; BURKE, D. S. Nutritional significance and measurement of carotenoids. Current Topics in Nutraceutical Research, 2:79-91, 2004.

SOUSA, E. T.; RODRIGUES, F. M.; MARTINS, C. C.; OLIVEIRA, F. S. de; PEREIRA, P. A. de P.; ANDRADE, J. B. de. Multivariate optimization and HS-SPME/GC-MS analysis of VOCs in red, yellow and purple varieties of *Capsicum chinense* sp. Peppers. Microchemical Journal, 82:142-149, 2006.

STEFFEN, A.; PAWLISZYN, J. Analysis of Flavor Volatiles Using Headspace Solid-Phase Microextraction. Journal of Agricultural and Food Chemistry, 44:2187-2193, 1996.

TAIZ, L.; ZEIGER, E. Plant physiology. 3. ed. Sunderland: Sinauer Associates, 2002. 690p.

TENA, M. T.; CARRILLO, J. D. Multiple solid-phasemicroextraction: Theory and applications. Trends in Analytical Chemistry, 26(3):206-214, 2007.

VAN RUTH, S. Methods for gas chromatography-olfactometry: a review. Biomolecular Engennering, 17:121-128, 2001.

VAN RUTH, S.; BOSCAINI, E.; MAYR, D.; PUGH, J.; POSTHUMUS, M. Evaluation of three gas chromatography and two direct mass spectrometry techniques

for aroma analysis of dried red bell peppers. International Journal of Mass Spectrometry. 223-224:55-65, 2003.

VAN RUTH, S.; ROOZEN, J. P.; COZIJNSEN, J.; POSTHUMUS, M. A. Volatile compounds of rehydrated French beans, bell peppers and leeks. Part II. Gas chromatography/sniffing port analysis and sensory evaluation. Food Chemistry. 54(1):1-7, 1995b.

VAN RUTH, S.; ROOZEN, J. P; COZIJNSEN, J. L. Volatile compounds of rehydrated French beans, bell peppers and leeks. Part 1. Flavour release in the mouth and in three mouth model systems. Food Chemistry. 53(1):15-22, 1995a.

WAGNER, C.M. Variabilidade e base genética da pungência e de caracteres do fruto: implicações no melhoramento de uma população de *Capsicum*. Tese de Doutorado: Escola Superior de Agricultura Luiz de Queiroz, 2003, 104p.

WHO, World Health Organization, Micronutrient Deficiencies: Vitamin A Deficiency, <http://www.who.int/nutrition/topics/vad/> acesso em 29 de novembro de 2010.

ZELLNER, B.; DUGO, P.; DUGO, G.; MONDELLO, L. Gas chromatography-olfactometry in food flavour analysis. Journal of Chromatography A, 1186:123-143, 2008.

ZHANG, Z.; PAWLISZYN, J. Solid phase microextraction, a solvent free alternative for simple preparation. Analytical Chemistry, 65:1843-1847, 1993.

ZIMMERMANN, M.; SCHIEBERLE, P. Important odorants of sweet bell pepper powder (*Capsicum annuum* cv. *annuum*): differences between samples of

Hungarian and Morrocan origin. European Food Research and Technology, 211:175-180, 2000.

CHAPTER II

ARTICLE

Fast method for capsaicinoids analysis from Capsicum chinense fruits

CHAPTER II

Fast method for capsaicinoids analysis from *Capsicum chinense* fruits

Marla Sganzerla[a], Janclei Pereira Coutinho[a], Arlete Marchi Tavares de Melo[b], Helena Teixeira Godoy[a*]

[a] *Department of Food Science, Faculty of Food Engineering, University of Campinas (UNICAMP), P.O. Box 6121, 13083-862 Campinas, SP, Brazil*

[b] *Horticultural Center, Agronomic Institute of Campinas (IAC), P.O. Box 28, 13012-970 Campinas, SP, Brazil*

[*] Corresponding author.

Phone: +55 19 3521 4024

Fax: +55 19 3521 2153

E-mail address: helena@fea.unicamp.br (H. T. Godoy)

***This manuscript was published in the journal "Food Research International".**

SGANZERLA, M.; COUTINHO, J. P.; MELO, A. M. T.; GODOY, H. T. Fast method for capsaicinoids analysis from *Capsicum chinense* fruits. **Food Research International**, v. 64, p. 718-725, 2014.

ABSTRACT

Chili peppers are widely utilized in the world as savory food additives due the pungency induced by the capsaicinoids. Also, these compounds have functional properties as antimutagenic, antitumoral, antioxidant and analgesic. These characteristics increase the interest in this compound class, hence the capsaicinoid analysis must be reproducible and accurate. This study aimed to develop and validate a fast, efficient and reproducible method to analyze capsaicinoids in Brazilian *Capsicum chinense* fruits. The extracts were obtained after an optimization step that indicated the condition 100% of methanol and 10 min in ultrasound assisted extraction. The analyses were carried out in an ultra high performance liquid chromatographic system with detection by a photo diode array and mass spectrometer. The analytical method developed permits the separation of 8 capsaicinoids in 4 min of time analysis expending only 2 mL of solvent as mobile phase. The validation parameters evaluated for the method show the effectiveness and satisfactory performance to answer the analytical needs of this research area, presenting low values to relative standard deviation in repeatability and reproducibility and recoveries ranged from 88 to 112% for capsaicin and 89 to 109% for dihydrocapsaicin. In the extracts from different accessions of *C. chinense* fruits analyzed, the contents of capsaicin and dihydrocapsaicin were in the range of 156-1442 μg g^{-1} and 26-478 μg g^{-1} of fresh fruit, respectively, showing the large application of this method for quantification of the two major capsaicinoids in fast routine analysis and may be used to determine the concentrations of other minor capsaicinoids once appropriate standards are available.

Keywords: chili pepper; method development; ultrasound assisted extraction; ultra high performance liquid chromatography.

2.1. Introduction

Fruits of chili pepper plants that belong to the family Solanaceae, genus *Capsicum* are among the most consumed spices throughout the world (Garcés-Claver, Arnedo-André, Avier Abadía, Gil-Ortega, & Álvarez-Fernández, 2006) and are very important commercially. Brazil, a center of genetic diversity, is one of the world's largest producers of *Capsicum* peppers. In the year 2005, chili peppers of this genus were the second-most exported vegetable from Brazil, with an exportation volume of 9222 t (Ribeiro, Lopes, Carvalho, Henz, & Reifschneider, 2008). Some of the most popular domesticated varieties of peppers cultivated in the Brazilian territory belong to the specie *Capsicum chinense*, wich includes innumerous morphotypes, which fruits have different characteristics of color and aroma and can be of low as well as high pungency.

The consumption of chili peppers is due mainly to their very pungent flavor. The pungency is caused by capsaicinoids and is proportional to the combined concentrations of the various vanillyl amides that are collectively referred to as capsaicinoids (Reilly, Crouch, & Yost, 2001). Among the most abundant of these components are capsaicin (trans-8 methyl-N-vanillyl-6-nonenamide) and dihydrocapsaicin (8 methyl-Nvanillylnonanamide), which are responsible for about 90% of the spiciness (Barbero, Liazid, Palma, & Barroso, 2008a; Laskaridou-Monnerville, 1999). Besides these two major capsaicinoids, other minor ones have

been shown to occur in peppers (Barbero, Liazid, Palma, & Barroso, 2008b; Garcés-Claver et al., 2006; Jin, Pan, Xie, Zhou, & Xia, 2009; Zewdie & Bosland, 2001), including nordihydrocapsaicin, norcapsaicin, homocapsaicin I and II, homodihydrocapsaicin I and II, nornorcapsaicin, nornornorcapsaicin, and nonivamide, among others. The relative concentrations of these analogues vary with taxa and genotype (Jarret et al., 2003; Zewdie & Bosland, 2001).

The interest in these compounds extends far beyond their roles as flavor ingredients in food; they have also medical, toxicological, and forensic implications. Capsaicinoids are known for their pharmacological properties for instance as chemoprotectors against mutagenesis or tumorigenesis (Surh et al., 1995), as antimicrobials (Careaga et al., 2003; Cichewicz, 1996; Graham, Anderson, & Lang, 1999; Molina-Torres,Garcia-Chavez, & Ramirez-Chavez, 1999), as antioxidants (Hendersen & Slickman, 1999), for their analgesic effects (Kaale, Van Schepdael, Roets, & Hoogmartens, 2002), their effect on the neuronal responsiveness for pain transmission and neurogenic inflammation (Szolcsányi, 2004), and their anticancer effect that is closely related to their ability to prevent cell proliferation and migration and to induce cell apoptosis (Luo, Peng, & Li, 2011). In addition, these compounds are discussed as a way to manage obesity (Mueller-Seitz, Hiepler, & Petz, 2008; Reilly et al., 2001) and capsaicin is currently used for the treatment of diabetic neuropathy, osteoarthritis, post-herpetic neuralgia, and psoriasis (Davis, Markey, Busch, & Busch, 2007).

Due their properties and current application in the food industry, in the medical area as pharmaceuticals, and in defensive sprays (Daood et al., 2002), capsaicinoid

compounds have been widely studied and for this purpose diverse procedures have been reported for the isolation and analysis of these secondary metabolites (Kozukue et al., 2005).

In the last decade, there has been an increasing demand for new analytical methods that are more reliable and accurate, with short operational time and reduced cost, as well as with minimized use and generations of hazardous substances. Accordingly, many studies have been published that report advances in the extraction techniques and instrumental analysis applied to the measurement of pungency (Barbero et al., 2008a; Ha et al., 2010; Thompson, Phinney, Welch, & White, 2005).

The extraction of capsaicinoids from chili peppers has been conducted using different techniques, including maceration (Kirschbaum-Titze, Hiepler, Mueller-Seitz, & Petz, 2002), magnetic stirring (ContrerasPadilla & Yahia, 1998), enzymatic extraction (Salgado-Roman et al., 2008), solid-phase microextraction (SPME) (Tapia, Garcia, Escamilla, Calva, & Rocha, 1993), accelerated solvent extraction (ASE) (Chantai, Juangsamoot, Ruangviriyachai, & Techawongstien, 2012), ultrasonic assisted extraction (UAE) (Barbero et al., 2008b), Soxhlet (Korel, Bagdatlioglu, Balaban, & Hisil, 2002), supercritical fluid extraction (SFE) (Duarte et al., 2004; Sato et al., 1999), pressurized liquids (Barbero, Palma, & Barroso, 2006a), and microwave-assisted extraction (MAE) (Barbero, Palma, & Barroso, 2006b). Among these extraction techniques, the UAE method is particularly commended for its simplicity and low equipment cost (Boonkird, Phisalaphong, & Phisalaphong, 2008; Deng, Gao, Huang, & Liu, 2012).

Techniques used to separate capsaicinoids include thin layer chromatography (Lee, Suzuki, Kobashi, Hasegawa, & Iwai, 1976), capillary gas chromatography (Ha et al., 2010), micellar electrokinetic capillary chromatography (Laskaridou-Monnerville, 1999), supercritical fluid chromatography (SFE/SFC) (Sato et al., 1999), and especially liquid chromatography (LC), the method most frequently used for analysis of capsaicinoids because of its rapidity, reliability, accuracy and precision (Barbero et al., 2008a,b; Chantai et al., 2012; Davis et al., 2007; Garcés-Claver et al., 2006).

Methods using liquid chromatography with ultraviolet (UV) detection have been used successfully, although they have limited selectivity and a correct identification of individual compounds solely based on chromatographic behavior and UV spectrophotometric data, due to the complexity of the matrix and structural similarity between the capsaicinoids, is impracticable. The most recent methods for the determination of capsaicinoids have used LC coupled to more selective techniques such as mass spectrometry (Alothman et al., 2012; Garcés-Claver et al., 2006; Jin et al., 2009; Kozukue et al., 2005; Schweiggert, Carle, & Schieber, 2006; Thompson et al., 2005).

Nowadays, high speed and low cost of analysis are increasingly being demanded in many areas where liquid chromatography is applied in order to increase throughput and reduce costs (Barbero et al., 2008a). In this connection, the ultra high performance liquid chromatography (UHPLC) technique has been known to be economical and environmentally friendly due to extremely rapid analysis and the low consumption of solvent for mobile phase, reduced up to 5 to 10 fold,

comparing with the conventional HPLC (Ha et al., 2010). Recently, the UHPLC method coupled with mass spectrometry has been adopted in many areas of food and pharmaceutical analysis.

This study reports a new method using the UHPLC technique, rapid and reproducible, completely validated and optimized since extraction step for capsaicinoid determination applied to Brazilian *C. chinense* fruits that have not yet been sufficiently investigated.

2.2. Material and methods

2.2.1. Plant material

For this study were used fruits from 9 accessions of *C. chinense* (Table 2.5) from a chili pepper germplasm bank of the Agronomic Institute of Campinas (IAC). The plants grown in field conditions during the 2011 summer season in IAC (Campinas, SP, Brazil, 22°54′S, 47°05′ W, 674 m of elevation). The fruits were harvested during spring season at the ripening stage and preserved in the freezer at - 20 °C until analysis. About 2 kg of ripe fruits was harvested from 40 plants of each accession. Of these *C. chinense* accessions, four accessions were the color orange, two the color red, and three the color yellow.

'Cumari do Pará' chili pepper (*C. chinense*) was used for the development of the UAE and UHPLC methods and fruits of 'Malagueta' chili pepper (*Capsicum frutescens*) purchased on the local market were used to show the separation of

minor capsaicinoids, because in the *C. chinense* specie only capsaicin and dihydrocapsaicin were found.

2.2.2. Chemical and reagents

The solvents methanol, acetone and acetonitrile (J.T. Baker, Phillipsburg, NJ, USA) utilized were of HPLC-grade. The water was obtained from a Milli-Q water bidistillation system (Millipore, Bedford, MA, USA). The reference standards of capsaicinoids, capsaicin and dihydrocapsaicin (more than 95% of purity) were obtained from Cayman Chemical Company (Arbor, MI, USA).

All solvents used as mobile phase were filtered and degassed using Millipore filters (0.22 μm pore size, filter type GV (Durapore) PVDF for water and FG (Fluoropore) PTFE for organic solvents).

2.2.3. Analysis of capsaicinoids

Analysis of the capsaicinoids was performed using an UHPLC–DAD–MS/MS Thermo LCQ Fleet system (Thermo Fisher, San Jose, CA, USA). The separation of capsaicinoids was achieved with a Hypersil Gold C18 column with pore size 175 Å (1.9 μm, 3 mm × 100 mm) (Part number: 0606943X9, Thermo Scientific, Waltham, MA, USA) and mobile phase consisting of water (A) and acetonitrile (B) (A:B (40:60, v/v)) in isocratic mode at 0.5 mL min^{-1} of flow rate. Capsaicinoids in the sample were indicated by the relative retention time to standards and by comparing the mass spectra between standards, library and samples. The MS was equipped with an APCI (atmospheric pressure chemical ionization) source in positive mode of

ionization, working with vaporizer temperature set at 300°C, sheath gas pressure at 50 units, auxiliary gas pressure at 5 units (arbitrary units of the equipment), a corona needle voltage of 6 kV and an ion trap detection system operating in selected monitoring mode for ions m/z 80–310 and the fragments for each capsaicinoid. Data handling was performed with the Xcalibur software package.

2.2.4. Validation of analytical procedures

To determine that the proposed method provides suitable aspects for quantitative analysis of the capsaicinoids, the following validation data are commonly investigated. The linearity of the UHPLC method was determined through external calibration curves obtained with a series of standard solution which were prepared covering a concentration range of 0.0055-66.0 μg mL^{-1} for capsaicin and 0.0044-60.0 μg mL^{-1} for dihydrocapsaicin by serial dilution of the stock standard solutions. The limit of detection (LOD) was calculated as the analyte concentration giving a signal to noise ratio (S/N) of 3 and limit of quantitation (LOQ) was determined giving a signal to noise ratio (S/N) of 6. The precision of the method was presented as the repeatability and reproducibility of retention time and peak area. The repeatability (intra-day precision) was deduced from ten replicates within a day (n = 10) and reproducibility (inter-day precision) was calculated from the experiments carried out in three consecutive days (n = 3 × 10). Recovery experiments were carried out with the standard addition in the sample matrix method using 'Cumari do Pará' pepper in three concentration levels for capsaicin (118.4, 236.8, and 355.2 μg mL^{-1}) and dihydrocapsaicin (42.7, 85.5, and 128.2 μg mL^{-1}).

2.2.5. Extraction procedure

2.2.5.1. Ultrasound assisted extraction (UAE) optimization

The influence of operating parameters (solvent type, solvent ratio methanol:acetone and time of ultrasonically assisted extraction) on extraction of capsaicinoids from 'Cumari do Pará' was studied, employing different extraction conditions according an experimental design (2^2, with central and axial points, totalizing 11 assays): where the variable 1 was methanol proportion in relation to acetone (0-100%); and variable 2 was the sample extraction time in ultrasound bath (0-20 min). Sample quantity (1 g) and solvent volume (25 mL) in the extraction are previously established according to the study of Barbero et al. (2008b). The UAE process was performed in a Unique UC1400 ultrasonic bath (Indaiatuba, SP, Brazil) working at a frequency of 40 kHz and at room temperature (24 °C ±1 °C), which allowed the water in the bath to be renewed.

2.2.5.2. Sample extraction

The experiment was carried out in triplicate. For the sample preparation chili pepper fruits were blended in a grinder Turratec TE102 (Tecnal, Piracicaba, SP, Brazil) for 3 min at 20,000 rpm until a homogeneous sample was obtained and, immediately, submitted to the extraction step using the conditions established in the UAE optimization step.

2.2.6. Quantification of capsaicinoids

Quantification was based on the UV response at 280 nm and recoveries from spiked samples in the UHPLC system working in the same conditions previously described for separation of compounds. Quantification was performed on the capsaicinoids (capsaicin and dihydrocapsaicin) present in nine accessions of peppers (*C. chinense*) through calibration curves obtained from the standard solutions. Since there are no commercial standards of another capsaicinoid as nordihydrocapsaicin, homocapsaicin, homodihydrocapsaicin, homocapsaicin and considering that these compounds are not representative in the samples valuated, the structural similarities between these molecules and standards were not considered for tentative quantification.

Calibration graphs were constructed by the external standard method by plotting the ratio between peak areas of analyte versus analyte concentration. The curves were prepared by injecting 10 µL in real triplicates of 0.0055, 13.2, 26.4, 39.6, 52.8 and 66 µg mL^{-1} for capsaicin and 0.0044, 12, 24, 36, 48, and 60 µg mL^{-1} for dihydrocapsaicin, and these solutions are prepared by dilution of capsaicin and dihydrocapsaicin stock solutions with methanol:water (70:30, v/v).

2.2.7. Statistical analysis

The Statistical analysis and design generation were performed using the software Statistica 7.0. The models were validated by means of the Analysis of Variance (ANOVA) at the 95% confidence interval ($p < 0.05$). A lack of fit test and regression tests for each calibration curve were performed.

2.3. Results and discussion

2.3.1. Analytical conditions

The optimization for UHPLC analysis of capsaicinoids was investigated by varying the composition of mobile phase whereas the other conditions used throughout were as follows: flow rate of 0.5 mL min^{-1}, ambient temperature, and PDA detector set at 280 nm.

In this study, the organic solvent selected for the preliminary experiments was methanol due to the solubility of capsaicinoids. The mobile phases containing various percent of methanol:water were investigated and with the addition of acetonitrile a better resolution of peaks was achieved. After, the separation of capsaicinoids in shorter time was obtained by excluding the methanol of mobile phase and the application of a mobile phase of less complexity that constituted only of water and acetonitrile (40:60, v/v) in mode of isocratic elution at 0.5 mL min^{-1} was verified.

Initially, methanol was employed as sample solvent injection for the analysis of capsaicinoids using the extract obtained immediately after the extraction procedure, however it was observed that the injection solvent had a greater chromatographic strength than mobile phase (water and acetonitrile, 40:60, v/v) causing an effect of enlargement of peak base. Therefore, water was added in the injection solvent, to decrease the chromatographic strength without reducing the capsaicinoid solubility. The increasing water percentage provided a decrease in the base peak and gain on detected signal and chromatographic resolution (Fig. 2.1).

Thus, a step for dilution of the capsaicinoid extract with water prior to the analysis was established, changing the composition of sample solvent injection for 70% of methanol and 30% of water. A partial insolubility of the capsaicinoids was observed in percentages of water greater than 30%.

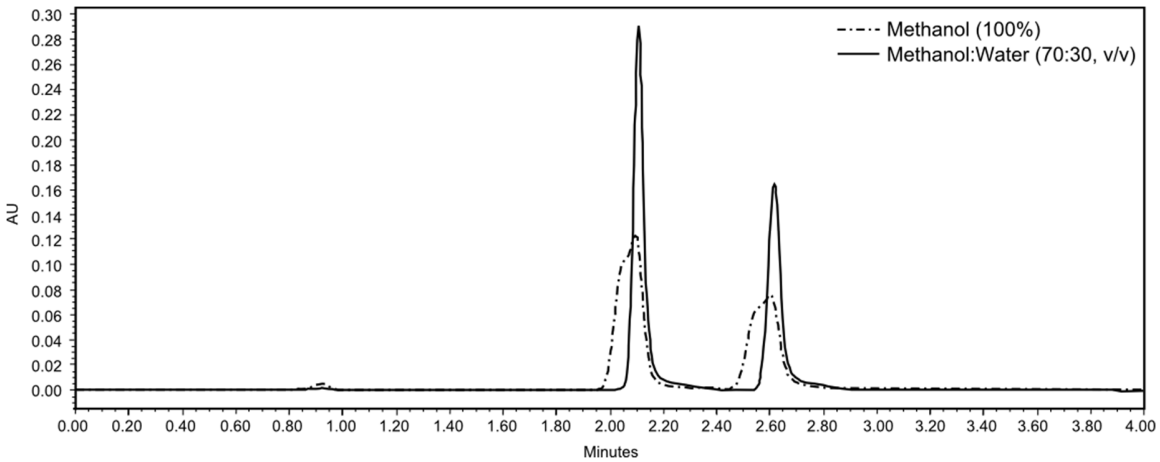

Figure 2.1. Effect of sample solvent injection composition.

The method developed is rapid and efficient with the separation of 8 capsaicinoids in a very short time of analysis (4 min). The speed of this method is evidenced for comparison with other methods in the literature in relation to time analysis and number of compounds separated (Table 2.1). The method proposed in this work also follows the trend of a decrease in the solvent consumption, expending 2 mL for each analysis and resulting in little generation of residue.

Table 2.1. Comparison among different methods of capsaicinoids analysis.

Method	Flow Rate (mL min^{-1})	Run Time (min)	Volume of MP for analysis (mL)	Mobile phase (MP) composition	Instrumentation devices	Compounds analyzed	References
1	0.5	4	2	(Acetonitrile: Water) (60:40) Isocratic	UHPLC-DAD-MS/MS Thermo LCQ Fleet system. Column Hypersil Gold C18 (100 x 3 mm, 1.9μm) (Thermo Scientific)	Capsaicin, Dihydrocapsaicin, Nornorcapsaicin, Nornordihydrocapsaicin, Nordihydrocapsaicin, Norcapsaicin, I-Dihydrocapsaicin, Homodihydorcapsaicin.	This work
2	1	14	14	A:B(50:50) Isocratic A(Acetonitrile) B(Water-Acetonitrile 90:10)	Waters LC616 System Spherisorb ODS2 C18 column (150 mm x 4.6mm, 5 μm).	Capsaicin, Dihydrocapsaicin	Estrada et al., (2002).
3	1	70	70	A:B (31:69) A (Acetonitrile) B (Water with 0.5% formic acid)	LC-MS Finnigan LCQ Advantage MAX. Zorbax Eclipse XDB-C18 column (4.6 x150 mm, 3.5 μm) (Agilent Technologies)	Capsaicin, Dihydrocapsaicin, Nordihydrocapsaicin, Homocapsaicin (I e II), Homodihydrocapsaicin (I e II), nonivamide.	Kozukue et al., (2005).
4	2	11	22	Gradient: 50% de A, changing to 80% of A in 7 min and return to 50% of A in 8 min, with 3 min of equilibrium.A(Acetonitrile) B (Water with 1% of acetic acid)	Hewlett-Packard liquid chromatograph (model 1090) Pinnacle II C-18 column (250 mm x 4.6 mm, 5 μm).	Capsaicin Dihydrocapsaicin	Davis et al., (2007).
5	0.2	6	1.2	Water: Acetonitrile (45:55) with 0.1% of acetic acid, isocratic.	LC-MS/MS (Thermo, USA). Zorbax SB-C18 column (100x2.1mm, 3.5μm Agilent, USA)	Capsaicin, Dihydrocapsaicin	Zhang et al.,

							(2010).
6	0.6	7	4.2	Water with 1% of formic acid: Acetonitrile (60:40)	U-HPLC (LaChromUltra Hitachi-High Technologies). C18 (2 mm ×50 mm, 2 µm) Hitachi-High Technologies column	Capsaicin, Dihydrocapsaicin	Ha et al., (2010).
7	6	15	90	Gradient: 10% of B, change to 100% of B in 15 min. A (Water/Acetic acid 0.01%) B (Methanol/ Acetic acid 0.01%)	The HPLC-fluorescence (Sunnyvale, CA, USA), (PDA-100), a fluorescence detector (RF 2000), C18 (100mm×4.6 mm Merck).	Capsaicin, Dihydrocapsaicin, Nordihydrocapsaicin, Homocapsaicin, Homonordihydrocapsaicin	Barbero et al., (2008b).
8	0.4	44	17.6	Gradient: 100% of A, changing to 100% B in 42 min. A (Water, 0.01% acetic acid) B(Methanol, 0.01% acetic acid)	HPLC Waters, with column C18 (Luna, 150 x 3mm, 5µm) Phenomenex	Capsaicin, Dihydrocapsaicin, Nordihydrocapsaicin, I-dihydrocapsaicin, Homodihydrocapsaicin	Barbero et al., (2006b).
9	0.4	30	12	Gradient: 20% of B changing to 100% of B in 24 min, A(Water/Acetonitrile 90:10 with 0.5% acetic acid) B(Acetonitrile/Water 90:10, with 0.5% acetic acid)	Agilent HPLC (Agilent, Germany) column C18 (150mm×3.0mm,4µm) Phenomenex LC-MS (Bruker - Bremen, Germany)	Nornorcapsaicin, Nornordihydrocapsaicin, N-Vanillyl-octamide, Norcapsaicin, Nordihydrocapsaicin, Capsaicin, N-Vanillyl-nonamide, Dihydrocapsaicin, N-Vanillyl-Decamide.	Schweiggert et al., (2006).
10	1	30	30	Gradient: 100% of A changing to 100% of B in 30 min A(Water/Acetonitrile 90:10) B (Acetonitrile/Water 90:10)	Knauer Chromatograph, detector UV. ColumnEurospher 80 (C18) (Dimensions not specified)	Capsaicin, Dihydrocapsaicin	Perucka&Oleszek (2000).
11	1	70	70	Acetonitrile/Water (40:60) with acetic acid (pH3.0)	HPLC Shimadzu series VP, PDA (UV-Vis). Column C18 250 x 4.6 mm.	Capsaicin, Dihydrocapsaicin, Nordihydrocapsaicin.	Poyrazoglu et al.,

							(2005).
12	0.8	30	24	Water/Acetonitrile/Tetrahydrofuran/ Acetic acid (55:40:5:1)	HPLC, pump M-510, auto sampler WISP – 712, fluorescence detector (Waters) Column YMC-Pack ODS-A (150x4.6mm, 3μm)	Capsaicin, Zucapsaicin	Lu & Cwik (1997).
13	1.5	8	12	Acetonitrile/Water (50:50) Isocratic	Thermo HPLC system, photodiode array (PDA) detector, column Betasil C18 (150 × 4.6 mm × 3 μm)	Capsaicin, Dihydrocapsaicin, Nordihydrocapsaicin	Al Othman et al., (2011).
14	0.5	8	4	Acetonitrile (A)/ Water 0.1% formic acid (B). Gradient started with 40% A changing to 50% of A in 8 minutes	UPLC-MS Acquity Waters Column BEH C18 (100 x 2.1mm; 1.7 μm)	Capsaicin, Dihydrocapsaicin, Nordihydrocapsaicin, Homocapsaicin, Homodihydrocapsaicin	Alothman et al., (2012)
15	0.2	4	0.8	Methanol/Water/Acetic Acid (90:9:1) Isocratic	HPLC (Waters GmbH, Eschborn, Germany) 3C8 column (125 x 2 mm, 3 μm).	Capsaicin, Dihydrocapsaicin	Wolf et al., (2007).

According to Table 2.1 , other works showed a similar short time of analysis (Ha et al., 2010; Wolf, Huschka, Raith, Wohlrab, & Neubert, 1999; Zhang, Hu, Sheng, & Li, 2010), but shown separation only for capsaicin and dihydrocapsaicin, minor capsaicinoids that cannot be separated in chromatographic stage, were detected with a mass spectrometer in the case of the study of Zhang et al. (2010). On the other hand, the methods able to separate also the minor compounds were performed in a larger time of analysis using the HPLC technique (Al Othman, Ahmed, Habila, & Ghafar, 2011; Barbero et al., 2006b; Schweiggert et al., 2006) and using the UHPLC technique, Alothman et al. (2012) achieved the separation of five compounds in 8 min of run time.

2.3.2. Validation of the analytical method

The method developed has been validated for capsaicin and dihydrocapsaicin with respect to limit of detection (LOD), limit of quantification (LOQ), linearity ranges, repeatability, reproducibility, and accuracy through analyte recoveries.

LOD and LOQ were estimated by signal to noise ratios of 3 and 6, respectively. In this condition, LOD was 0.0027 μg mL^{-1} for capsaicin and 0.0022 μg $mL^{-1}$for dihydrocapsaicin. LOQ was 0.0055 μg mL^{-1} and 0.0044 μg mL^{-1} for capsaicin and dihydrocapsaicin, respectively. The linearity was determined for LOQ up to 66 and 60 μg mL^{-1} for capsaicin and dihydrocapsaicin, respectively. The analytical curves were constructed with six different concentrations of standard solution for capsaicin and dihydrocapsaicin, each solution was prepared and injected in triplicate and the results were submitted to ANOVA (Table 2.2).

Table 2.2. Calibration curves of capsaicin (CAP) and dihydrocapsaicin (DHC).

		CAP	DHC
Equation		y=13257x-2337.3	y=16255x – 3741
R^2		0.999	0.998
Anova (p≤0.05)	Regression	$F_{(1,4)}$ = 15800.22 Significant	$F_{(1,4)}$ =11474.08 Significant
	Lack of fit	$F_{(4,12)}$ = 0.66 No significant	F(4,12) = 0.62 No significant
Linearity ($\mu g.mL^{-1}$)		0.0055 – 66	0.0044 – 60

The precision was evaluated by RSD (Relative Standard Deviation) of peak area and retention time, using solution standard in three levels of concentration. Repeatability (Intra-day precision) was determined by 10 injections on the same day, and reproducibility (Inter-day precision) by the 10 injections in each day for three consecutive days. The low RSD of peak area (less than 6.11%) and retention time (less than 0.32%) showed a good repeatability and reproducibility. The accuracy was carried out by recovery of capsaicin and dihydrocapsaicin with the standard addition method on the sample 'Cumari do Pará' in three concentration levels. Recoveries ranged from 88 to 112% for capsaicin and 89 to 109% for dihydrocapsaicin, indicating satisfactory accuracy of the method (Table 2.3).

Table 2.3. Precision and accuracy of method developed for capsaicinoids determination.

Parameter of Validation		Capsaicin levels (µg mL^{-1})	Dihydrocapsaicin levels (µg mL^{-1})	Capsaicin (%)*	Dihydrocapsaicin (%)*
Repeatability (Intra-day precision) (RSD %) n=10	Area	0.0055	0.0044	3.58	5.86
		33	30	0.40	0.27
		66	60	0.40	0.21
	Retention time	0.0055	0.0044	0.16	0.12
		33	30	0.025	0.02
		66	60	0.024	0.01
Reproducibility (Inter-day precision) (RSD %) n=30	Area	0.0055	0.0044	5.28	6.11
		33	30	0.9	0.97
		66	60	0.8	0.73
	Retention time	0.0055	0.0044	0.30	0.32
		33	30	0.12	0.14
		66	60	0.09	0.13
Accuracy n=3 (%, mean value ± sd)	Quantity on sample (µg)	940.59	155.43		
	Quantity added (µg)	118.4	42.7	103.27±7.72	100.3±8.42
		236.8	85.5	100.86±6.48	96.59±9.84
		355.2	128.2	92.66±3.57	95.79±7.95

* Values of % RSD to Repeatability and Reproducibility parameters and % recovery to Accuracy, expressed through mean values ± standard deviation (sd) to triplicate analysis.

2.3.3. Extraction optimization

The experimental results obtained by this initial model demonstrated how the variable concentration of methanol:acetone and time extraction in ultrasound bath influenced the extraction efficiency. Other parameters considered in the extraction were kept constant, namely the amount of sample (1.0 g) and the solvent volume (25 mL) according Barbero et al. (2008b).

The extraction yields were found in the ranges of 1.55 to 2.88 mg g^{-1}for capsaicin in fresh weight of 'Cumari do Pará', with RSD less than 0.5% in the central points. Capsaicin and dihydrocapsaicin showed a similar behavior in the extraction assays in accordance to Chantai et al. (2012), thereby different conditions do not

give priority to either compound. The experimental design parameters and the elemental response are shown in Table 2.4.

Table 2.4. Experimental design and capsaicin amount extracted.

Assay	Extraction conditions		Capsaicin Concentration*
	% of Methanol in acetone (v/v)	Ultrasound time (minutes)	
1	14.5 (-1)	2.9 (-1)	1.87
2	14.5 (-1)	17.1 (+1)	2.88
3	85.5 (+1)	2.9 (-1)	1.78
4	85.5 (+1)	17.1 (+1)	2.72
5	0 (-1.41)	10 (0)	1.55
6	100 (+1.41)	10 (0)	2.88
7	50 (0)	0 (-1.41)	2.04
8	50 (0)	20 (+1.41)	2.07
9	50 (0)	10 (0)	2.21
10	50 (0)	10 (0)	2.22
11	50 (0)	10 (0)	2.23

* mg g^{-1} of fresh 'Cumari do Pará' pepper.

The quadratic model was shown to be more appropriate to represent the responses of optimization of extraction (Eq. (1)).

$$= + + + + + \quad (1)$$

where, is the predicted response, is the coefficient of model and is the variable values. The indexes and correspond to values of methanol and time, respectively. The last term of Eq. (1) was not considered in the predictions of this model since the effect of the interaction between the two variables was not

significant. Analysis of variance (ANOVA) was performed on the experimental design to assess the significance of the model. The generated model showed a lack of fit with a value of $F_{(3,2,95\%)}$ calculated of 3359.78, greater than $F_{(3,2,95\%)}$ tabulated, which was 19.16. As a model cannot be used to predict the optimum point of extraction, the optimum point of extraction was defined by the analysis of real results and practical considerations as in the results reported by Meinhart et al. (2010).

According to the assays performed in the extraction optimization step, an increase in the amount of capsaicin extracted in assays 2 and 6 (Table 4) was observed, the condition of assay 6 using 100% methanol and 10 min of ultrasound extraction was selected to carried out the experiments based on the fact that methanol used as solvent extractor for capsaicinoids from chili pepper fruits showed an important advantage because it reduces the amounts of pigments and oils extracted simultaneously with the capsaicinoids according to Attuquayefio and Buckle (1987) and is compatible with the UAE process and subsequently chromatographic analysis. In relation to extraction time, these experiments indicated that an intermediary value (10 min) of the range studied is the most suitable. Besides making working with a shorter time of sample preparation possible, this fact can be supported by authors that suggest the possibility of degradation occurrence in function of a long time of conventional liquid–solid extraction (Ya-Qin, Jian-Chu, Dong-Hong, & Xing-Qian, 2009).

Thus, the suitable condition for extraction of capsaicinoids by indirect sonication in an ultrasonic bath was at a ratio of 1 g of sample material: 25 mL of methanol, with 10 min of extraction time. These conditions were very similar to those

established by Barbero et al. (2008b), which in his study of extraction optimization, also found methanol as the most efficient extractor solvent with an optimal range time of ultrasound between 10 and 20 min.

With regard to extraction solvent for capsaicinoids, the mostly used organic solvents that are reported as the most efficient in the capsaicinoid extraction are methanol and acetone (Attuquayefio & Buckle, 1987; Barbero et al., 2008b; Boonkird et al., 2008; Deng et al., 2012), but these results were found in studies where these two organic solvents are not confronted or had their performance evaluated in the form of mixture. This is the first time that these solvents were assessed in the same study. The effectiveness of UAE depends on the capacity of the extraction solvent for absorbing and transmitting the energy of ultrasounds (Barbero et al., 2008b). Thus, selecting an appropriate solvent for analyte extraction from matrix of samples is an important step in the development of the UAE method (Deng et al., 2012). Finally, 100% of methanol and 10 min in ultrasound bath were chosen as the extraction conditions for the following experiments.

The enhancement of extraction efficiency of organic compounds by ultrasound is attributed to the phenomenon of cavitation produced in the solvent by the passage of an ultrasonic wave. Ultrasound also exerts a mechanical effect, allowing greater penetration of solvent into the sample matrix, increasing the contact surface area between solid and liquid phases (Barbero et al., 2008b; Deng et al., 2012).

2.3.4. Identification of capsaicinoids

The identification of capsaicinoids was carried out in 'Malagueta' pepper (*C. frutescens*) sample. Capsaicin and dihydrocapsaicin were identified by the comparison of retention time, UV–vis and mass spectrum obtained with commercial standards. The minor capsaicinoids were identified according to their chromatographic behavior and by comparison of mass spectrum with library data. Fig. 2.2 shows the chromatogram for separation of capsaicinoids present in the extract from "Malagueta" pepper.

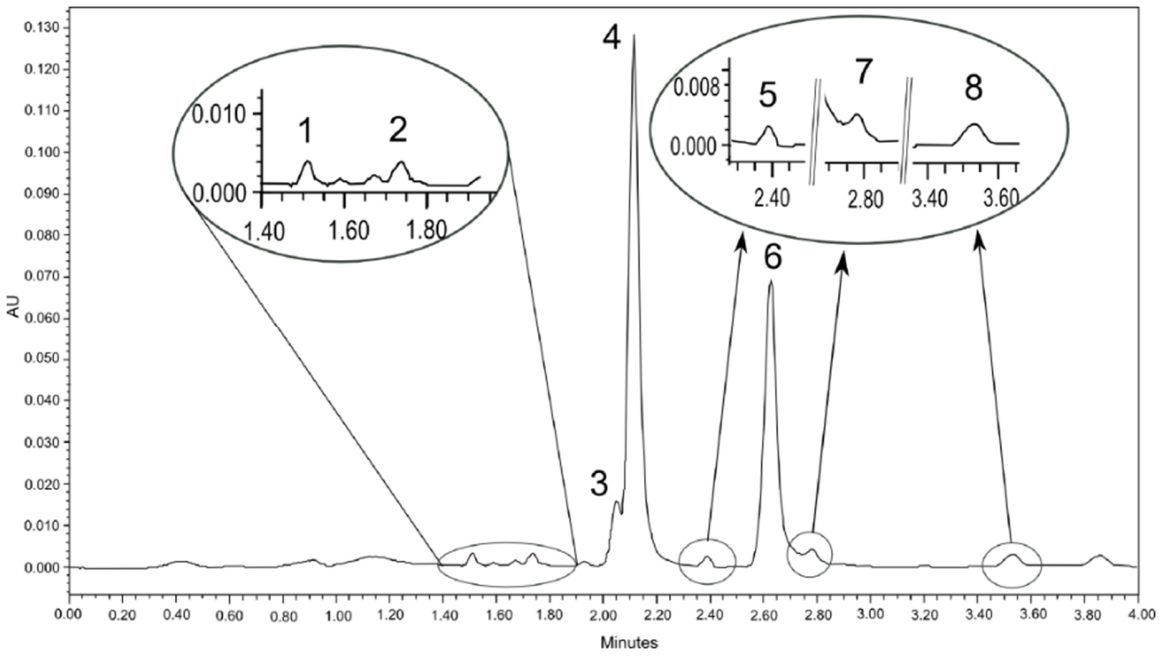

Figure 2.2. Separation profile of capsaicinoids from 'Malagueta' chili pepper (*C. frutescens*). 1- nornorcapsaicin, 2- nornordihydrocapsaicin, 3- nordihydrocapsaicin, 4- capsaicin, 5- norcapsaicin, 6- dihydrocapsaicin, 7- i-dihydrocapsaicin, 8- homocapsaicin.

The protonated molecule [M + H] + for the capsaicinoids found presented the following m/z ratios: nornorcapsaicin, 278; nornordihydrocapsaicin, 280; nordihydrocapsaicin, 294; capsaicin, 306; norcapsaicin, 292; dihydrocapsaicin, 308; isomer of dihydrocapsaicin, 308; and homodihydrocapsaicin, 322. In the mass spectra of these eight capsaicinoids, the m/z peak (137) characteristic of the fragmentations of capsaicinoids appears clearly. The [M + H]+ and fragments identified were compatible to standards of capsaicin and dihydrocapsaicin and literature data (Kozukue et al., 2005; Barbero et al., 2006a; Schweiggert et al., 2006).

2.3.5. Quantification of capsaicinoids in C. chinense fruits

The capsaicin and dihydrocapsaicin levels were determined in 9 different access ions of *C. chinense*. The levels of capsaicin and dihydrocapsaicin were between 156 and 1442 μg g^{-1} and 26 and 478 μg g^{-1} of fresh pepper, respectively (Table 2.5).

Table 2.5. Amounts of capsaicin and dihydrocapsaicin in the *C. chinense* chili peppers

Sample	Capsaicin* (μg g^{-1} fresh sample)	Dihydrocapsaicin* (μg g^{-1} fresh sample)
IAC 1573 'Cumari-do-Pará'	798±56	143±9
IAC 1552 'Murupi amarela'	1270±80	206±9
IAC 1638 'De cheiro'	656±13	153±12
IAC1642 'Habanero'	1442±3	478±5
IAC 1644 'Fidalga'	156±24	26±9
IAC 1647 'De cheiro'	182±41	36±2
IAC 1648 'De cheiro'	267±59	53±6
IAC 1641 'Murupi vermelha'	303±40	82±10
IAC 1643 'Biquinho'	nd	nd

* Mean values ± standard deviation to triplicate analysis. nd = no detected.

The chili pepper capsaicinoid contents showed varied pungency levels in the peppers used for this study that evidences the large application of the method developed. The contents of capsaicin and dihydrocapsaicin found in this work for the different chili pepper accessions are in good agreement with those found by other authors. According to our results, 'Habanero' chili pepper, one of the most pungent varieties (Garcés-Claver et al., 2006), had the highest capsaicinoid content.

2.4. Conclusions

The method described for capsaicinoid extraction, chromatographic separation is fast, efficient and reliable. In addition, the method shows large application for the quantification of capsaicin and dihydrocapsaicin in different concentration levels. Total sample preparation takes about 15 min, with reduced requirements for sample, solvents and instrumentation, also resulting in reduced chromatographic interferences. UAE, by means of the method developed, allows the quantitative and reproducible extraction of the capsaicinoids present in chili peppers, employing methanol as solvent extractor.

Due to its simplicity and its analytical capabilities, the method developed can be applied for the fast routine analysis of capsaicinoids in chili peppers and would be particularly suitable to routinely analyze capsaicinoids in breeding programs and may be used to determine the concentrations of other minor capsaicinoids once appropriate standards are available.

2.5. Acknowledgments

The authors are grateful to CAPES - Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Process number 201879/2012-3) and FAPESP - Fundação de Amparo a Pesquisa do Estado de São Paulo (Process numbers 2010/19863-0 and 2011/17094-2) for providing the fund for current research activities and a scholarship was awarded to one author (Process number 2011/15431-1).

2.6. References

Al Othman, Z. A., Ahmed, Y. B. H., Habila, M.A., & Ghafar, A. A. (2011). Determination of capsaicin and dihydrocapsaicin in *Capsicum* fruit samples using high performance liquid chromatography. **Molecules**, 16(10), 8919–8929.

Alothman, Z. A., Wabaidur, S. M., Khan, M. R., Ghafar, A. A., Habila, M.A., & Ahmed, Y. B. H. (2012). Determination of capsinoids in *Capsicum* species using ultra performance liquid chromatography–mass spectrometry. **Journal of SeparationScience**, 35(21), 2892–2896.

Attuquayefio, V. K., & Buckle, K. A. (1987). Rapid sample preparation method for HPLC analysis of capsaicinoids in *Capsicum* fruits and oleoresins. **Journal of Agricultural and Food Chemistry**, 35, 777–779.

Barbero, G. F., Liazid, A., Palma, M., & Barroso, C. G. (2008a). Fast determination of capsaicinoids from peppers by high-performance liquid chromatography using a reversed phase monolithic column. **Food Chemistry**, 107(3), 1276–1282.

Barbero, G. F., Liazid, A., Palma, M., & Barroso, C. G. (2008b). Ultrasound-assisted extraction of capsaicinoids from peppers. **Talanta**, 75(5), 1332–1337.

Barbero, G. F., Palma, M., & Barroso, C. G. (2006a). Determination of capsaicinoids in peppers by microwave-assisted extraction–high-performance liquid chromatography with fluorescence detection. **Analytica Chimica Acta**, 578(2), 227–233.

Barbero, G. F., Palma, M., & Barroso, C. G. (2006b). Pressurized liquid extraction of capsaicinoids from peppers. **Journal of Agricultural and Food Chemistry**, 54(9), 3231–3236.

Boonkird, S., Phisalaphong, C., & Phisalaphong, M. (2008). Ultrasound-assisted extraction of capsaicinoids from *Capsicumfrutescens* on a lab- and pilot-plant scale. **Ultrasonics Sonochemistry**, 15(6), 1075–1079.

Careaga, M., Fernández, E., Dorantes, L., Mota, L., Jaramillo, M. E., & Hernandez-Sanchez, H. (2003). Antibacterial activity of *Capsicum* extract against Salmonella typhimurium and Pseudomonas aeruginosa inoculated in raw beef meat. **International Journal of Food Microbiology**, 83(3), 331–335.

Chantai, S., Juangsamoot, J., Ruangviriyachai, C., & Techawongstien, S. (2012). Determination of capsaicin and dihydrocapsaicin in some chilli varieties using

accelerated solvent extraction associated with solid-phase extraction methods and RP–HPLC– fluorescence. **E-Journal of Chemistry**, 9(3), 1550–1561.

Cichewicz, R. H. (1996). The antimicrobial properties of chile peppers (*Capsicum* species) and their uses in Mayan medicine. **Journal of Ethnopharmacology**, 52(2), 61–70.

Contreras-Padilla, M., & Yahia, E. M. (1998). Changes in capsaicinoids during development, maturation, and senescence of chile peppers and relation to peroxidase activity. **Journal of Agricultural and Food Chemistry**, 46(6), 2075–2079.

Daood, H. G., Illes, V., Gnayfeed, M. H., Meszaros, B., Horvath, G., & Biacs, P. A. (2002). Extraction of pungent spice paprika by supercritical carbon dioxide and subcritical propane. **The Journal of Supercritical Fluids**, 23(2), 143–152.

Davis, C. B., Markey, C. E., Busch, M.A., & Busch, K. W. (2007). Determination of capsaicinoids in Habanero peppers by chemometric analysis of UV spectral data. **Journal of Agricultural and Food Chemistry**, 55(15), 5925–5933.

Deng, X. Y., Gao, K., Huang, X., & Liu, J. (2012). Optimization of ultrasonic-assisted extraction procedure of capsaicinoids from Chili peppers using orthogonal array experimental design. **African Journal of Biotechnology**, 11(67), 13153–13161.

Duarte, C., Moldão-Martins, M., Gouveia, A. F., Beirão da Costa, S., Leitão, A. E., & Bernardo Gil, M. G. (2004). Supercritical fluid extraction of red pepper (*Capsicumfrutescens* L.). **Journal of Supercritical Fluids**, 30(2), 155–161.

Estrada, B., Bernal, M.A., Pomar, F., & Merino, F. (2002). Capsaicinoids in vegetative organs of *Capsicumannuum* L. in relation to fruiting. **Journal of Agricultural and Food Chemistry**, 50(5), 1188–1191.

Garcés-Claver, A., Arnedo-André, M. S., Avier Abadía, R., Gil-Ortega, S. J., & Álvarez Fernández, A. (2006). Determination of capsaicin and dihydrocapsaicin in *Capsicum* fruits by liquid chromatography–electrospray/time-of-flight mass spectrometry. **Journal of Agricultural and Food Chemistry**, 54(25), 9303–9311.

Graham, D. Y., Anderson, S. Y., & Lang, T. (1999). Garlic or jalapeno peppers for treatment of Helicobacter pylori infection. **The American Journal of Gastroenterology**, 94(5), 1200–1202.

Ha, J., Seo, H. Y., Shim, Y. S., Seo, D. W., Seog, H., Ito, M., et al. (2010). Determination of capsaicinoids in foods using ultra high performance liquid chromatography. **Food Science and Biotechnology**, 19(4), 1005–1009.

Hendersen, D. E., & Slickman, A.M. (1999). Quantitative HPLC determination of the antioxidant activity of capsaicin on the formation of liquid hydroperoxides of linoleic acid: A comparative study against BHT and melatonin. **Journal of Agricultural and Food Chemistry**, 47(7), 2563–2570.

Jarret, R. L., Perkinsb, B., Fanc, T., Princec, A., Guthrieb, K., & Skoczenski, B. (2003). Using EIA to screen *Capsicum* spp. germplasm for capsaicinoid content. **Journal of Food Composition and Analysis**, 16(2), 189–194.

Jin, R., Pan, J., Xie, H., Zhou, B., & Xia, X. (2009). Separation and quantitative analysis of capsaicinoids in chili peppers by reversed-phase argentation LC. **Chromatographia**, 70(5–6), 1011–1013.

Kaale, E., Van Schepdael, A., Roets, E., & Hoogmartens, J. (2002). Determination of capsaicinoids in topical cream by liquid–liquid extraction and liquid chromatography. **Journal of Pharmaceutical and Biomedical Analysis**, 30(4), 1331–1337.

Kirschbaum-Titze, P., Hiepler, C., Mueller-Seitz, E., & Petz, M. (2002). Pungency in paprika (*Capsicumannuum*). 1. Decrease of capsaicinoid content following cellular disruption. **Journal of Agricultural and Food Chemistry**, 50(5), 1260–1263.

Korel, F., Bagdatlioglu, N., Balaban, M.O., & Hisil, Y. (2002). Ground red peppers: Capsaicinoids content, Scoville scores, and discrimination by an electronic nose. **Journal of Agricultural and Food Chemistry**, 50(11), 3257–3261.

Kozukue, N., Han, J., Lee, S., Kim, J., Lee, K., Park, M., et al. (2005). Analysis of seven capsaicinoids in peppers and pepper-containing foods by liquid chromatography– mass spectrometry. **Journal of Agricultural and Food Chemistry**, 53(23), 9172–9181.

Laskaridou-Monnerville, A. (1999). Determination of capsaicin and dihydrocapsaicin by micellar electrokinetic capillary chromatography and its application to various species of *Capsicum*, Solanaceae. **Journal of Chromatography**. A, 838(1–2), 293–302.

Lee, K. R., Suzuki, T., Kobashi, M., Hasegawa, K., & Iwai, K. (1976). Quantitative microanalysis of capsaicin, dihydrocapsaicin and nordihydrocapsaicin using mass fragmentography. **Journal of Chromatography**. A, 123(1), 119–128.

Lu, J., & Cwik, M. (1997). Determination of capsaicin and zucapsaicin in human serum by high-performance liquid chromatography with fluorescence detection. Journal of Chromatography B, 701(1), 135–139.

Luo, X. J., Peng, J., & Li, Y. J. (2011). Recent advances in the study on capsaicinoids and capsinoids. **European Journal of Pharmacology**, 650(1), 1–7.

Meinhart, A.D., Bizzotto, C. S., Ballus, C. A., Prado, M.A., Bruns, R. E., Teixeira Filho, J., et al. (2010). Optimisation of a CE method for caffeine analysis in decaffeinated coffee. **Food Chemistry**, 120(4), 1155–1161.

Molina-Torres, J., Garcia-Chavez, A., & Ramirez-Chavez, E. (1999). Antimicrobial properties of alkamides present in flavouring plants traditionally used in Mesoamerica: Affinin and capsaicin. **Journal of Ethnopharmacology**, 64(3), 241–248.

Mueller-Seitz, E., Hiepler, C., & Petz, M. (2008). Chili pepper fruits: Content and pattern of capsaicinoids in single fruits of different ages. **Journal of Agricultural and Food Chemistry**, 56(24), 12114–12121.

Perucka, I., & Oleszek, W. L. (2000). Extraction and determination of capsaicinoids in fruit of hot pepper *Capsicumannuum* L. by spectrophotometry and high-performance liquid chromatography. **Food Chemistry**, 71 (2), 287–291.

Poyrazoğlu, E. S., Yemis, O., Kadakal, Ç., & Artik, N. (2005). Determination of capsaicinoid profile of different chilli peppers grown in Turkey. Journal of the **Science of Food and Agriculture**, 85(9), 1435–1438.

Reilly, C. A., Crouch, D. J., & Yost, G. S. (2001). Quantitative analysis of capsaicinoids in fresh peppers, oleoresin *Capsicum* and pepper spray products. **Journal of Forensic Science**, 46(3), 502–509.

Ribeiro, C. S.C., Lopes, C. A., Carvalho, S. I. C., Henz, G. P., & Reifschneider, F. J. B. (2008). Pimentas *Capsicum*. Brasília-DF, **Embrapa Hortaliças**, Brazil, (Chapter 1) (pp. 1 –14) (1st ed.).

Salgado-Roman, M., Botello-Alvarez, E., Rico-Martinez, R., Jimenez-Islas, H., Cardenas Manriquez, M., & Navarrete-Bolanos, J. L. (2008). Enzymatic treatment to improve extraction of capsaicinoids and carotenoids from chili (*Capsicumannuum*) fruits. **Journal of Agricultural and Food Chemistry**, 56(21), 10012–10018.

Sato, K., Sasaki, S. S., Goda, Y., Yamada, T., Nunomura, O., Ishikawa, K., et al. (1999). Direct connection of supercritical fluid extraction and supercritical fluid chromatography as a rapid quantitative method for capsaicinoids in placentas of *Capsicum*. **Journal of Agricultural and Food Chemistry**, 47(1 1), 4665–4668.

Schweiggert, U., Carle, R., & Schieber, A. (2006). Characterization of major and minor capsaicinoids and related compounds in chili pods (*Capsicumfrutescens* L.) by high performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry. **Analytica Chimica Acta**, 557(1–2), 236–244.

Surh, Y. J., Lee, R. C. J., Park, K. K., Mayne, S. T., Liem, A., & Miller, J. A. (1995). Chemoprotective effects of capsaicin and diallyl sulfide against mutagenesis or tumorigenesis by vinyl carbamate and N-nitrosodimethylamine. **Carcinogenesis**, 16, 2467–2471.

Szolcsányi, J. (2004). Forty years in capsaicin research for sensory pharmacology and physiology. **Neuropeptides**, 38(6), 377–384.

Tapia, J. C., Garcia, R., Escamilla, E. M., Calva, G., & Rocha, J. A. (1993). Capsaicin recovery from a cell culture broth. **Industrial Engineering and Chemical Research**, 32, 2242–2246.

Thompson, R. Q., Phinney, K. W., Welch, M. J., & White, E. (2005). Quantitative determination of capsaicinoids by liquid chromatography–electrospray mass spectrometry. **Analytical and Bioanalytical Chemistry**, 381(7), 1441–1451.

Wolf, R., Huschka, C., Raith, K., Wohlrab, W., & Neubert, R. H. H. (1999). Rapid quantification of capsaicin and dihydrocapsaicin in human skin extracts after dermal administration using HPLC–ESI–MS. **Journal of Liquid Chromatography & Related Technologies**, 22(4), 531–539.

Ya-Qin, M., Jian-Chu, C., Dong-Hong, L., & Xing-Qian, Y. (2009). Simultaneous extraction of phenolic compounds of citrus peel extracts: Effect of ultrasound. **Ultrasonics Sonochemistry**, 16(1), 57–62.

Zewdie, Y., & Bosland, P. W. (2001). Capsaicinoid profiles are not good chemotaxonomic indicators for *Capsicum* species. **Biochemical Systematics and Ecology**, 29(2), 161–169.

Zhang, Q., Hu, J., Sheng, L., & Li, Y. (2010). Simultaneous quantification of capsaicin and dihydrocapsaicin in rat plasma urine HPLC coupled with tandem mass spectrometry. **Journal of Chromatography B**, 878(24), 2292–2297.

CHAPTER III

ARTICLE

Optimization of headspace solid-phase microextraction (HS-SPME) parameters for the analysis of the volatile fraction from Brazilian chili pepper (Capsicum chinense)

CHAPTER III

Optimization of headspace solid-phase microextraction (HS-SPME) parameters for the analysis of the volatile fraction from Brazilian chili pepper (*Capsicum chinense*)

Marla Sganzerla[a], Cristiano Augusto Ballus[a], Carlo Bicchi[b], Arlete Marchi Tavares de Melo[c], Helena Teixeira Godoy[a]

[a] *Laboratory of Food Analysis, Department of Food Science, Faculty of Food Engineering, University of Campinas (UNICAMP), P.O. Box 6121, CEP 13083-862, Campinas, SP, Brazil*

[b] *Laboratory of Phytochemical Analysis, Department of Pharmaceutical Science and Technology, Faculty of Pharmacy, University of Turin (UNITO), CAP 10125, Turin, TO, Italy*

[c] *Horticultural Center, Agronomic Institute of Campinas (IAC), P.O. Box 28, CEP 13012-970 Campinas, SP, Brazil*

***Manuscript prepared for submission to the journal "Food Chemistry"**.

ABSTRACT

Characterization of aroma profile is an important and challenging analysis because of the number and variety of volatile compounds and the complexity of the food matrices. Headspace Solid-Phase Microextraction (HS-SPME) is one of the techniques most employed to the extraction of volatile compounds from different samples. The varieties of *Capsicum chinense* peppers are very important for different regions of Brazil, since its typical aroma is fundamental for several traditional dishes. The aim of this study was to optimize the HS-SPME extraction temperature and extraction time for the analysis of volatile fraction in Brazilian *Capsicum chinense* fruits, belonging to the 'Habanero' type, by GC-MS. Central composite design and Derringer's desirability function strategies were used for the optimization, in order to evaluate simultaneously the 'total sum peak areas' and 'number of extracted compounds' responses. The maximized results for both responses were obtained using PDMS/DVB/CAR fiber, temperature of 40 °C and extraction time of 30 min. Eighty-two compounds were identified in the volatile fraction of the 'Habanero' pepper, and the most abundant were hexyl isovalerate, cis-hexenyl isovalerate, hexyl 3-methylbutanoate, 3,3-dimethylcyclohexanol, longifolene, and 2-methyl-1-tetradecene. The compound 2,3-dimethylcyclohexanol and longifolene were reported for the first time in 'Habanero' pepper.

Keywords: habanero pepper; GC-MS; HS-SPME; aroma compounds; central composite design.

3.1. Introduction

Aroma compounds are volatile chemicals that attach great importance to the assessment of food quality especially in fresh fruits and vegetables, and, recently, these volatile compounds have received more attention (Guadayol et al., 1997, Taylor et al., 2001). The aroma substances consist of several diversified classes of compounds, some of them being highly reactive and are present in food in extremely low concentrations.

The analysis of aroma profile is a difficult task that comprises the investigation of individual compounds belonging to several classes in complex matrices (Bicchi et al., 2000). The fraction of aroma compounds of a simple fresh fruit or vegetable may be composed of 50-300 constituents. This large number of aroma chemicals complicates the task even further. Other difficulties are associated with identification of aroma compounds, elucidation of their chemical structure and characterization of sensory properties.

The study of volatile fraction has benefited from the progress in the sample preparation and instrumentation techniques over the last decades, which led to long lists of volatiles compounds determined in several foods. The analytical steps involved in the volatile fraction characterization consist on isolation by different techniques, followed by separation, identification and quantification by gas chromatography-mass spectrometry (GC-MS) (Bogusz et al., 2011). Different sample preparation methods have been developed to isolate compounds that contribute to aroma, such as solvent extraction-simultaneous distillation extraction

(Chitwood et al., 1983; Korany et al., 2002) and dynamic and static headspace (Luning et al., 1994, Bogusz et al., 2011).

Solid-phase microextraction (SPME) is a robust and sensitive technique for volatile headspace sampling introduced in 1990's by Arthur and Pawliszyn (1990). It is an equilibrium-based technique that relies on the adsorption of volatiles on an inert fiber from which compounds can be thermally desorbed inside a GC inlet. In 1993, Zhang and Pawliszyn (1993) applied SPME to static headspace (S-HS) sampling (HS-SPME). SPME has been shown to be an excellent sampling method, allowing simultaneous extraction and concentration of analytes from sample matrices (Lambropoulou, Konstantinou, Albanis, 2007) and is one of the most popular methods for volatile compounds isolation (Olivieira, Pereira, Marsaioli, & Augusto, 2004). SPME is considered the most suitable technique for the study of high volatile compounds and the volatile profile obtained using it represents better the smell that is perceived by the consumer (Yang; Peppard, 1994). In addition, SPME eliminates the problems associated with chemically and thermally unstable food samples, where generation of artifacts can be problematic, since it avoids the lengthy use of organic solvents or high temperatures in the extraction and concentration stages.

SPME is reliable and offer high sensitivity, providing detection limits in parts per billion by volume (Tholl et al., 2006). Fibers are available with combinations of different coating materials. Fiber coatings fall into two categories, liquid polymers with high molecular weight or high porosity solids, and their combination has been shown to be the most effective at collecting a broader spectrum of compounds ranging in volatility (Bicchi, Drigo and Rubiolo, 2000). The quantity of captured

volatiles is regulated by two equilibrium constants, the rate of volatile release from the sample matrix into the surrounding air and the partitioning of airborne volatiles to the SPME fiber (partition ratio). SPME analysis is also quite sensitive to experimental conditions such as fiber coating, sample volume and concentration, temperature and time of adsorption, ionic strength, among others (Lord and Pawliszyn, 2000; Nongonierma et al., 2006; Pawliszyn, 2009). Finding the optimal experimental conditions in SPME is an important task, since the kinetics and thermodynamics of extraction depend on several experimental conditions (Medina, Satue-Gracia and Frankel, 1999; Ribeiro, Teófilo, Augusto and Ferreira, 2010).

Chili peppers are one of the most popular vegetables in the world (Kollmannsberger, Rodríguez-Burruezo, Nitz and Nuez, 2011) and are economically important because of the vast quantity consumed and the diverse varieties used (Garcés-Claver, Arnedo-André, Avier Abadía, Gil-Ortega, and Álvarez-Fernandéz, 2006; Pino, Sauri-Duch and Marbot, 2006). Chili peppers are all the fruits from a group of five cultivated species (*Capsicum annuum*, *C. frutescens*, *C. baccatum*, *C.pubescens* and *C.chinense*), and even several relatives included in the genus *Capsicum*.

In Brazil, the varieties of *Capsicum chinense* peppers, also known as “scented peppers”, are essential ingredients of several typical cookery recipes from different regions of the country and the consumption of this kind of spice represents a relevant characteristic of the local culture (Souza et al., 2006). The typical aroma of *C. chinense* peppers is one of the most attractive properties and consist in a quality parameter for the consumer (Pino et al., 2007).

Considering that the volatile profile of pepper is highly complex and many factors can influence the response of the system, optimization of the extraction procedure can be carried out using multivariate statistical tools. The multivariate optimization provide secure information concerning the best analytical conditions, the existence of experimental errors, as well as shows any interactions that might exist between the factors involved. In opposition, traditional methods as univariate method optimization, where only one variable is analyzed at a time, leaving the others fixed, generally require a large number of experiments, and do not allow to investigate possible interactions among variables or fully explore the experimental domain for optimization (Souza et al., 2006; Ferreira, Bruns and Silva et al., 2007).

Optimization of HS-SPME technique for analysis of volatile compounds finding the optimal experimental conditions has been reported focusing in maximize the total sum peak areas as the only one response. Unfortunately, the experimental conditions resulting from the maximization of the extraction based on total sum peak areas may simultaneously results in losses of certain volatile compounds of low or high molar mass, depending, for example, of the set conditions of time and/or temperature extraction. Thus, the optimal condition obtained could not be representative of the total aroma profile of the investigated matrix. As a result, it would be important to also consider the number of extracted compounds. Optimization of HS-SPME technique for analyzing volatile compounds of *Capsicum* spp. has been previously reported (Souza et al. 2006; Bogusz et al. 2011). However, their effort focused only on total sum peak areas as the response to obtain the optimal conditions for the extraction time and temperature. As far as we know, there

are no studies evaluating the total sum peak areas and the number of extracted compounds simultaneously, when optimizing HS-SPME for volatile compounds analysis for peppers.

The aim of this study was to optimize the parameters that can influence the efficiency of HS-SPME for the analysis of volatile fraction in Brazilian *Capsicum chinense* fruits, applying a strategy based on central composite design (CCD) and Derringer's desirability function in order to simultaneously evaluate total sum peak areas and the number of compounds obtained as responses from the experimental design.

3.2. Material and methods

3.2.1. Plant material and chemicals

One accession (IAC 1648) of *Capsicum chinense* peppers belonging to the 'Habanero' type was employed in this study. The plants belonging to this accession were grown in the IAC-Agronomic Institute of Campinas (Campinas, SP, Brazil, 22°54'S, 47°05'W, 674 m of elevation) and the fully ripe fruits from several plants were harvested during spring season in 2011. Two hundred grams of the whole pepper fruits were blended using a grinder Turratec TE102 (Tecnal, Piracicaba, SP, Brazil) for three minutes at 20,000 rpm until homogeneous sample was achieved. Then, 1 g was immediately placed in a headspace vial (20 mL) sealed with a stainless steel cap with PTFE/silicone septum (Supelco, Bellefonte, PA, USA) for extraction to prevent loss of the volatile compounds. 3-Octanol with a purity of 99%

(Sigma-Aldrich, St. Louis, MO, USA) was added as internal standard in all extraction assays. Linear retention indices (LRI) of individual volatiles were calculated according to Van der Dool and Kratz method (Van der Dool and Kratz, 1963) using a n-alkane standard solution (Supelco, Bellefonte, PA, USA) ranging from heptane to triacontane.

Other reference volatile compounds (all with purity higher than 98%) were obtained from PolyScience Corp. (Niles, WI, USA): 1-hexanol, 2-methyldodecane, 2-methyltridecane, 2-methyltetradecane, 2-methylhexadecane; from Sigma-Aldrich (St. Louis, MO, USA): cis-3-hexenyl isovalerate, hexyl 3-methyl butanoate, hexyl butanoate, hexyl pentanoate, hexyl hexanoate, hexyl 3-methyl butenoate, heptyl pentanoate, α-ionone, trans-β-ionone, α-cubebene; and caryophyllene was coming from clove essencial oil extracted in laboratory.

3.2.2. Optimization strategy

Optimization of the HS-SPME conditions applied to volatile compounds from *Capsicum chinense* fruits was carried out using a 2^2 factorial central composite design (CCD), with four axial points ($\alpha = 1.4142$) and five central points (Box and Hunter, 1978). The full design was carried out in duplicate and randomly, resulting in 26 experiments.

The variables chosen were the temperature (T, °C) and extraction time (t, min). The temperature varied from 26 °C to 54 °C and time varied from 16 to 50 min. The responses adopted to evaluate the experimental design were the total sum peak

areas and the number of extracted compounds. The detailed experimental design is shown in Table 3.1.

Usually, the studies of the effectiveness of analyte extraction using HS-SPME technique are conducted evaluating the following parameters: fiber type, amount of sample; incubation time; extraction time; incubation/extraction temperature, agitation speed and others that may interfere on the result (Kataoka et al., 2000; Wardencki, Michulec and Curylo, 2004). On the other hand, according to some authors (Vichi et al. 2003; Risticevic, Carasek and Pawliszyn, 2008), temperature and time of extraction are two important factors that control sample recovery by the fibers. Recently, Cuevas-Glory and co-workers (2014) carried out a study covering a complete optimization of HS-SPME condition applied to the characterization of volatile compounds in Habanero pepper and concluded that the main factors related to the efficiency of the analyte pre-concentration on the fiber were extraction time, extraction temperature and salt addition. The significance of salt addition is solely due to the fact that sample preparation in this study was done with suspension of pepper material in water prior the extraction step. Souza et al. (2006) achieved the same conclusion about significant factors evaluating HS-SPME applied to *Capsicum chinense* peppers by means of a screening factorial prior the completion of the optimization purpose. Aware of these reports, only temperature and extraction time were studied, in order to establish the optimum extraction conditions in the present study.

The other parameters, such as amount of sample (1 g), headspace vial volume (20 mL) and equilibrium time (10 minutes at the extraction temperature) were fixed by the authors.

The 50/30 mm DVB/Car/PDMS (divinylbenzene/carboxen/polydimethylsiloxane) coated fiber, 1 cm (Supelco, Bellefonte, PA, USA) was the type of fiber employed in this study, in combination with a manual holder (Supelco, Bellefonte, PA, USA). The fiber was conditioned according to the manufacturer's recommendations prior to its first use. The choice for this triple phase type of fiber for the analysis of volatile compounds from different matrices, including peppers, is supported by several authors (Mondello et al., 2004; Mazida, Salleh and Osman, 2005; Bianchi et al., 2007; Bogusz et al., 2011; Cuevas-Glory et al., 2014).

A useful approach for the simultaneous optimization of several response variables is the Derringer's desirability function (Ribeiro, Teófilo, Augusto and Ferreira, 2010, Ballus et al., 2014) that allows consider different scalings for the responses selected in the study. Thus, after calculating the models for the two responses and validate them through Analysis of Variance (95% confidence level), the two models were combined by using Derringer's desirability function to simultaneously optimize the total peak sum areas and the number of extracted compounds, in order to obtain an optimal condition where the two responses were maximized.

Data treatment was performed using Design Expert 6.0.10 software (StatEasy Corp., Minneapolis, MN, USA).

3.2.3. Volatile compounds analysis

Following the volatile compounds extraction procedure for all the assays of the experimental design and those performed after finding the optimal conditions of extraction time and extraction temperature for the 'Habanero' pepper sample (in triplicate), the SPME fiber containing the absorbed compounds was immediately inserted into the GC injector port and the fiber was thermally desorbed for 5 minutes at 230 °C (splitless mode). Before each sampling procedure, the SPME fiber was reconditioned for 10 minutes at 230 °C to avoid 'carry over' of compounds or 'memory effect' on the fiber and, consequently, interferences in the subsequent sample extraction (Holt, 2011).

A system of gas chromatography coupled to a mass spectrometry - GC7890A/MS5975C MSD (Agilent Technologies, Wilmington, DE, USA) was employed in the analysis. The injector operated at constant temperature of 230 °C in splitless mode using a SPME Injection Sleeve 0.75 mm ID (Supelco, Bellefonte, PA, USA). The carrier gas, helium of purity 5.0 (White Martins/Praxair Inc., Rio de Janeiro, RJ, Brazil), was used at a constant flow rate of 0.6 $mL.min^{-1}$. The separation of the volatile compounds was performed in a HP-5MS fused silica capillary column (5% phenyl/95% polidimethylsiloxane as stationary phase, 30 m length, 0.250 mm internal diameter, 0.25 µm film thickness) (Agilent Technologies, Wilmington, DE, USA). The oven temperature program applied in the analysis consisted of utilizing 50 °C initial temperature for 1 min, after which the temperature was raised at 3 $°C.min^{-1}$ rate to 200 °C, then increased to 280 °C at 20 $°C.min^{-1}$ for a total GC run

time of 55 min. The transfer line temperature was held at 250 °C. The column eluent was submitted to MS detection in EI (electron ionization) mode (ionization energy 70 eV), while the ion source temperature was kept at 230 °C. Acquisition was carried out in scanning mode (mass range m/z 50-500). Volatile compounds were identified by comparison of their obtained mass spectra with those reported by NIST 2011 database (considering a minimum similarity value of 80%) and with those from authentic standard compounds, as well as by also comparing the linear retention index (LRI) values with those of the standards and literature.

To evaluate the composition of the samples, the semi-quantitative determination in equivalents was carried out by using the method of the internal standard addition, in which ratios between the response of the individual compounds and that of the internal standard were considered. In this case, 3-octanol was used as an internal standard. The optimum amount of 3-octanol added to the sample vial was adjusted to 3.451 ng to avoid saturation of the fiber device and also to prevent it from masking peaks of interest.

All analysis of the experimental design and those performed to elucidate the composition of the sample were carried out in a system of GC-MS since when comparing the techniques GC-MS and GC-FID applied to the analyses of volatile compounds in peppers, Saskia van Ruth et al. (2003) have verified no occurrence of significant differences in the proportions of aroma compounds analyzed. In addition, the FID detector does not give any structural information, which is fundamental for studies with complex matrices.

3.3. Results and discussion

3.3.1. HS-SPME optimization

The results obtained performing the experimental design proposed are summarized in Table 3.1, with the responses for total sum peak areas and total number of compounds extracted. The experimental design was performed with five central point and one replication for each experiment of the CCD, in order to have more degrees of freedom to estimate the pure error and detect a possible lack of fit of the models.

Table 3.1. Experimental design with coded and uncoded variables and the results obtained in the HS-SPME optimization for volatile compounds analysis from *Capsicum chinense* fruits.

Experiment number	Coded factors		Uncoded factors		CCD Responses	
	X_1:T	X_2:t	Temperature (°C)	Time (min)	Total sum peak areas	Number of compounds
1	-1	-1	30	16	2,52E+10	85
1	-1	-1	30	16	2,53E+10	85
2	1	-1	50	16	4,14E+10	82
2	1	-1	50	16	4,22E+10	82
3	-1	1	30	44	3,59E+10	85
3	-1	1	30	44	4,08E+10	85
4	1	1	50	44	6,07E+10	80
4	1	1	50	44	5,50E+10	80
5	-1,41	0	26	30	3,20E+10	85
5	-1,41	0	26	30	3,43E+10	85
6	1,41	0	54	30	3,11E+10	80
6	1,41	0	54	30	2,62E+10	80
7	0	1,41	40	50	2,30E+10	85
7	0	1,41	40	50	2,24E+10	83
8	0	-1,41	40	10	3,16E+10	85
8	0	-1,41	40	10	2,92E+10	85
9	0	0	40	30	4,47E+10	85
9	0	0	40	30	4,38E+10	85
10	0	0	40	30	4,37E+10	85
10	0	0	40	30	4,33E+10	85
11	0	0	40	30	4,98E+10	85
11	0	0	40	30	4,76E+10	85
12	0	0	40	30	4,41E+10	85
12	0	0	40	30	4,66E+10	85
13	0	0	40	30	4,42E+10	85
13	0	0	40	30	4,58E+10	85

T, temperature ; t, time.

The extraction time affects the mass transfer of the analytes onto the fiber, thus, optimum time is required for the fiber to reach its equilibrium. The temperature directly affects how fast this equilibrium is reached, favoring the diffusion, and exerts

an influence on the composition of the volatile phase. Higher temperatures increase the rate of compounds with low vapor pressure (semi-volatiles) adsorbed on the SPME fiber.

The responses, total sum peak areas and total number of extracted compounds, were chosen to avoid the discrimination of low molar mass volatile compounds. An increase of the extraction temperature, although related to an increase in the rate of analyte transfer towards the fiber (Setkova, Risticevic, Pawliszyn, 2007) and in the proportion of semi-volatile compounds extracted, resulting in increase of total peak areas, is directly associated with the effect of decreasing distribution constant, which is more critical for lighter compounds (Risticevic, Carasek and Pawliszyn, 2008). As a consequence, a high total sum peak areas can hide the loss of some volatile compounds. The use of the number of extracted compounds in combination with the total sum peak areas allowed overcoming this drawback.

The values obtained for the total sum peak areas and the total number of extracted compounds responses were used to obtain the models for each response. Linear and quadratic models were calculated for both responses and applied in the optimization process. Analysis of variance (ANOVA), at 95% of confidence level, was performed to obtain the regression significance, the statistical significance of each effect and to verify the occurrence of lack of fit. These results, only with the significant coefficients, are given on the Table 3.2.

Table 3.2. ANOVA summary, significant model coefficients and their standard errors, considering the statistical significance of the regression and the lack of fit[a]

Responses	Indicated model	Significant coefficients[b] ± standard error						Regression significance ($p < 0.05$)	Model fit ($p > 0.05$)
		Intercept	T	t	T^2	t^2	Tt		
Total sum peak areas	Quadratic	4,54E+10 ± 2,76E+09	-	-	-	-6,4E+09 ± 2,34E+09	-	0.049	**< 0.0001**
Number of compounds	Quadratic	85 ± 0,13	-1,88 ± 0,10	-0,43 ± 0,10	-1,37 ± 0,11	-0,37 ± 0,11	-0,50 ± 0,14	< 0.0001	**0.0327**

T, temperature; t, time.

[a] Bold values in regression significance means this model does not present significant regression. Bold values in model fit means this model presents lack of fit.

[b] The significance of each coefficient was calculated using the residual mean square values.

The models obtained for the two responses presented statistical significance of the regressions, but also presented significant lack of fit (ANOVA, 95%).

For the regression significance, in the model for the total sum peak areas response, the ratio between the mean square of regression and the mean square of residual (MS_R/MS_r), or $F_{calculated}$, was 2.73, that compared with the value for $F_{tabulated(5,20,95\%)}$, 2.71, showed that $F_{calculated} > F_{tabulated}$, indicating the significance of the model regression. The model for number of extracted compounds presented a $F_{calculated}$ value of 107.27, then $F_{calculated} > F_{tabulated}$ by about forty times, indicating also the existence of an adequate significance of the model regression.

In relation to the lack of fit, $F_{calculated}$ is given by the ratio between the mean square lack of fit and the mean square pure error (MS_{lof}/MS_{pe}), and for a good fit, the model should present $F_{calculated} < F_{tabulated}$. For both the models generated in this optimization procedure, the values for $F_{calculated}$, equivalent to 95.48 for total sum peak areas and 3.68 for number of extracted compounds, were greater than the $F_{tabulated(3,17,95\%)}$ value (3.2), indicating a significant lack of fit. These results make it inappropriate to apply these models to predict the optimum condition of extraction time and extraction temperature. The $F_{calculated}$ for the total sum peak area model was almost thirty times higher than the $F_{tabulated}$, so, the prediction using this model can be very erroneous. In the contrary, the $F_{calculated}$ for the number of extracted compounds was just slightly higher than the $F_{tabulated}$, which can lead to a lower error if this model is used to predict an optimal condition.

A significant lack of fit was also observed by Bogusz and co-workers (2011) for their total sum of peak areas model. As a result, the authors defined the optimal

conditions by choosing those who gave the greatest values for the total sum of peak area, based solely on the experimental results. Subsequently, the authors successfully applied these optimum conditions of extraction time and extraction temperature to the analysis of the volatile fraction from pepper fruits. However, in our work, the greatest experimental values for total sum peak areas and total number of extracted compounds do not converged to the same conditions of extraction time and extraction temperature. An example is the experiment number 4 (Table 3.1), who presented the highest total sum peak areas, but one of the lowest number of extracted compounds. Consequently, it was decided to use the desirability function approach to achieve the optimal conditions for analysis of the volatile compounds from *Capsicum chinense* fruits.

3.3.1.1. Desirability function

In spite of both models have presented significant lack of fit, an attempt to combine the two models and search for a possible optimal condition was investigated. By applying the Derringer's desirability function approach, the two models were combined to maximize the response values (in order to obtain the highest total sum peak areas and the maximum number of extracted compounds), and then avoid the discrimination among the volatile compounds.

Initially, lower and upper limits for each response were specified, considering the lower and upper experimental results obtained in the CCD experiments. Since the model for the number of extracted compounds presented a $F_{calculated}$ value slightly higher than the $F_{tabulated}$, and also because it is the most important response

concerning the characterization of the volatile fraction of a sample, a high level of importance was set for this model. Based on these parameters, three possible combinations of the two variables, with maximum value for the two responses, were predicted. All the three combinations of variables predicted by the use of Derringer's desirability function were slightly variations of the values for extraction time and extraction temperature corresponding to the central points of the CCD. Data regarding the Derringer's desirability function simultaneous optimization is presented in Table 3.3, only for the chosen condition. In fact, inspecting the experimental results in Table 3.1, it is clear that the central point conditions indeed represent the best compromise between the total sum peak areas and number of extracted compounds. As the central point experiments were executed ten times in the CCD, it was not necessary to perform any additional confirmatory experiments. In addition, the response values predicted are included in the interval of experimentally observed values for each response. The selected solution achieved with Derriger’s desirability function showed a global desirability of 0.92, which represents a good fit with the parameters defined for the optimization.

Table 3.3. Desirability criteria, predicted optimal variables and responses, and experimentally observed responses for the predicted variables

Variables and responses	Desirability criteria for variables and responses				Predicted variables (coded)	Predicted responses	Observed responses (Mean ± sd, n = 10)[a]
	Goal	Lower Limit	Upper Limit	Level of Importance			
Temperature	is in range	-1.41	1.41	3	-0.02	-	-
Time	is in range	-1.41	1.41	3	0.1	-	-
Total sum peak areas	maximize	2.24E+10	6.07E+10	1	-	4.54E+10	4.54E+010 ± 2.10E+09
Number of extracted compounds	maximize	80	85	5	-	85	85 ± 0

[a]sd = standard deviation

Based on these results, the optimal conditions for the extraction time and extraction temperature were defined as the same of those applied in the central point of the experimental design, corresponding to 30 minutes at 40 °C, respectively.

The extraction time is a parameter that strongly influences the trapping of the volatile compounds on the SPME fiber. The extraction time optimal set in 30 minutes was efficient to reach the equilibrium for all the volatile compounds from *Capsicum chinense* pepper evaluated in this study. In a long extraction time, the less-volatile compounds possibly displace those compounds with higher volatility previously adsorbed. Moreover, the reduction of total sum peak areas with the increase in the extraction time can be explained if all active sites have been occupied, which occasionally leads to compound desorption (Zhang and Pawliszyn 1993).

The equilibrium constants involved in HS-SPME technique depends also on the extraction temperature, that here was set in 40 °C. Souza et al. (2006) and Cuevas-Glory et al. (2014) observed that higher extraction temperatures (above 64 °C and 53 °C, respectively) applied to the volatile compounds from peppers motivate the desorption of analytes from the fiber due to a competing mechanism of thermal desorption of the volatile compounds. In a study to elucidate the aroma composition of coffee, which is also a complex matrix comprising volatile compounds of interest with large differences on their volatility, it was observed that the optimal extraction temperature for the suitable extraction of more volatile analytes should also be a maximum of 40 °C (Risticevic, Carasek and Pawliszyn, 2008).

3.3.2. HS-SPME–GC–MS optimized method applied to the analysis of volatile fraction from Brazilian Capsicum chinense fruits

The headspace volatile compounds from Brazilian *Capsicum chinense* fruits (‘Habanero’ type) were evaluated using SPME after the optimization step. By using the optimized HS-SPME technique, it was possible to identify 82 volatile compounds from ‘Habanero’ peppers, while other three volatile compounds could not be identified, due to the low purity of the spectra data generated. All the compounds are listed in Table 3.4, together with their semi-quantitative amounts. A typical chromatogram of the volatile fraction from Brazilian ‘Habanero’ pepper is shown in Fig. 3.1.

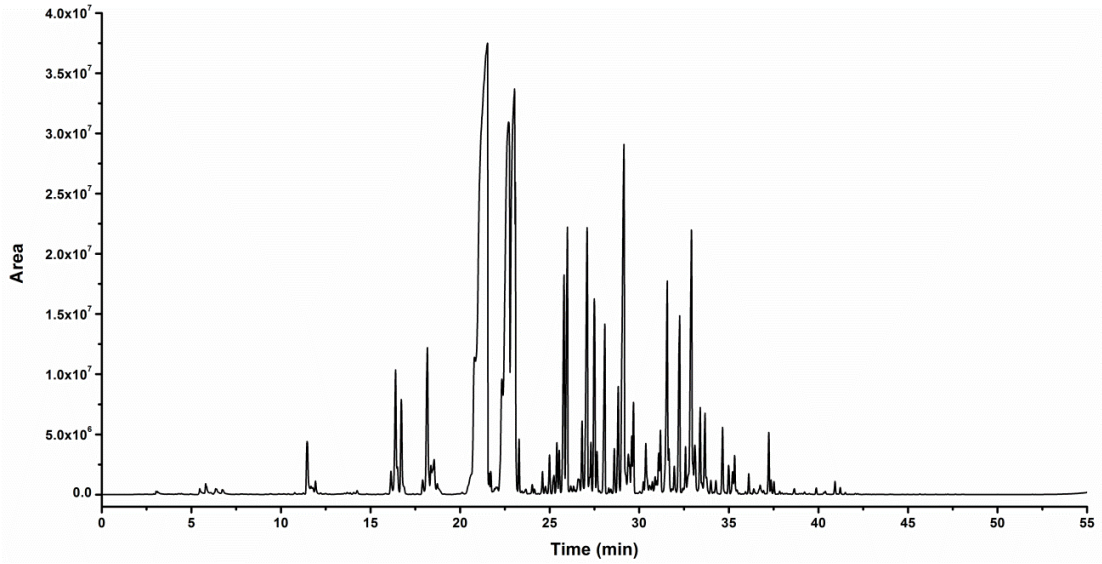

Figure 3.1. Typical chromatogram of Brazilian 'Habanero' pepper volatiles obtained in HP-5MS column according to the conditions summarized in the item 3.2.3.

The yield of total volatile compounds responsible for the aroma in *C. chinense* 'Habanero' peppers, estimated by the addition of a measured quantity of internal standard to the pepper sample, was equivalent to 255.36 $mg.kg^{-1}$. This content is in accordance with the range (110.71 - 302.53 $mg.kg^{-1}$) reported for *C. chinense* 'Cachucha' peppers (Pino, Fuentes and Barrios, 2011) and slightly higher than those reported in commercial Habanero pepper (Pino, Sauri and Marbot, 2006).

The number of volatile compounds reported in this study was higher than those previously reported for Brazilian *Capsicum chinense* peppers. For red, yellow, and purple *C. chinense* peppers, 28, 31, and 23 volatiles were found, respectively, by analysis performed with PDMS 100 µm fiber (Souza et al. 2006). In 'Murupi' pepper (*C. chinense*), with the use of PDMS/Car/DVB 50/30 µm fiber, 77 volatile compounds were identified (Bogusz et al., 2012). In addition, the number of volatile

compounds here identified for Brazilian ‘Habanero’ peppers is higher than 53 volatile compounds reported by Cuevas-Glory et al. (2014) in ‘Habanero’ green and orange peppers from Yucatan, Mexico, using PDMS/Car/DVB 50/30 µm fiber following an optimization study. In opposition, for *C. chinense* ‘Farolillo’ red pepper from Ecuador using PDMS/Car/DVB 50/30 µm fiber, Rodriguez-Burruezo et al. (2010) reported a large number of compounds, corresponding to 155 volatiles. Pino, Fuentes and Barrios (2011) identified 136 compounds in Cachucha mature peppers (*Capsicum chinense* Jacq.) by steam-distillation-continuous-extraction isolation technique.

HS-SPME has also been the technique more largely used for the volatile analysis of other varieties of *Capsicum* such as *C. annuum* ‘Jalapeño’ (76 volatile compounds), *C. annuum* ‘Serrano’ (86 volatiles), *C. frutescens* ‘Tabasco’ (124 volatiles), *C. frutescens* ‘Malagueta’ (83 volatiles), *C. baccatum* var. pendulum ‘Dedo-de-moça’ (49 volatiles) and *C. pubescens* (63 volatiles) (Rodriguez-Burruezo et al. 2010; Bogusz et al. 2011; Kollmannsberger et al. 2011).

Table 3.4. Volatile compounds (mg.kg^{-1}) in Brazilian *C. chinense* 'Habanero' pepper by HS-SPME and GC-MS

Compounds[a]	Rt	LRI_{exp}[b]	LRI_{ref}[c]	Δ	TI[d]	Content (mg.kg^{-1})[e]	RSD (%)
Alcohols							
Isohexanol	5.797	828	851	23	C	0.51	5.31
3-Hexenol	6.356	848	848	0	B	0.2	4.34
Trans-3-hexenol	6.407	850	853	-3	B	0.16	9.56
1-Hexanol	6.727	861	860	1	A	0.29	10.2
2,3-Dimethylcyclohexanol	28.829	1383	1390	7	B	2.42	6.14
3,3-Dimethylcyclohexanol	29.150	1391	1392	1	B	12.27	5.67
2-Ethylcyclohexanol	30.590	1425	1390	35	C	0.25	9.63
2,7-Octadien-1-ol	30.235	1417	1260	157	C	0.24	9.54
cis-5-Octen-1-ol	31.086	1438	1608	-170	C	0.85	2.49
Hidrocarbons							
2-Methyldodecane	23.671	1266	1265	1	A	0.19	4.45
Tridecane	25.233	1301	1313	-12	A	0.53	3.35
2-Methyltridecane	28.076	1366	1364	2	A	4.14	9.75
3-Methyltridecane	28.302	1371	1372	-1	B	0.12	9.26
Tetradecane	29.581	1400	1400	0	A	1.09	1.46
2-Methyl-1-tetradecene	31.568	1450	1445	5	B	5.69	1.64
2-Methyltetradecane	32.248	1466	1465	1	A	4.24	2.13
Pentadecane	33.670	1502	1500	2	A	1.86	2.56
2-Methylpentadecane	36.117	1564	1564	0	B	0.36	7.47
3-Methylpentadecane	36.397	1571	1568	3	B	0.12	2.26
Hexadecane	37.523	1599	1600	-1	A	0.22	1.47
2-Methyl-E-7-hexadecene	39.226	1646	1655	-9	B	0.07	1.55
2-Methylhexadecane	39.882	1664	1664	0	A	0.11	1.73
Heptadecane	41.224	1695	1700	-5	A	0.12	4.39
Esters							
Isobutyl isovalerate	11.926	1007	989	18	B	0.49	11.32
Isovaleric acid, 3-methylbutyl-2 ester	14.252	1059	1033	26	C	0.20	5.23
3-Methylbutyl 2-methylbutanoate	16.144	1100	1091	9	B	0.71	13.96
Iso-Amil isovalerate	16.399	1106	1094	12	B	4.63	1.92
Hexyl isobutyrate	16.724	1113	1118	-5	B	3.04	1.38
Pentyl 2-methylbutyrate	17.914	1139	1126	13	B	0.43	1.12
Pentyl 3-methylbutyrate	18.171	1145	1121	24	C	5.2	5.41
Hexyl butanoate	18.382	1150	1176	-26	A	0.84	2.16
3-Methyl-3-butenyl isovalerate	18.547	1153	1122	31	C	1.44	8.06
Hexyl 2-methylbutyrate	20.814	1203	1218	-15	B	7.83	11.19
Hexyl isovalerate	21.532	1219	1218	1	B	68.33	3.49

Pentyl 4-methylpentanoate	21.632	1221	1218	3	B	0.35	13.45
Heptyl isobutyrate	21.718	1223	1218	5	B	0.45	12.43
5-Hexenyl pentanoate	22.074	1231	1231	0	B	0.18	9.22
cis-3-Hexenyl valerate	22.336	1237	1243	-6	B	4.83	7.52
cis-3-Hexenyl isovalerate	22.711	1245	1231	14	A	30.2	1.80
Hexyl 3-methylbutanoate	23.023	1252	1237	15	A	29.72	2.14
Hexyl pentanoate	23.292	1258	1248	10	A	1.01	5.01
Methyl 4-decenoate	24.031	1274	1290	-16	B	0.2	8.28
Hexyl 3-methyl-2-butenoate	24.601	1287	1271	16	B	0.45	4.25
Methyl 8-methyl-nonanoate	24.768	1291	1218	73	C	0.2	2.76
Octyl (E)-2-methylbut-2-enoate	24.994	1296	1348	-52	C	0.86	1.82
Heptyl pentanoate	25.406	1305	1372	-67	A	1.06	0.45
Pentyl pivalate	25.537	1308	1228	80	C	0.89	2.23
Heptyl pivalate	25.800	1314	1297	17	B	5.83	2.96
Hexyl hexanoate	25.983	1318	1353	-35	A	6.69	1.72
Hexyl 3-methyl-2-butenoate	26.184	1323	1271	52	A	0.23	6.84
(Z)-Hex-3-enyl (E)-2-methylbut-2-enoate	26.342	1326	1325	1	B	0.23	3.77
Heptyl 2-methylbutanoate	26.812	1337	1332	5	B	1.57	2.69
Heptyl isopentanoate	27.089	1343	1338	5	B	7.49	3.19
cis-3-Hexenyl Hexanoate	27.307	1348	1365	-17	B	1.23	4.51
2-Methylcyclohexyl pentanoate, (E)-	27.645	1356	1407	-51	C	0.97	5.69
Benzyl isovalerate	29.395	1396	1394	2	B	0.96	6.9
Octyl 2-methylbutanoate	29.459	1398	1417	-19	B	0.47	5.7
Octyl isovalerate	29.679	1403	1417	-14	B	1.65	6.64
Octyl pentanoate	31.187	1440	1418	22	C	1.25	8.97
Hexyl benzoate	36.755	1580	1558	22	C	0.30	9.77
Butyl 9-decenoate	37.232	1592	1570	22	C	1.1	6.29
Isoamyl decanoate	37.838	1608	1615	-7	B	0.07	4.92
Hexyl decanoate	41.506	1703	1763	-60	C	0.05	5.38
Norcarotenoids							
β-Cyclocitral	22.007	1229	1204	25	C	0.27	3.10
α-Ionone	30.737	1429	1429	0	A	0.28	7.92
Trans-β-Ionone	33.111	1488	1489	-1	A	1.38	9.81
Terpenes							
α-Cubebene	27.498	1353	1344	9	A	4.79	4.67
Ylangene	28.418	1374	1392	-18	B	0.12	5.62
α-Copaene	28.614	1378	1402	-24	C	0.95	5.99
β-Cubebene	29.198	1392	1384	8	B	0.55	5.4
Cedrene	30.886	1433	1415	18	B	0.56	3.52
α-Himachalene	31.653	1452	1447	5	B	1.02	3.38
β-Farnesene (E)	31.962	1459	1448	11	B	0.69	2.55
Caryophyllene	32.437	1471	1453	18	A	0.17	11.30

β-Cadinene	32.768	1479	1491	-12	B	0.46	1.18
Longifolene	32.913	1483	1436	47	C	7.89	8.59
γ-Cadinene	34.278	1517	1515	2	B	0.33	2.39
δ-Cadinene	34.651	1527	1514	13	B	1.4	10.45
Naphthalene, 1,2,3,4,6,8a-hexahydro-1-isopropyl-4,7-dimethyl-	34.992	1535	1515	20	B	0.57	9.80
α-Muurolene	35.215	1541	1525	16	B	0.48	13.7
Others							
Phoracantholide	24.156	1277	1308	-31	C	0.11	1.42
2,6,10-trimethyl-9-undecenal	33.402	1495	1458	37	C	1.93	11.64
Not identified	30.373	1420				1.34	8.84
Not identified	38.656	1630				0.13	2.99
Not identified	40.929	1693				0.23	2.41

[a]Tentative identification. [b]Linear retention index on HP-5MS obtained experimentally. [c]Linear retention index from literature, Δ = differences between the experimental linear retention indexes and those from the references. [d]TI: tentative identification, the reliability of the identification proposal is indicated by the following: A, mass spectrum and retention index agreed with standards; B, mass spectrum and retention index agreed with database or literature; C, mass spectrum agreed with mass spectral database (considering a minimum similarity value of 80%). [e]The results are presented as the mean of triplicate analysis.

The most abundant chemical classes of volatile compounds detected in *C. chinense* 'Habanero' pepper were as follows: esters, hydrocarbons, alcohols, terpenes and, at last, norcarotenoids. The abundance of esters in *C. chinense* has been reported by different authors (Souza et al. 2006; Rodriguez-Burruezo et al. 2010; Pino et al. 2011; Bogusz et al., 2012; Cuevas-Glory et al., 2014). In total, thirty-nine esters were identified, representing around 45% of the total composition in Braziliam *C. chinense* 'Habanero" pepper. The most abundant esters found were hexyl isovalerate, cis-hexenyl isovalerate and hexyl 3-methyl butanoate. Cuevas-Glory et al. (2014) also describe hexyl isovalerate as the major compound in the volatile composition of 'Habanero' pepper from Yucatan, and this compound was considered a volatile with a powerful fruity odor notes (Forero et al. 2009).

Hydrocarbons were the second important class in terms of total amount, and 14 hydrocarbons were identified, highlighting 2-methyltridecane, 2-methyl-1-tetradecene and 2-methyltetradecane. Alcohols were another class of compounds with large contribution to the total aroma compounds and 3,3-dimethylcyclohexanol was the most representative in this class, totalizing more than 70% of the alcohols, in agreement with data reported for Mexican 'Habanero' pepper in green and orange stages by Cuevas-Glory et al. (2014). In this study, the isomer of 3,3-dimethylcyclohexanol, the 2,3-dimethylcyclohexanol, was reported for the first time in 'Habanero' pepper type and was previously found in *C. chinense* 'Cachucha' peppers (Pino, Fuentes and Barrios, 2011).

Terpenoids comprise mainly mono and sesquiterpene hydrocarbons. In this class, longifolene and α-cubebene were the major compounds. Longifolene was reported in 'Habanero' (*C. chinense*) fruits for the first time in our study, despite the large difference between LRI_{ref} and LRI_{exp} (Δ value) presented for this compound, the mass spectra data is in agreement with those reported by the library, achieving similarity value of 95%. Another reference about this compound in other type of *Capsicum chinense* fruits can be found in the study with the different *Capsicum* fruits (Bogusz et al., 2014). Oxygenated terpenes were not detected in our study with the Brazilian *C. chinense* 'Habanero' peppers. Trans-β-ionone was the main norcarotenoid detected. According to Rodríguez-Burruezo et al. (2010), this compound is especially important for the aroma intensity and quality in *Capsicum* peppers.

3.4. Conclusions

The strategy of multivariate experimental design made possible, with a reduced number of experiments, the identification of the optimal extraction temperature and time conditions for application of HS-SPME sampling method in the analysis of the volatile fraction from *C. chinense* 'Habanero' peppers. HS-SPME optimal conditions for 'Habanero' pepper by using PDMS/Car/DVB fiber were extraction time of 30 min and extraction temperature of 40 °C. Eighty-two compounds were tentatively identified in the volatile fraction. The most abundant volatile compounds in 'Habanero' pepper were hexyl isovalerate, cis-hexenyl isovalerate, hexyl 3-methylbutanoate, 3,3-dimethylcyclohexanol, longifolene, and 2-methyl-1-tetradecene. 2,3-Dimethylcyclohexanol and longifolene were reported for the first time in 'Habanero' pepper (*C. chinense*).

3.5. Acknowledgments

This work was supported by grants from CAPES and FAPESP (Project number 2011/17094-2). The authors also acknowledge the Agronomic Institute of Campinas for supplying the samples. The author M. Sganzerla would like to thank CAPES for the exchange fellowship (Institutional program PDSE, BEX process number 1262/12-2) and the author C. A. Ballus would like to thank FAPESP by the post-doctoral fellowship (process no. 13/25242-7).

3.6. References

Arthur, C.; Pawliszyn, J. (1990). Solid phase microextraction with thermal desorption using fused silica optical fibers. **Analytical Chemistry**, 62, 2145 – 2148.

Ballus, C. A.; Meinhart, A. D.; Campos Jr., F. A. S.; Bruns, R. E.; Godoy, H. T. (2014). Doehlert design-desirability function multi-criteria optimal separation of 17 phenolic compounds from extra-virgin olive oil by capillary zone electrophoresis. **Food Chemistry**, 146, 558 – 568.

Bianchi,F.; Careri, M.; Conti, C.; Musci, M.; Vreuls, R. (2007). Comparison of comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry and gas chromatography-mass spectrometry for the qualitative characterisation of roasted barley by solid-phase microextraction. **Journal of Separation Science**, 30(4), 527 – 533.

Bicchi, C.; Drigo, S.; Rubiolo, P. (2000) Influence of fibre coating in headspace solid-phase microextraction–gas chromatographic analysis of aromatic and medicinal plants. **Journal of ChromatographyA**, 892, 469 – 485.

Bogusz Junior, S.; Tavares de Melo, A; Zini, C. A.; Godoy, H. T. (2011). Optimization of the extraction conditions of the volatile compounds from chili pepper by headspace solid phase micro-extraction. **Journal of ChromatographyA.** 1218, 3345 – 3350.

Bogusz Junior, S.; Tavares, A. M.; Zini, C. A.; Godoy, H. T.; Teixeira Filho, J. (2012). Analysis of the volatile compounds of Brazilian chilli peppers (*Capsicum* spp.) at two stages of maturity by solid phase micro-extraction and

gas chromatography-mass spectrometry. **Food Research International**, v. 48, p. 98 – 107.

Bogusz Junior, Stanislau; Março, Paulo Henrique; Valderrama, Patrícia; Damasceno, Flaviana Cardoso; Aranda, Maria Silvana; Zini, Cláudia Alcaraz; Caramão, Elina Bastos; Melo, Arlete Marchi Tavares; Teixeira Filho, José; Godoy, Helena Teixeira. (2014). Analysis of volatile compounds in *Capsicum* spp. by headspace solid-phase microextraction and GCXGC-TOFMS. **Analytical Methods**, 1 – 9.

Box, G. E. P.; Hunter, S.; Hunter, W. G. (1978). Statistics for experiments. 1 st edition, John Wiley & Sons, Inc. 653 p.

Chitwood, R. L.; Pangborn, R. M.; Jenning, W. (1983). GC/MS and sensory analysis of volatiles from three cultivars of *Capsicum*. **Food Chemistry**, 11, 201 – 216.

Cuevas-Glory, L. F.; Sosa-Moguel, O.; Pino, J.; Sauri-Duch, E. (2014). GC-MS Characterization of volatile compounds in Habanero pepper (*Capsicum chinense* Jacq.) by optimization of headspace solid-phase microextraction conditions. **Food Analytical Methods**, 1 – 9.

Ferreira, S. L. C.; Bruns, R. E.; da Silva, E. G. P.; dos Santos, W. N. L.; Quintella, C. M.; David, J. M.; de Andrade, J. B.; Breitkreitz, M. C.; Jardim, I. C. S. F.; Barros Neto, B. B. (2007). Statistical designs and response surface techniques for the optimization of chromatographic systems. **Journal of Chromatography A**, 1158(1), 2 – 14.

Forero, D.; Quijano, C. E.; Pino, J. A. (2009). Volatile compounds of chile pepper (*Capsicum annuum* L. var. *glabriusculum*) at two ripening stages. **Journal of Flavor Fragrance**, 24: 25 – 30.

Garcéz-Claver, A.; Arnedo-André, M. S.; Avier Abadía, R.; Gil-Ortega, S. J.; Álvarez-Fernández, A. (2006). Determination of capsaicin and dihydrocapsaicin in *Capsicum* fruits by liquid chromatography-electrospray/time-of-flight mass spectrometry. **Journal of Agricutural and Food Chemistry**, 54(25), 9303 – 9311.

Guadayol, J. M.; Caixach, J.; Ribé, J.; Cabañas, J.; Rivera, J. (1997). Extraction, separation and identification of volatile organic compounds from paprika oleoresin (Spanish type). **Journal of Agricultural Food Chemistry**, 45, 1868 – 1872.

Holt, R.U., (2001). Mechanisms effecting analysis of volatile flavour components by solid phase microextraction and gas chromatography. **Journal of Chromatography A**, 937, 107 – 114.

Kataoka, H.; Lord, H.; Pawliszyn, J. (2000). Application of solid phase microextraction in food. **Journal of Chromatography A** 880 (1-2), 35 – 62.

Kollmannsberger, Hubert; Rodrıguez-Burruezo, Adrián; Nitz, Siegfried; Nuez, Fernando. (2011). Volatile and capsaicinoid composition of ají (*Capsicum baccatum*) and rocoto (*Capsicum pubescens*), two Andean species of chile peppers. **Journal of the Science of Food and Agriculture**, 91, 1598 – 1611.

Korany, K.; Kocsis, N.; Amtmann, M.; Mednyanszky, Z. (2002). GC-MS investigation of aroma compounds of Hungarian red paprika (*Capsicum annuum*) cultivars. **Journal of Food Composition and Analysis**, 15, 195 – 203.

Lambropoulou D. A.; Konstantinou I. K.; Albanis T. A. (2007). Recent developments in headspace microextraction techniques for the analysis of environmental contaminants in different matrices. **Journal of ChromatographyA**, 1152, 70 – 96.

Lord, Heather; Pawliszyn, Janusz. (2000). Evolution of solid-phase microextraction technology. **Journal of Chromatography A**, 885(1), 153 – 193.

Luning, P. A.; Rijk, T. D.; Harry, J.; Wichers, H. J.; Roozen, J. P. (1994). Gas chromatography, mass spectrometry, and sniffing port analyses of volatile compounds of fresh bell peppers (*Capsicum annuum*) at different ripening stages. **Journal of Agricultural and Food Chemistry**, 42, 977 – 983.

Mazida M. M.; Salleh M. M.; Osman H. (2005). Analysis of volatile aroma compounds of fresh chilli (*Capsicum annuum*) during stages of maturity using solid phase microextraction (SPME). **Journal of Food Composition and Analysis** 18, 427 – 437.

Medina, I.; Satue-Gracia, M. T.; Frankel, E. N. (1999). Static Headspace gas chromatographic analyses to determine oxidation of fish muscle lipids during thermal processing. **Journal of American Oil Chemistry Society**, 76 (2), 231 – 236.

Mondello, Luigi; Casilli, Alessandro; Tranchida, Peter Quinto; Dugo, Paola; Costa, Rosaria; Festa, Saverio; Dugo, Giovanni. (2004) Comprehensive

multidimensional GC for the characterization of roasted coffee beans. **Journal of Separation Science**, 27, 442 – 450.

Nongonierma, A.; Voilley, A.; Cayot, P.; Quéré, J. L.; Springett, M. (2006). Mechanisms of extraction of aroma compounds from foods, using adsorbents. Effect of various parameters. **Food Reviews International**, 22(1), 51 – 94.

Oliveira, A. M.; Pereira, N. R.; Marsaioli, A., Jr.; Augusto, F. (2004). Studies on the aroma of cupuaçu liquor by headspace solid-phase microextraction and gas chromatography. **Journal of CrhomatographyA**, 1025, 115 – 124.

Pawliszyn, J. Handbook of Solid Phase Microextraction. **Chemical Industry Press**, Beijing, China, 2009.

Pino, J.; Sauri-Duch, E; Mabot, R. (2006). Change in volatile compounds of Habanero chile pepper (Capsicum Chinense Jack. Cv. Habanero) at two ripening stages. **Journal of Food Chemistry**, 94: 394 – 396.

Pino, Jorge; Fuentes, Víctor; Barrios, Odalys. (2011). Volatile constituents of Cachucha peppers (*Capsicum chinense* Jacq.) grown in Cuba. **Food Chemistr**y, 125, 860 – 864.

Pino, Jorge; González, Marilú; Ceballos, Liena; Centurión-Yah, Alma Rosa;, Trujillo-Aguirre, Jorge; Latournerie-Moreno, Luis; Sauri-Duch, Enrique. (2007). Characterization of total capsaicinoids, colour and volatile compounds of Habanero chilli pepper (*Capsicum chinense* Jack.) cultivars grown in Yucatan. **Food Chemistry**, 104, 1682 – 1686.

Ribeiro, J. S.; Teófilo, R. F.; Augusto, F; Ferreira, M. M. C. (2010). Simultaneous optimization of the microextraction of coffee volatiles using response surface

methodology and principal component analysis. **Chemometrics and Intelligent Laboratory Systems**, 102, 45 – 52.

Risticévic, Sanja; Carasek, Eduardo; Pawliszyn, Janusz. (2008). Headspace solid-phase microextraction–gas chromatographic–time-of-flight mass-spectrometric methodology for geographical origin verification of coffee. **Analytica Chimica Acta**, 617, 72 – 84.

Rodríguez-Burruezo, A.; Kollmannsberger, H.; González-Mas, M. C.; Nitz, S.; Nuez, F. (2010). HS-SPME comparative analysis of genotypic diversity in volatile fraction and aroma contributing compounds of *Capsicum* fruits from the annuum-chinense-frutescens complex. **Journal of Agricultural and Food Chemistry**, 58, 4388 – 4400.

Saskia van Ruth, Elena Boscaini; Mayrb, Dagmar; Pugh, Jonathan; Posthumus, Maarten. (2003). Evaluation of three gas chromatography and two direct mass spectrometry techniques for aroma analysis of dried red bell peppers. **International Journal of Mass Spectrometry**, 223 – 224, 55 – 65.

Setkova, L.; Risticevic, S.; Pawliszyn, J. (2007). Rapid headspace solid-phase microextraction-gas chromatographic-time-of-flight mass spectrometric method for qualitative profiling of ice wine volatile fraction. I. Method development and optimization. **Journal of Chromatography A**, 1147(2), 213 – 223.

Souza, E. T.; Rodrigues, F. M.; Martins, C. C.; Oliveira, F. S.; Pereira, P. A. P.; Andrade, J. B. (2006). Multivariate optimization and HS-SPME/GC-MS

analysis of VOCs in red, yellow and purple varieties of *Capsicum chinense* sp. peppers. **Microchemical Journal**, 82, 142 – 149.

Taylor, A. J.; Besnard, S.; Puaud, M.; Linforth, R. S. T. (2001). In vivo measurement of flavour release from mixed phase gels. Biomol Eng , 17, 143 – 150.

Tholl, D.; Boland, W.; Hansel, A.; Loreto, F.; Rose, U.S.R.; Schnitzler, J.P. (2006). Practical approaches to plant volatile analysis. **Plant Journal**, 45, 540 – 560.

Van der Dool, H.; Kratz, P. D. (1963). A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. **Journal of Chromatography**, 11, 463 – 471.

Vichi, S.; Castellote, A. I.; Pizzale, L.; Conte L. S.; Buxaderas S.; Lopez-Tamames E. (2003). Analysis of virgin olive oil volatile compounds by headspace solid-phase microextraction coupled to gas chromatography with mass spectrometric and flame ionization detection. **Journal of ChromatographyA** 983 (1-2): 19-33.

Wardencki, Waldemar; Michulec, Magdalena; Curylo, Janusz. (2004). A review of theoretical and practical aspects of solid-phase microextraction in food analysis. **International Journal of Food Science & Technology**, 39(7), 703-717.

Yang, X.; Peppard, T. (1994). Solid-phase microextraction for flavor analysis. J. Agric. **Food Chemistry**, 42, 1925 – 1930.

Zhang, Z.; Pawliszyn, J. (1993). Headspace solid-phase microextraction. **Analytical Chemistry**, 65(14), 1843 – 1852.

CHAPTER IV

ARTICLE

Characterization of capsaicinoids and volatile profile in Brazilian Capsicum chinense 'Habanero' peppers grown under different conditions of water availability

CHAPTER IV

Characterization of capsaicinoids and volatile profile in Brazilian *Capsicum chinense* 'Habanero' peppers grown under different conditions of water availability

Marla Sganzerla[a], Cristiano Augusto Ballus[a], Carlo Bicchi[b], Arlete Marchi Tavares de Melo[c], Helena Teixeira Godoy[a]

[a] Laboratory of Food Analysis, Department of Food Science, Faculty of Food Engineering, University of Campinas (UNICAMP), P.O. Box 6121, CEP 13083-862, Campinas, SP, Brazil.

[b] Laboratory of Phytochemical Analysis, Department of Pharmaceutical Science and Technology, Faculty of Pharmacy, University of Turin (UNITO), CAP 10125, Turin, TO, Italy.

[c] Horticultural Center, Agronomic Institute of Campinas (IAC), P.O. Box 28, CEP 13012-970 Campinas, SP, Brazil.

***Manuscript prepared for submission to the "Journal of Agricultural and Food Chemistry"**.

ABSTRACT

Pungency and aroma are the most important sensory attributes for *Capsicum chinense* fruits. The 'Habanero' peppers varieties are just renowned to be highly aromatic and the hottest peppers in the world. The pungency of the peppers is due to the presence of substances called capsaicinoids. The volatile fraction of 'Habanero' peppers comprise mainly the esters and terpenes chemical classes. These secondary metabolism derivatives compounds are influenced by two factors: genetics and plant-environment interactions and are susceptible to water availability conditions. The aim of this research was to evaluate the susceptibility of the Brazilian 'Habanero' pepper to water deficit treatments and their effects on the composition of capasaicinoids and volatile fraction. The higher values of capsaicin and dihydrocapsaicin were found in the mature peppers, achieving ranges from 2.85 - 3.33 $mg.g^{-1}$ for capsaicin and 1.06 - 1.71 $mg.g^{-1}$ for dihydrocapsaicin. The mainly volatile compounds were esters, terpenes and alcohols. Total volatile compounds presented higher values of concentration in the peppers in green stage of maturity with ranges from 195.47 – 298.94 $mg.kg^{-1}$. The PCA performed allowed to classify the 'Habanero' peppers in function of the degree of maturity. In addition, PCAs apllied separately for green and mature peppers showed a clear separation in function of the harvest date. The peppers were clustered in function of the water availability treatments received, but a new study strategy, with more frequent monitoring, would be more adequate to better understand the effects on the peppers composition.

Keywords: chili pepper; water stress; pungency; aroma; HS-SPME; GC-MS; UHPLC.

4.1. Introduction

The fruits of chili peppers belonging to the specie *Capsicum chinense* (family *Solanaceae*) vary widely in size, shape, color, aroma, and pungency. The varieties of chili peppers are very important commercially, since large quantities of these fruits are consumed around the world (Luo, Peng and Li, 2011). Chilies are grown worldwide, with Asia producing the most, followed by Mexico and the United States (Chinn, Sharma-Shivappa and Cotter, 2011). The popularity of chili peppers has been rising over the years, with a large number of growers emerging also all over Brazil, especially in the Minas Gerais, São Paulo, Goiás and Rio Grande do Sul states, totalizing a production of approximately 75,000 tons/year (Ribeiro Lopes, Carvalho, Henz and Reifschneider, 2008).

Capsicum chinense fruits are very popular in several typical dishes from Bahia state, in Brazil, and their consumption represents a relevant characteristic of the local culture (Souza et al., 2006). The principal varieties recognized as the representative for this species around the world are the 'Habanero' and 'Scotch Bonnet' peppers. In addition, the 'Habanero' and 'Scotch Bonnet' peppers varieties are just renowned to be highly aromatic and the hottest peppers in the world (Kurian and Starks, 2002). These characteristics are the most important sensory attributes for this species.

Pungency is the best know quality indicator trait in peppers, and it has been studied for decades under many points of view (e.g., food chemistry, food technology, pharmaceutical, plant genetics and breeding programs) (Borruezo, Kollmannsberger, González-Mas, Nitz and Nuez, 2010). In another hand, the quantity of studies about the volatile fraction is still much lower and, among the studies found, most of them only investigate the aroma composition from 'Bell' peppers type (*Capsicum annuum*) (Pino, González, Ceballos, Centurión-Yah, Trujillo-Aguirre, Lantournerie-Moreno and Saury-Duch, 2007).

The pungency of the peppers is due to the presence of substances called capsaicinoids that are biosynthesized by condensation of fatty acids and vanillyllamine. Capsaicin (CAP) and dihydrocapsaicin (DHC) are generally the most abundant capsaicinoids in chilies and both frequently account for more than 95% of the total capsaicinoids present (Bennett & Kirby, 1968; Jarret et al., 2003). Although typically present in very low concentrations, numerous analogues of CAP have been found in several varieties of peppers (Garcéz-Claver et al., 2006; Barbero, Liazid, Palma and Barroso, 2008; Jin, Pan, Xie, Zhou and Xia, 2009). These minory compounds vary widely in their relative pungencies (Perkins et al., 2002). The concentration of these analogues in peppers is related to taxa and genotype (Zewdie and Bosland, 2001) and can be absent in some varieties of peppers as was showed for 9 accessions of *Capsicum chinense*, including fruits from a hot pepper (IAC 1642 'Habanero') and a sweet pepper (IAC 1643 'Biquinho') (Sganzerla, Coutinho, Melo and Godoy, 2014).

Chili peppers, like the 'Habanero' (*Capsicum chinense*), are a rich source of valuable capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide). Capsaicin has been extensively studied via experimental and clinical investigations, due to its pharmaceutical and antioxidant properties (Rosa et al., 2002). The capsaicinoids are synthesized exclusively in the epidermal cells of the placenta of *Capsicum* fruits and are accumulated in blisters along the epidermis (Garcés-Claver et al., 2006). According to Cisneros-Piñeda et al. (2007), in the fruit of "Habanero orange' (*Capsicum chinense*) from Yucatan, more than 85% of its composition in fresh weight corresponds to pericarp fraction, whereas about 7.84% is placenta which concenter more than 90% of the capsaicin content in the pepper fruit.

Finally, the pungency of *Capsicum chinense* peppers has been widely investigated for years to answer requirements of several research areas, whereas, the range of studies about the volatile fraction of peppers is much lower and most of them are committed to elucidate the volatile composition of 'Bell' peppers (*Capsicum annuum*) varieties. In general, there are few studies regarding to the aroma composition of *Capsicum chinense*. Consequently, the volatile fraction has been studied only for a reduced part of the diversity comprised in *Capsicum* genus.

Relative to the aroma composition of *C. chinense* fruits, the major part of the studies were performed with peppers cultivated or commercialized in Mexico, focusing in hot varieties like the 'Habanero' (Pino, Sauri, & Marbot, 2006; Cisneros-Piñeda et al., 2007; Pino et al., 2007; Cuevas-Glory et al., 2014) which are composed predominantly by esters and terpenes chemical classes.

Current trends, according to FAO - Food and Agriculture Organization of the United States (Alexandratos and Bruinsma, 2012) indicate that the world population is around 7 billion and is expected to reach 8.3 billion in 2030. It makes clear that agricultural productivity must increase in order to feed the growing world population (Howell, 2001). The increment of production must be achieved considering sustainable agriculture principles mainly regarding to water requirements, since the water is a scarce resource in many countries worldwide. Considerable scope remains for improving irrigation management practices, avoiding percolation losses and increasing water productivity at the farm level around the world. Current irrigation practices are generally based on local farmers' experience and most of them irrigate without monitoring the soil or plant parameters of water status. Thus, this creates the need for continuous improvement in irrigation practices, especially in the commercial vegetable production. Water saving irrigation methods should be followed in order to save water and maximize yield (Bouwer, 2000; Antony and Singandhupe, 2004).

The quality of irrigation water affects primarily plant development and secondly, the yield and quality of peppers (Yildirim, 2010). Several studies evaluated the effects of irrigation conditions on the productivity yield specialy for 'Bell' pepper (*Capsicum annuum*). Sezen et al. (2006) reported that the yield of 'Bell' pepper obtained by applying water between 293.6 to 540.4 mm during irrigation was 21.01 to 35.29 tons by hectare. Other researchers have reported on the accumulation of capsaicinoids in *Capsicum* fruits in relation to fruit age, size, and stage of

development (Iwai et al., 1979; Salgado-Garciglia and Ochoa-Alejo, 1990; Estrada et al., 1997). Estrada et al. (1999) reported that the amount of capsaicinoids, mainly capsaicin and dihydrocapsaicin, in 'Padrón' pepper (*C. annuum*) fruits from water-stressed plants was higher than in control plants, especially under low-water treatments. Ruiz-Lau et al., (2011) investigated the accumulation of capsaicinoids in 'Habanero' fruits grown in Yucatan (Mexico) and the water deficit stress significantly increased capsaicin and dihydrocapsaicin concentrations in pepper fruits.

These results reflect the fact that pungency levels in peppers, as well as, the whole secondary metabolism derivatives compounds, are determined by two factors: genetics and plant-environment interactions (Estrada et al., 1997; Contreras-Padilla and Yahia, 1998). Thus, the water availability conditions during the cultivation of 'Habanero' peppers directly affects the most important sensorial properties of these fruits: pungency and aroma. So far, the volatile constituents in *Capsicum* peppers obtained under water stress have not been studied.

The aim of this research was to evaluate the susceptibility of the Brazilian 'Habanero' pepper to water deficit treatments and the effect of this abiotic stress on the composition of capasaicinoids and volatile fraction.

4.2. Material and methods

4.2.1. Chemicals

The solvents methanol, acetone and acetonitrile (J. T. Baker, Phillipsburg, NJ, USA) utilized were of HPLC-grade. The water was obtained from a Milli-Q water bidistillation system (Millipore, Bedford, MA, USA). All solvents used as mobile phase in UHPLC analysis were filtered using a Millipore filters (0.22 μm pore size, filter type GV (Durapore) PVDF for water and FG (Fluoropore) PTFE for organic solvents) and degassed by ultrassonication.

The reference standards of capsaicinoids, capsaicin and dihydrocapsaicin (more than 95% of purity) were purchased from Cayman Chemical Company (Arbor, MI, USA). 3-Octanol with a purity of 99% (Sigma-Aldrich, St. Louis, MO, USA) was added as internal standard in all extraction assays for analysis of volatile compounds. Linear retention indices (LRI) of individual volatiles were calculated according to Van der Dool and Kratz method (Van der Dool and Kratz, 1963) using a n-alkane standard solution (Supelco, Bellefonte, PA, USA) ranging from heptane to triacontane (C7-C30). Other reference volatile compounds (all with purity higher than 98%) were obtained from PolyScience Corp. (Niles, WI, USA): 1-hexanol, 2-methyldodecane, 2-methyltridecane, 2-methyltetradecane, 2-methylhexadecane; from Sigma-Aldrich (St. Louis, MO, USA): cis-3-hexenyl isovalerate, hexyl 3-methyl butanoate, hexyl butanoate, hexyl pentanoate, hexyl hexanoate, 2-methoxy-3-isobutylpyrazine, hexyl 3-methyl butenoate, heptyl pentanoate, α-ionone, trans-β-

ionone, α-cubebene; and caryophyllene has come from clove essencial oil extracted in laboratory.

4.2.2. Plant material and grown conditions

The treatments under different conditions of water availability were carried out in the Horticultural Center in the IAC – Agronomic Institute of Campinas (Campinas, SP, Brazil, 22°54'S, 47°05'W, 674 meters of elevation) during the spring season of 2011. Plants belonging to the accession of *Capsicum chinense* peppers, 'Habanero' type (IAC 1648) from the collection of local germplasm bank, were employed in this study and the green and fully ripe fruits from two harvest steps (H1 at October 19; and H2 at November 21) were evaluated. The experiment was performed in a factorial 2^2 using randomized complete block design with four treatments and four replications. Each replicate included six plants in the plot. The variables of the factorial design were the cycles of watered suspension (presence and absence) during the seedling phase and levels of water refilled (45 and 90% of the field capacity) along the phase of plant in the pot.

To determine the effect of the water supply on some quality parameters like capsaicinoids and volatile composition of the 'Habanero' pepper fruits, firstly the pepper seeds were placed in polystyrene trays with 64 cells to germination. The cells were filled with commercial substrate and in each one was deposited 3 seeds and a layer of vermiculite that allow the soil to hold the water and oxygen. The trays were kept under irrigation and fertilization controlled conditions in the nursery. The

seedlings were fed with a fertilizer solution containing 48% N, 26% Ca, and 45% K and water in the dosage of 1.5 $g.L^{-1}$. For each plant in the experiment, the same substrate was used and the same amount of fertilizer was given.

In the stage of four leaves development, the irrigation and fertilization were suspended and the cycles of presence and absence of water deficit stress treatments began. In the treatment without water deficit stress, the seedlings were manually watered daily whereas in the treatment with presence of hydric stress, the seedlings were watered after measurement of water potential in leaves and three cycles of watered suspension were applied. Water potential of leaves was determined in the morning using a pressure chamber 'Scholander' type (PMS Instrumental, Model 1000, Albany, OR, USA).

When the seedlings presented four pairs of leaves, they were transplanted to 12 L polyethylene pots filled with soil and peat moss (2:1, v/v) and the pots were placed in a polyethylene covered greenhouse. Manually irrigations corresponding to 45 and 90% of field capacity were applied at 2-day intervals up to the second harvest. Finally, seedlings without cycles of watered suspension and, sequentially, plants with 90% of the field capacity of water refilled were considered the control treatment (samples codified P11). Otherwise, the pepper seedlings with cycles of suspension of the irrigation that have become plants with 45% of the field capacity of water refilled were considered the complete water deficit stress treatments (samples codified P22) as can be seen in the following scheme (Figure 4.1).

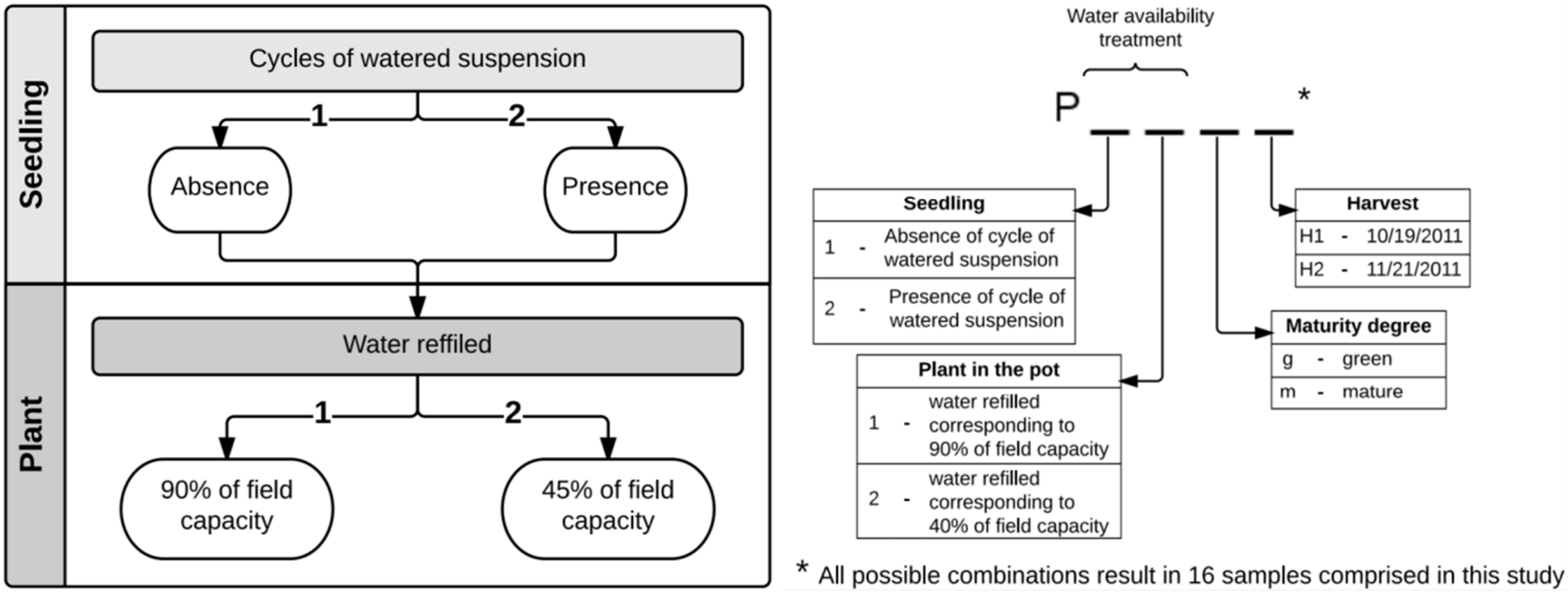

Figure 4.1. Scheme of availability water treatments in the peppers growth and code composition of the code for the sample name obtained.

4.2.3. Biometric and moisture content analysis

The peppers were characterized using biometrical parameters and moisture content of the fruits. For each availability water treatment, the length (mm), width (mm) and weight (g) of the pepper fruits produced (n = 12) were measured. The moisture content was determined in triplicate at 105 °C according to the method 934.01 published by the Association of the Official Analytical Chemists (AOAC, 2000).

4.2.4. Capsaicinoids analysis

4.2.4.1. Extraction procedure

The samples from each water availability treatment, in green and mature stages obtained in two harvests dates, were analyzed. Each sample was prepared by grinding the pepper fruits using a Turratec TE102 (Tecnal, Piracicaba, SP, Brazil) for 3 minutes at 20,000 rpm until a homogeneous sample was obtained. Immediately, according to Sganzerla et al. (2014), 1 g of the sample and 25 mL of methanol were submitted to ultrasound assisted extraction – UAE step, using an ultrasonic bath UC1400 (Unique, Indaiatuba, SP, Brazil) operating at frequency of 40 kHz and room temperature. Prior to the analysis was performed a step of dilution, changing the sample solvent injection composition to 70% of methanol and 30% of water. The analysis were carried out in triplicate for each sample.

4.2.4.2. Instrumental conditions

Analysis of the capsaicinoids was performed using an UPLC-PDA Acquity™ Waters system (Waters, Arvada, CO, USA). The separation of capsaicinoids was achieved with a Hypersil Gold C18 with pore size 175 Å (1.9 µm, 3 mm x 100 mm) column (Thermo Scientific, Waltham, MA, USA) and mobile phase consisting of water (A) and acetonitrile (B) (40:60, v/v) in isocratic mode at 0.5 mL min^{-1} flow rate.

Capsaicinoids in the sample were identified by comparison of the relative retention time and full absorption spectra data (200 – 500 ηm) obtained with those resulting from injection of the reference standards in the same analytical conditions. For quantification, the signal from the absorbance at 280 ηm was employed. Data handling was performed with Empower 2 software package.

4.2.4.3. Method validation

Limits of detection (LOD) and quantification (LOQ) of the UPLC-PDA method were estimated to be 3 and 6 times the signal-to-noise ratio, respectively. Intra-day instrumental precision for peak area was determined by injecting a solution containing the capsaicin and dihydrocapsaicin standards 10 consecutive times in one day. Inter-day instrumental precision was determined by injecting the same standards solution applied in the intra-day assay procedure in triplicate for three consecutive days. The linearity was individually verified for each compound, with analytical curves made from six equally spaced points in triplicate. About the extraction method, intra-day precision and inter-day precision were performed

trhough ten extrctions procedures whitin a day for the first parameter and in triplicate for three consecutive days, using the 'Habanero' pepper P11mH1 (control treatment). Recovery experiments were carried out with the standard addition in the sample matrix method using 'Habanero' pepper (P11mH2 – control treatment) in three concentration levels for capsaicin (65.88, 36.66, and 5.5 $\mu g.mL^{-1}$) and dihydrocapsaicin (60.48, 31.36, and 4.5 $\mu g.mL^{-1}$).

4.2.5. Volatile compounds analysis

4.2.5.1. Sample preparation

The whole pepper fruits were blended by a grinder Turratec TE102 (Tecnal, Piracicaba, SP, Brazil) for three minutes at 20,000 rpm until homogeneous sample was achieved. Then, 1 g was immediately placed in a headspace vial (20 mL) sealed with a stainless steel cap with PTFE/silicone septum (Supelco, Bellefonte, PA, USA) for extraction to prevent loss of the volatile compounds. The extraction procedure was performed, after 10 minutes of equilibrium time at 40 °C, using a 50/30 mm DVB/Car/PDMS (divinylbenzene/carboxen/polydimethylsiloxane) coated fiber, 1 cm (Supelco, Bellefonte, PA, USA) in combination with a manual holder (Supelco, Bellefonte, PA, USA). The optimal extraction conditions were established in a previous study (Chapter 3) and corresponding to 30 minutes of extraction time and 40 °C of extraction temperature.

Prior to the first use, the fiber was conditioned according to the manufacturer's recommendations. Before each sampling procedure, the SPME fiber was reconditioned for 10 minutes at 230 °C to avoid 'carry over' of compounds or 'memory effect' on the fiber and, consequently, interferences in the subsequent sample extraction (Holt, 2011).

4.2.5.2. Instrument conditions

Following the volatile compounds extraction procedure for all the assays, the SPME fiber containing the absorbed compounds was immediately inserted into the GC injector port and the fiber was thermally desorbed for 5 minutes at 230 °C (splitless mode).

A system of gas chromatography coupled to a mass spectrometry - GC7890A/MS5975C MSD (Agilent Technologies, Wilmington, DE, USA) was employed in the analysis. The injector operated at constant temperature of 230 °C in splitless mode using a SPME Injection Sleeve 0.75 mm ID (Supelco, Bellefonte, PA, USA). The carrier gas, helium of purity 5.0 (White Martins/Praxair Inc., Rio de Janeiro, RJ, Brazil), was used at a constant flow rate of 0.6 $mL.min^{-1}$. The separation of the volatile compounds was performed in a HP-5MS fused silica capillary column (5% phenyl/95% polidimethylsiloxane as stationary phase, 30 m length, 0.250 mm internal diameter, 0.25 µm film thickness) (Agilent Technologies, Wilmington, DE, USA). The oven temperature program applied in the analysis consisted of utilizing 50 °C initial temperature for 1 min, after which the temperature was raised at 3

°C.min^{-1} rate to 200 °C, then increased to 280 °C at 20 °C.min^{-1} for a total GC run time of 55 min. The transfer line temperature was held at 250 °C. The column eluent was submitted to MS detection in EI (electron ionization) mode (ionization energy 70 eV), while the ion source temperature was kept at 230 °C. Acquisition was carried out in scanning mode (mass range m/z 50-500).

Volatile compounds were identified by comparison of their obtained mass spectra with those reported by NIST 2011 database (considering a minimum similarity value of 80%) and with those from authentic standard compounds, as well as by also comparing the linear retention index (LRI) values with those of the standards and literature. For evaluate the composition of the samples, the semi-quantitative determination in equivalents was carried out by using 3-octanol as internal standard. The optimum amount of 3-octanol added to the sample vial was adjusted to 3.451 ng to avoid saturation of the fiber device and to prevent it from masking peaks of interest.

4.2.6. Statistical analysis

Individual capasaicinoids (in mg.g^{-1}) and volatile compounds contents (in mg.kg^{-1}) were used to perform a principal component analysis (PCA), to better visualize the sample behaviors towards the analyzed compounds and their correlations. PCA will cluster similar samples together in the scores graph, and also will show, in the loadings graph, which variables were responsible for this clustering. Thus, it will be possible to find out if the capsaicinoids content and the volatile profile

of the pepper samples could be used to classify them according to the water availability treatments during the growth of the plants, degree of maturity or harvest date.

All data were auto-scaled before the analysis so that each column data matrix was mean-centred and scaled to unit variance. This procedure is important to avoid the effects of different scales of variables. The PCA was performed using Pirouette 3.11 software (Infometrix, Inc., Bothell, WA).

4.3. Results and discussion

4.3.1. Biometrical characterization and moisture content of the peppers

The quality of the Habanero pepper is determined by the appearance and weight of the fruit, firmness and color are also important (Ruiz-Lau et al., 2011). Biometrical characterization and moisture content of the 'Habanero' peppers (*C. chinense*) obtained under different conditions of water availability are showed in the Table 4.1.

The 'Habanero' peppers obtained in this study presented elongated shape pointed at the end, characterized for ratios between length and width from 1.26 – 1.74, in accordance with Pino et al. (2011), which describe Habanero like a variety of elongated, pendant, lantern-shaped or campanulate form. Moreover, the peppers originated from the same water availability treatment showed a large variation in the biometric parameters evaluated. The relative standard deviation (RSD) achieved

values around 60% for weight parameter. Lower variations among the measured biometrical values were observed for width parameter.

Table 4.1. Biometrical characterization and moisture content of the 'Habanero' peppers (*C. chinense*) obtained under different conditions of water availability

Samples[1]	Biometrical parameters (n = 12)[2]			Moisture content
	Length (mm)	Width (mm)	Weight (g)	(%, w/w)[3]
P11gH1	38.11±4.71	23.33±3.06	5.02±1.77	88.02±0.92
P11mH1	35.64±6.65	20.37±2.00	4.28±1.38	84.48±0.52
P12gH1	36.09±5.47	25.00±2.80	4.50±1.17	89.21±0.15
P12mH1	30.67±8.58	20.53±4.98	3.67±1.90	84.09±0.12
P21gH1	39.31±2.86	25.53±2.29	5.75±1.28	89.19±0.33
P21mH1	34.42±11.85	20.66±3.00	4.98±2.22	82.94±0.37
P22gH1	33.35±5.03	26.44±2.54	4.36±1.21	89.28±0.07
P22mH1	30.57±11.96	20.18±3.77	4.07±2.29	83.23±0.31
P11gH2	34.81±5.84	23.36±2.54	4.39±1.09	86.91±0.97
P11mH2	22.63±10.13	17.49±3.90	2.42±1.32	80.65±0.18
P12gH2	34.67±5.58	24.93±2.90	4.89±1.56	87.99±2.17
P12mH2	25.47±7.98	18.05±2.48	2.62±1.32	83.50±0.10
P21gH2	35.08±10.49	25.70±3.13	5.49±2.17	86.74±0.46
P21mH2	29.40±10.71	19.67±5.89	3.87±2.36	80.94±0.22
P22gH2	36.42±3.46	28.70±3.92	6.02±1.28	87.04±0.32
P22mH2	28.93±5.41	22.65±5.58	3.94±1.78	84.96±0.52

[1] The samples are identified according the code schematized in the code composition presented in the Figure 4.1, regarding to the availability water treatments, degree of maturity and harvest date.
[2] The values represent the mean ± standard deviation of the analysis carried out in 12 peppers fruit for each sample.
[3] The values represent the mean ± standard deviation of the analysis carried out in triplicate.

Fruit weight is highly influenced by the lack of soil water in the root zone. According to Sezen et al. (2011), when soil water deficit in the root zone increases, occurs a loss in turgidity, and a reduction in growth and fruit weight; however, this effect was not clearly observed in the present study. The heaviest fruits were the peppers in green stage, which also showed greater moisture contents. During ripening of the peppers, moisture and fruit weight reduced in a similar way for all water availability treatments. In cases like this, it would be also interesting to correlate productivity data, since it is known that during the flowering and fruit formation periods, the pepper is more sensitive to limited water supply (Yldirim et al., 2012), which can result in lower number of fruits per plant and, by consequence, higher resources available to development of the formed pepper fruits.

The peppers in green stage from all treatments with cycles of hydric stress, as well as those from the control condition, harvested earlier (H1), had higher moisture contents.

4.3.2. Capsaicinoids analysis

4.3.2.1. Results for the method validation

Table 4.2 presents the merit data evaluated during the UHPLC method validation and recovery results. The intra-day and inter-day instrumental precisions

performed using a solution with the standards of capsaicin and dihydrocapsaicin (CAP and DHC, respectivelly) in three levels of concentration were satisfactory because the results of relative standard deviation for peak areas remained lower than 5%. The accuracy was carried out by standard addition of CAP and DHC in three levels of concentration on the sample P11mH2. The recovery percentages obtained ranged from 90.33 – 104.99% for capsaicin and 95.69 – 116.13% for dihydrocapsaicin. In spite of both limits of detection and quantification were low and suitable to this method application, they are higher than those reported by Sganzerla et al. (2014) using a UHPLC-PDA system coupled to a mass spectrometer from another manufacturer.

The intra-day and inter-day method precisions performed using a 'Habanero' pepper and considering the level concentration ($mg.g^{-1}$) of capsaicin and dihydrocapsaicin (CAP and DHC, respectivelly) in the sample were satisfactory. The results of relative standard deviation were equivalent to 4.19% and 3.77% (for CAP and DHC, respectivelly) in the extraction procedures (n=10) whitin a day and 2.39% and 2.08% (for CAP and DHC, respectivelly) in the extraction procedures along three consecutive days (n=3).

Table 4.2. Figures of merit for the UPLC-PDA method validation for capsaicinoids analysis

Validation parameter	**Compounds**			
	CAP		**DHC**	
Linearity (μg.mL^{-1})	5.5 – 65.88		4.5 – 60.48	
Equation	y = 15081x - 26902		y = 14060x + 5169.5	
r^2	0.9969		0.9981	
Intra-day precision (n =10)	*CAP level (μg.mL^{-1})*	*(%RSD)*	*DHC level (μg.mL^{-1})*	*(%RSD)*
	65.88	0.84	60.48	1.02
	36.66	0.42	31.36	0.45
	5.5	0.87	4.5	1.13
Inter-day precision (n = 3)	*CAP level (μg.mL^{-1})*	*%RSD*	*DHC level (μg.mL^{-1})*	*%RSD*
	65.88	2.68	60.48	3.14
	36.66	0.68	31.36	0.75
	5.5	1.54	4.5	1.58
Accuracy (n = 3)	*CAP added (μg.mL^{-1})*	*%Recovery**	*DHC added (μg.mL^{-1})*	*%Recovery**
	65.88	94.21±7.59	60.48	109.01±6.84
	36.66	96.68±7.52	31.36	110.54±6.53
	5.5	92.83±4.34	4.5	97.72±10.39
LOD (μg.mL^{-1})	0.055		0.045	
LOQ (μg.mL^{-1})	0.016		0.014	

CAP: capsaicin and DHC: dihydrocapsaicin. * Values of % recovery for accuracy parameter are expressed by mean values ± standard deviation (sd) for triplicate analysis.

4.3.2. Capsaicinoids composition

The contents of capsaicinoids in the 'Habanero' peppers showed varied pungency levels in the fruits from different conditions of water availability. Regarding the pattern of capsaicinoids composition, in all the 'Habanero' peppers analyzed, capsaicin was the most abundant compound, corresponding to about 70% of the capsaicinoids, followed, to a lesser extent, by the dihydrocapsaicin, as can seen in the chromatograms in Figure 4.2. This capsaicinoids composition is usual in hot peppers *C. chinense*, *C. frutescens* and *C. annuum* (Kollmannsberger et al., 2011).

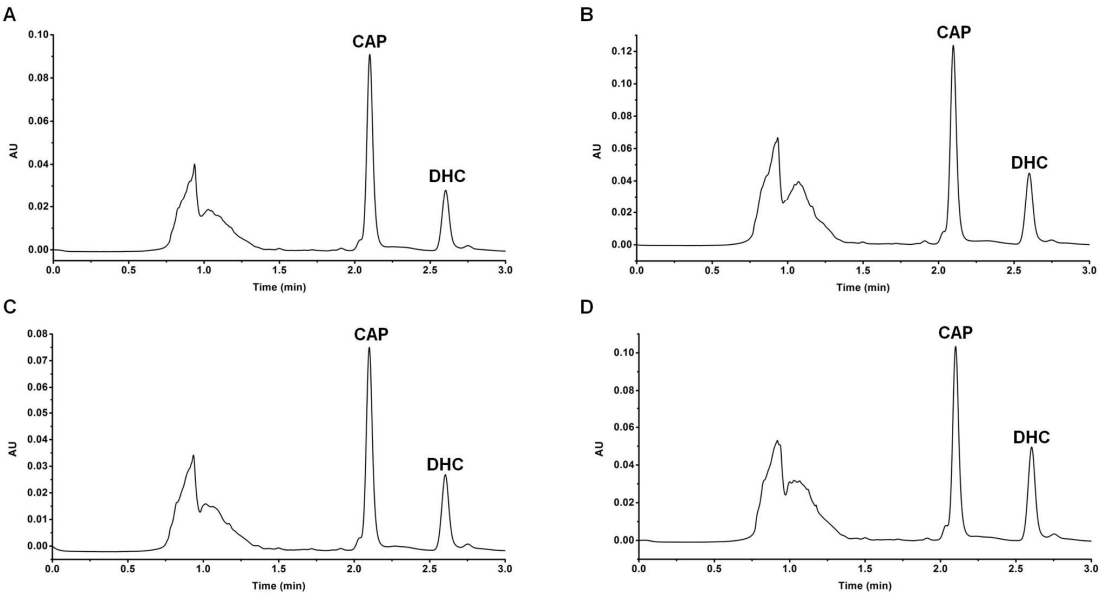

Figure 4.2. Chromatograms of capsaicinoids analysis with separation of compounds in a C18 column, mobile phase composed of water (40%, v/v) and acetonitrile (60%, v/v) at flow of 0.5 mL min^{-1} (absorbance at 280 ηm). CAP: capsaincin; DHC: dihydrocapsaicin. A: P11gH2 and B: P11mH2 (green and mature pepper from control treatment); C: P22gH2 and D: P22mH2 (green and mature pepper from complete water deficit stress treatments).

The concentration levels of capsaicin and dihydrocapsaicin found in this study for the 'Habanero' peppers (Table 4.3) are in good agreement with those found by other authors, Xiang-Yuan Deng et al. (2012) and Cisneros-Pineda et al. (2007) which reported values for capsaicin around 13.56 $mg.g^{-1}$ in dry weight for Mexican 'Habanero' peppers. In the other hand, Canto-Flick et al. (2008) reported values that ranged from 30.28 to 49.30 $mg.g^{-1}$ of capsaicin (expressed in dry weight of whole fruit) for different accessions of 'Habanero' pepper from Mexico.

Table 4.3. Capsaicin and dihydrocapsaicin contents in 'Habanero' peppers (*C.chinense*) grown under different conditions of water availability

Treatments[1]	Capsaicinoids[2] ($mg.g^{-1}$)	Degree of maturity			
		Green		Mature	
		Harvest 1	Harvest 2	Harvest 1	Harvest 2
P11	CAP	1.68 ± 0.07 a*, B**	2.70 ± 0.14 a, A	2.21 ± 0.08 a, B	3.24 ± 0.10 a, A
P12		1.03 ± 0.03 b, B	2.16 ± 0.04 b, A	2.14 ± 0.17 a, B	3.31 ± 0.13 a, A
P21		1.77 ± 0.16 a, A	1.73 ± 0.08 c, A	2.01 ± 0.29 a, B	2.85 ± 0.04 b, A
P22		1.26 ± 0.13 b, B	2.24 ± 0.08 b, A	2.48 ± 0.05 a, B	3.24 ± 0.11 a, A
P11	DHC	0.60 ± 0.06 a, B	0.90 ± 0.00 a, A	0.92 ± 0.04 a, B	1.35 ± 0.01 b, A
P12		0.25 ± 0.01 b, B	0.84 ± 0.02 a, A	0.97 ± 0.09 a, B	1.39 ± 0.05 b, A
P21		0.69 ± 0.07 a, A	0.52 ± 0.02 b, B	0.92 ± 0.15 a, A	1.08 ± 0.05 c, A
P22		0.37 ± 0.05 b, B	0.88 ± 0.07 a, A	1.10 ± 0.04 a, B	1.69 ± 0.07 a, A

[1] The samples are identified only with the code regarding to the availability water treatments (Fig. 4.1.). [2] The values represent the mean ± standard deviation of the analysis carried out in triplicate. * Significant differences in the same column (for each capsaicinoid) are indicated with different lowercase letters (comparison among the water supply treatments). ** Significant differences in the same row (inside the same maturity degree) are indicated with different uppercase letters (comparison between the harvests), $p < 0.05$.

The peppers in green stage of maturity showed lower contents of capsaicinoids in the fruits from all treatments in the two harvest procedures. In addition, the green peppers from the treatment with hydric stress during seedling and plant phase had lower capsaicin contents when compared with those green peppers from the control treatment.

To the peppers in green stage of maturity from the first harvest, the treatments with 90% of fiel capacity of water refilled in the plant phase showed higher contents of capasaicinoids.

Considering the mature peppers, seemingly, in the first harvest was observed higher values of capsaicin and dihydrocapsaicin concentration in the peppers from the completely water stress treatment in comparison to the fruits from control treatment, but these results do not showed significant differences at $p < 0.5$ among the treatments evaluated. Regarding to the diferent treatments of water availability, there were not significant differences in the contents of CAP and DHC for the mature peppers from the first harvest.

The hydric stress applied in the seedling phase followed by the water-refilled equivalent to 90% of field capacity, for mature peppers from second harvest, led to levels of capsaicinoids lower than those levels observed in the peppers from treatments with hydric stress during plant phase and control.

Significant differences in the content of CAP and DHC were observed for all the peepers in green stage of maturity when compare the harvest dates, achieving higher values in the second harvest with exception for the treatment P21. The same

tendency was identified for the mature peppers, which the fruit from the second harvest showed hiher contents of capsaicinoids, also with exception for the treatment P21.

The higher values of capsaicin and dihydrocapsaicin were found in the mature peppers from the second harvest, in all treatments evaluated, achieving ranges from 2.85 – 3.33 $mg.g^{-1}$ for capsaicin and 1.06 – 1.71 $mg.g^{-1}$ for dihydrocapsaicin.

4.3.3. Volatile compounds composition

The volatile compounds in the 'Habanero' samples were analyzed by solid-phase microextraction using triple phase fiber composed of PDMS/Car/DVB. This choice for the fiber was based in our previous study (Chapter III) and in data reported by innumerous authors (Mondello et al., 2004; Mazida, Salleh and Osman, 2005; Bianchi et al., 2007; Bogusz et al., 2011; Bogusz et al., 2014; Cuevas-Glory et al., 2014) which applied this technique to evaluate volatile compounds from different matrices, including peppers.

The results, based on the semi-quantification (Table 4.4), showed higher values of total volatile compounds for the peppers in green stage of maturity in the two harvests monitored with ranges from 195.47 – 298.94 $mg.kg^{-1}$. By using of the HS-SPME technique was possible to identify 85 and 82 volatile compounds in green and mature 'Habanero' peppers, respectively. Other 3 volatile compounds detected in all 'Habanero' pepper samples could not be identified. The profile of the peppers at green and mature stages are in the Figure 4.3.

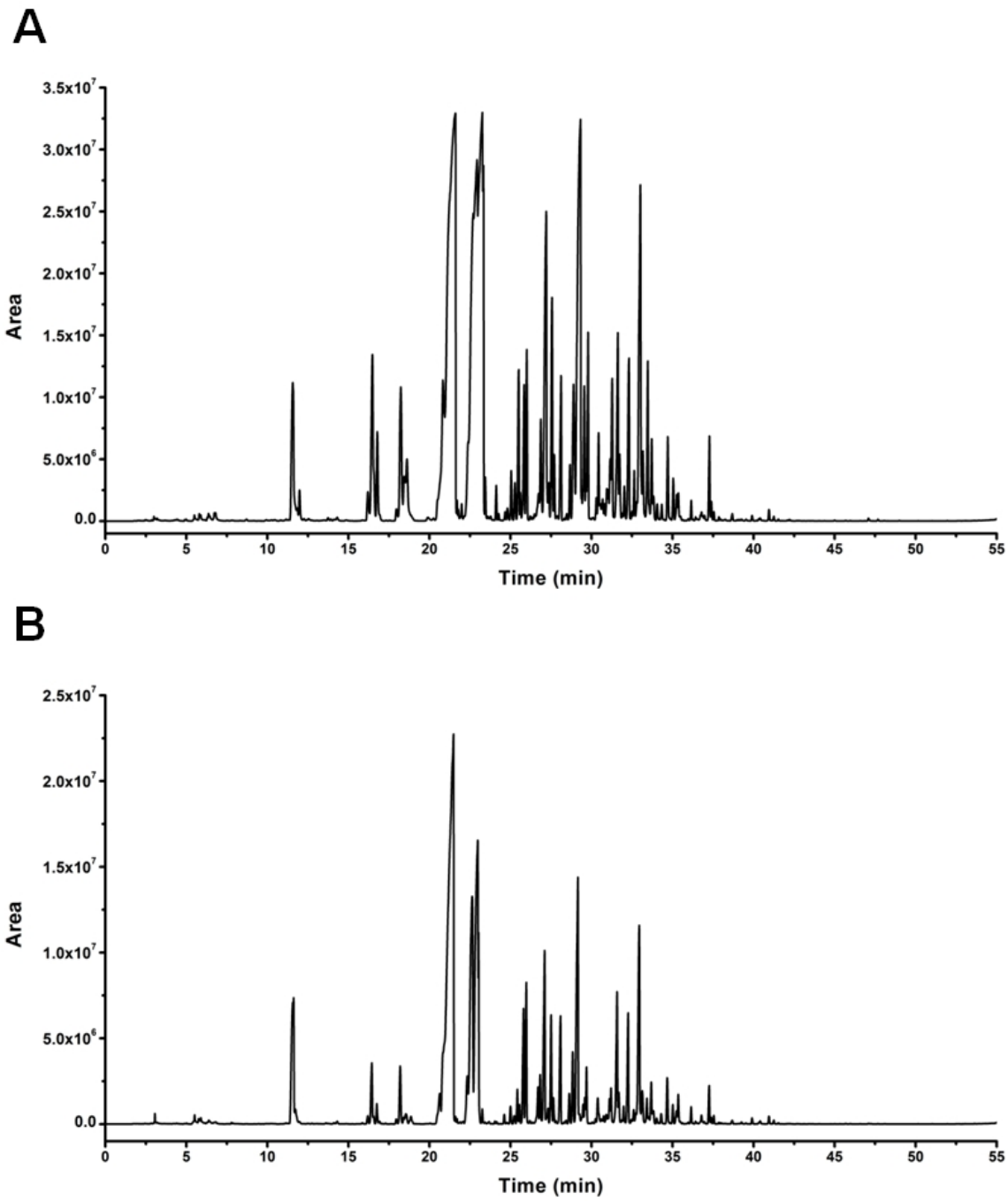

Figure 4.3. Typical chromatogram of Brazilian 'Habanero' green and mature peppers volatiles obtained in HP-5MS column according to the conditions summarized in the item 4.2.5.2. A: green peppers; B: mature peppers.

The samples of ‘Habanero’ peppers in green stage showed higher values of alcohols, which are responsible by powerful aromatic notes descripted as green or herbaceous, typical for peppers in this stage of maturity. The mainly alcohols found were isohexanol, 3-hexenol, trans-3-hexenol, 1-hexanol 2-3-dimethylciclohexanol and 3-3-dimethylcyclohexanol. The contents of these alcohols were found in lower quantities in the green peppers from the second harvest and, also, in the mature peppers, indicating that the closeness to the mature degree lead to the decay of alcohols in the aroma composition of ‘Habanero’ peppers. 2-Methoxy-3-isobutylpirazine was detected only in the green peppers, as well as, the compounds: 1,7-octadiene, 2,7-dimethyl, trans-hexenyl valerate, α-humulene, and β-himachalene.

The major compounds in the ‘Habanero’ peppers in green stage were esters: hexyl isovalerate, cis-3-hexenyl isovalerate, hexyl-3-methylbutanoate and heptyl isopentanoate; terpenes: longifolene and α-cubebene; and 3-3-dimethylcyclohexanol. These results are in accordance with Cuevas-Glory et al. (2014), regarding to the abundance of esters and 3-3-dimethylcyclohexanol; in relation to terpene composition, these authors have not found the longifolene, however, they have indicated the presence, in large quantity, of germacrene, which was not detected in the present study.

Also in the ‘Habanero’ peppers of mature degree, the esters were the major compounds. The same trend was reported by Cuevas-Glory et al. (2014) for the mature Mexican ‘Habanero’ peppers. In addition, Cuevas-Glory et al. (2014)

reported higher values of 3-3-dimethylcyclohexanol in mature peppers in relation to the green peppers that they have analyzed. About the 3-3-dimethylcyclohexanol, an opposite behavior was observed in the present study wich presented higher levels of this compound in the peppers of green degree of maturity.

Table 4.4. Volatile compounds (mg.kg^{-1}) in Brazilian *C. chinense* 'Habanero' pepper by HS-SPME and GC-MS

			Volatile contents in 'Habanero' peppers from the first harvest (H1)[4]							
Compounds[1]	**LRI$_{exp}$[2]**	**TI[3]**	***P11g***	***P11m***	***P12g***	***P12m***	***P21g***	***P21m***	***P22g***	***P22m***
Isohexanol	828	C	0.19	0.23	0.14	0.16	0.16	0.19	0.11	0.34
3-Hexenol	848	B	0.21	0.07	0.24	0.05	0.24	0.09	0.08	0.14
Trans-3-hexenol	850	B	0.26	0.11	0.19	0.09	0.21	0.11	0.12	0.17
1-Hexanol	861	A	0.64	0.10	0.60	0.08	0.50	0.08	0.30	0.15
Isobutyl isovalerate	1007	B	0.76	0.10	1.07	0.09	0.63	0.12	0.37	0.17
1,7-Octadiene, 2,7-dimethyl-	1020	C	0.22	nd	0.19	nd	0.29	nd	0.09	nd
Isovaleric acid, 3-methylbutyl-2 ester	1059	C	0.11	0.08	0.16	0.06	0.16	0.09	0.09	0.07
3-Methylbutyl 2-methylbutanoate	1100	B	0.97	0.23	1.17	0.20	0.82	0.25	0.48	0.31
Iso-Amil isovalerate	1106	B	4.37	1.55	5.45	1.44	3.39	1.67	2.09	2.53
Hexyl isobutyrate	1113	B	1.10	0.64	1.05	0.57	0.70	0.72	0.27	0.96
Pentyl 2-methylbutyrate	1139	B	0.28	0.14	0.27	0.13	0.18	0.13	0.12	0.15
Pentyl 3-methylbutyrate	1145	C	3.23	1.61	3.30	1.47	1.87	1.57	1.38	2.05
Hexyl butanoate	1150	A	0.88	0.26	1.17	0.19	1.13	0.21	0.59	0.28
3-Methyl-3-butenyl isovalerate	1153	C	2.08	0.50	2.29	0.57	1.26	0.56	1.27	0.69
2-methoxy-3-isobutylpyrazine	1183	A	0.32	nd	0.28	nd	0.37	nd	0.20	nd
Hexyl 2-methylbutyrate	1203	B	7.93	3.91	7.56	5.20	18.28	4.20	7.03	4.51
Hexyl isovalerate	1219	B	35.26	26.86	28.42	22.52	26.03	27.15	19.82	34.52

			Volatile contents in 'Habanero' peppers from the first harvest (H1)[4]							
Compounds[1]	**LRI_{exp}[2]**	**TI[3]**	***P11g***	***P11m***	***P12g***	***P12m***	***P21g***	***P21m***	***P22g***	***P22m***
Pentyl 4-methylpentanoate	1221	B	0.13	0.13	0.09	0.11	0.08	0.12	0.05	0.17
Heptyl isobutyrate	1223	B	0.10	0.10	0.09	0.10	0.07	0.09	0.04	0.09
β-Cyclocitral	1229	C	0.30	0.09	0.27	0.13	0.34	0.11	0.23	0.11
5-Hexenyl pentanoate	1231	B	0.10	0.07	0.10	0.07	0.14	0.07	0.14	0.08
cis-3-Hexenyl valerate	1237	B	3.07	1.82	1.61	1.65	3.75	1.68	2.79	1.70
cis-3-Hexenyl isovalerate	1245	A	32.38	11.25	34.33	9.97	36.71	10.38	31.06	11.57
Hexyl 3-methylbutanoate	1252	A	27.30	13.33	30.97	12.15	27.73	11.82	26.08	15.06
Trans-2-hexenyl valerate	1257	B	5.17	nd	6.86	nd	7.15	nd	5.65	nd
Hexyl pentanoate	1258	A	0.27	0.31	0.25	0.27	0.12	0.28	0.12	0.34
2-Methyldodecane	1266	A	0.07	0.07	0.09	0.07	0.07	0.07	0.05	0.07
Methyl 4-decenoate	1274	B	0.75	0.07	1.05	0.08	0.19	0.05	0.59	0.12
Phoracantholide	1277	C	0.16	0.06	0.16	0.04	0.09	0.04	0.13	0.05
Hexyl 3-methyl-2-butenoate	1287	B	0.07	0.16	0.06	0.14	0.04	0.13	0.05	0.14
Methyl 8-nonoate	1291	C	0.22	0.07	0.32	0.06	0.10	0.05	0.17	0.07
Octyl (E)-2-methylbut-2-enoate	1296	C	0.87	0.24	1.22	0.36	0.91	0.23	0.79	0.30
Tridecane	1301	A	0.63	0.22	0.95	0.13	1.01	0.14	0.69	0.19
Heptyl pentanoate	1305	A	2.72	0.40	4.11	0.34	3.73	0.33	3.19	0.57
Pentyl pivalate	1308	C	0.36	0.30	0.54	0.32	0.39	0.25	0.25	0.24

Volatile contents in 'Habanero' peppers from the first harvest (H1)[4]

Compounds[1]	**LRI_{exp}[2]**	**TI[3]**	***P11g***	***P11m***	***P12g***	***P12m***	***P21g***	***P21m***	***P22g***	***P22m***
Heptyl pivalate	1314	B	1.92	1.82	2.56	2.15	1.63	1.62	1.42	1.79
Hexyl hexanoate	1318	A	1.20	2.83	0.68	2.29	0.39	2.54	0.26	3.13
Hexyl 3-methyl-2-butenoate	1323	A	0.07	0.08	0.07	0.07	0.06	0.05	0.06	0.06
(Z)-Hex-3-enyl (E)-2-methylbut-2-enoate	1326	B	0.05	0.07	0.08	0.09	0.06	0.03	0.05	0.04
Heptyl 2-methylbutanoate	1337	B	2.07	0.64	3.21	0.65	2.05	0.57	2.00	0.74
Heptyl isopentanoate	1343	B	9.56	2.80	13.85	2.85	8.18	2.36	9.72	3.48
cis-3-Hexenyl Hexanoate	1348	B	0.31	0.49	0.34	0.42	0.29	0.38	0.21	0.42
α-Cubebene	1353	A	3.65	2.10	6.54	1.91	5.51	1.66	3.15	2.22
2-Methylcyclohexyl pentanoate, (E)-	1356	C	0.84	0.38	1.39	0.39	0.54	0.31	0.93	0.41
2-Methyltridecane	1366	A	2.29	1.82	2.90	1.47	1.16	1.37	1.76	2.12
3-Methyltridecane	1371	B	nd	0.04	nd	0.04	nd	0.03	nd	0.04
Ylangene	1374	B	0.15	0.05	0.21	0.05	0.22	0.05	0.15	0.07
α-Copaene	1378	C	0.93	0.41	1.44	0.43	1.42	0.39	0.84	0.57
2,3-Dimethylcyclohexanol	1383	B	3.91	0.92	5.50	1.01	6.64	0.89	4.78	1.18
3,3-Dimethylcyclohexanol	1391	B	21.36	4.62	29.49	5.00	27.31	4.20	26.04	6.58
β-Cubebene	1392	B	nd	0.26	nd	0.37	nd	0.25	nd	0.29
Benzyl isovalerate	1396	B	0.99	0.28	2.21	0.35	1.88	0.20	1.34	0.16
Octyl 2-methylbutanoate	1398	B	1.07	0.20	1.50	0.21	1.41	0.18	1.28	0.28

Volatile contents in 'Habanero' peppers from the first harvest (H1)[4]

Compounds[1]	**LRI_{exp}[2]**	**TI[3]**	***P11g***	***P11m***	***P12g***	***P12m***	***P21g***	***P21m***	***P22g***	***P22m***
Tetradecane	1400	A	0.31	0.40	0.32	0.33	0.16	0.27	0.14	0.34
Octyl isovalerate	1403	B	3.30	0.61	5.74	0.59	4.56	0.51	4.96	0.81
2,7-Octadien-1-ol	1417	C	0.31	0.10	0.55	0.07	0.66	0.05	0.49	0.06
Not identified	1420	-	1.45	0.56	1.93	0.52	2.60	0.39	2.16	0.45
2-Ethylcyclohexanol	1425	C	0.42	0.10	0.60	0.08	0.63	0.07	0.59	0.09
α-Ionone	1429	A	0.30	0.12	0.32	0.15	0.38	0.12	0.31	0.10
Cedrene	1433	B	0.60	0.16	0.90	0.16	0.87	0.13	0.83	0.16
cis-5-Octen-1-ol	1438	C	1.04	0.33	1.66	0.36	1.21	0.25	1.50	0.29
Octyl pentanoate	1440	C	2.33	0.45	3.55	0.46	3.66	0.38	3.66	0.48
2-Methyl-1-tetradecene	1450	B	3.43	2.05	5.05	2.27	3.72	1.84	3.98	2.67
α-Himachalene	1452	B	1.20	0.39	1.69	0.47	2.26	0.41	1.28	0.51
α-Humulene	1458	B	0.13	nd	0.20	nd	0.23	nd	0.14	nd
β-Farnesene (E)	1459	B	0.46	0.25	0.91	0.33	1.00	0.27	0.64	0.26
2-Methyltetradecane	1466	A	2.64	1.69	3.61	1.71	2.39	1.31	2.99	1.84
Caryophyllene	1471	A	0.16	0.06	0.28	0.09	0.35	0.07	0.21	0.10
β-Cadinene	1479	B	0.35	0.22	0.64	0.42	0.45	0.21	0.33	0.33
Longifolene-(V4)	1483	C	10.62	3.31	15.26	4.25	19.54	3.55	12.44	4.13
Trans-β-Ionone	1488	A	1.50	0.63	1.66	0.81	2.14	0.64	1.50	0.59

Volatile contents in 'Habanero' peppers from the first harvest (H1)[4]

Compounds[1]	**LRI_{exp}[2]**	**TI[3]**	***P11g***	***P11m***	***P12g***	***P12m***	***P21g***	***P21m***	***P22g***	***P22m***
2,6,10-trimethyl-9-undecenal	1495	C	2.80	0.65	4.20	0.60	3.62	0.46	4.20	0.60
Pentadecane	1502	A	0.84	0.72	1.25	0.60	0.94	0.59	0.95	0.63
β-Himachalene	1505	B	0.39	nd	0.76	nd	0.79	nd	0.49	nd
γ-Cadinene	1517	B	0.31	0.13	0.62	0.14	0.60	0.09	0.40	0.14
δ-Cadinene	1527	B	1.69	0.57	2.61	0.57	2.79	0.42	2.37	0.73
Naphthalene, 1,2,3,4,6,8a-hexahydro-1-isopropyl-4,7-dimethyl-	1535	B	0.77	0.22	1.35	0.21	1.33	0.17	1.16	0.29
α-Muurolene	1541	B	0.42	0.18	0.86	0.20	0.65	0.15	0.64	0.16
2-Methylpentadecane	1564	B	0.28	0.18	0.58	0.20	0.20	0.13	0.43	0.19
3-Methylpentadecane	1571	B	0.08	0.05	0.17	0.06	0.09	0.04	0.11	0.04
Hexyl benzoate	1580	C	0.22	0.18	0.68	0.16	0.33	0.14	0.42	0.14
Butyl 9-decenoate	1592	C	0.89	0.52	1.36	0.47	0.69	0.34	1.23	0.52
Hexadecane	1599	A	0.11	0.08	0.14	0.09	0.08	0.06	0.12	0.07
Isoamyl decanoate	1608	B	0.06	0.04	0.08	0.02	0.07	0.02	0.05	0.04
Not identified	1630	-	0.09	0.06	0.10	0.06	0.08	0.05	0.08	0.06
2-Methyl-E-7-hexadecene	1646	B	0.04	0.02	0.05	0.04	0.04	0.02	0.06	0.03
2-Methylhexadecane	1664	A	0.07	0.05	0.13	0.07	0.08	0.04	0.11	0.05
Not identified	1693	-	0.08	0.10	0.10	0.09	0.06	0.05	0.05	0.08

Heptadecane	1695	A	0.06	0.05	0.08	0.04	0.06	0.04	0.06	0.03
Hexyl decanoate	1703	C	0.02	0.02	0.04	0.02	0.04	0.01	0.03	0.02
Total			222.05	102.30	268.62	95.11	256.91	93.96	195.76	118.50

			Volatile contents in 'Habanero' peppers from the second harvest (H2)[4]							
Compounds[1]	**LRI_{exp}[2]**	**TI[3]**	***P11g***	***P11m***	***P12g***	***P12m***	***P21g***	***P21m***	***P22g***	***P22m***
Isohexanol	828	C	0.11	0.26	0.16	0.22	0.18	0.23	0.25	0.29
3-Hexenol	848	B	0.14	0.07	0.28	0.08	0.11	0.06	0.18	0.08
Trans-3-hexenol	850	B	0.15	0.10	0.21	0.09	0.14	0.06	0.20	0.07
1-Hexanol	861	A	0.33	0.11	0.43	0.10	0.25	0.06	0.45	0.09
Isobutyl isovalerate	1007	B	0.47	0.07	0.54	0.08	0.39	0.09	0.83	0.08
1,7-Octadiene, 2,7-dimethyl-	1020	C	0.08	nd	0.09	nd	0.08	nd	0.06	nd
Isovaleric acid, 3-methylbutyl-2 ester	1059	C	0.10	0.05	0.19	0.09	0.11	0.06	0.12	0.09
3-Methylbutyl 2-methylbutanoate	1100	B	0.75	0.13	0.69	0.19	0.55	0.12	0.94	0.14
Iso-Amil isovalerate	1106	B	3.26	1.24	3.91	1.61	3.55	0.98	6.83	1.29
Hexyl isobutyrate	1113	B	0.78	0.25	0.96	0.38	1.94	0.60	2.58	0.36
Pentyl 2-methylbutyrate	1139	B	0.38	0.08	0.25	0.09	0.27	0.09	0.37	0.08
Pentyl 3-methylbutyrate	1145	C	3.00	0.91	3.21	1.25	2.99	1.01	5.16	1.10
Hexyl butanoate	1150	A	1.17	0.10	0.87	0.14	1.42	0.13	1.80	0.12
3-Methyl-3-butenyl isovalerate	1153	C	1.05	0.27	1.73	0.40	0.71	0.28	2.62	0.30
2-Methoxy-3-isobutylpyrazine	1183	A	0.17	nd	0.22	nd	0.13	nd	0.18	nd

Volatile contents in 'Habanero' peppers from the second harvest (H2)[4]

Compounds[1]	**LRI_{exp}[2]**	**TI[3]**	***P11g***	***P11m***	***P12g***	***P12m***	***P21g***	***P21m***	***P22g***	***P22m***
Hexyl 2-methylbutyrate	1203	B	8.63	3.66	6.90	4.33	6.88	4.46	9.58	4.13
Hexyl isovalerate	1219	B	31.36	18.71	41.96	31.38	36.56	19.46	58.26	28.06
Pentyl 4-methylpentanoate	1221	B	0.13	0.06	0.22	0.12	0.13	0.06	0.27	0.10
Heptyl isobutyrate	1223	B	0.08	0.04	0.11	0.10	0.11	0.05	0.20	0.08
β-Cyclocitral	1229	C	0.28	0.04	0.30	0.06	0.15	0.07	0.40	0.05
5-Hexenyl pentanoate	1231	B	0.10	0.03	0.13	0.08	0.30	0.05	0.14	0.07
cis-3-Hexenyl valerate	1237	B	2.55	0.86	2.43	1.42	2.52	1.04	2.74	1.23
cis-3-Hexenyl isovalerate	1245	A	29.48	6.13	30.74	10.73	26.39	6.35	41.69	8.80
Hexyl 3-methylbutanoate	1252	A	22.76	8.41	27.57	14.67	22.50	7.93	31.38	12.63
Trans-2-hexenyl valerate	1257	B	4.25	nd	5.35	nd	3.36	nd	4.68	nd
Hexyl pentanoate	1258	A	0.22	0.10	0.40	0.20	0.31	0.17	0.65	0.20
2-Methyldodecane	1266	A	0.07	0.04	0.09	0.07	0.06	0.04	0.12	0.06
Methyl 4-decenoate	1274	B	0.23	0.03	0.78	0.04	0.43	0.09	0.60	0.04
Phoracantholide	1277	C	0.08	0.04	0.14	0.04	0.09	0.03	0.15	0.03
Hexyl 3-methyl-2-butenoate	1287	B	0.07	0.12	0.10	0.11	0.08	0.11	0.19	0.13
Methyl 8-nonoate	1291	C	0.19	0.02	0.26	0.04	0.16	0.03	0.32	0.03
Octyl (E)-2-methylbut-2-enoate	1296	C	0.45	0.13	0.56	0.27	1.49	0.35	1.02	0.26
Tridecane	1301	A	0.54	0.14	0.47	0.17	0.58	0.11	0.89	0.16

Volatile contents in 'Habanero' peppers from the second harvest (H2)[4]

Compounds[1]	LRI_{exp}[2]	TI[3]	*P11g*	*P11m*	*P12g*	*P12m*	*P21g*	*P21m*	*P22g*	*P22m*
Heptyl pentanoate	1305	A	1.41	0.26	1.61	0.50	1.78	0.20	3.34	0.46
Pentyl pivalate	1308	C	0.34	0.15	0.32	0.29	0.33	0.19	0.46	0.25
Heptyl pivalate	1314	B	1.46	1.14	2.03	2.22	1.72	1.18	3.04	1.80
Hexyl hexanoate	1318	A	1.21	1.35	2.05	2.37	1.51	1.69	3.69	2.18
Hexyl 3-methyl-2-butenoate	1323	A	0.07	0.04	0.08	0.06	0.08	0.04	0.12	0.05
(Z)-Hex-3-enyl (E)-2-methylbut-2-enoate	1326	B	0.05	0.02	0.08	0.04	0.08	0.02	0.11	0.05
Heptyl 2-methylbutanoate	1337	B	1.97	0.44	1.58	0.73	1.53	0.45	2.41	0.69
Heptyl isopentanoate	1343	B	6.59	1.79	7.45	3.40	6.05	1.66	12.23	3.18
cis-3-Hexenyl Hexanoate	1348	B	0.47	0.16	0.46	0.31	0.42	0.21	0.76	0.28
α-Cubebene	1353	A	4.11	1.18	3.79	1.96	3.30	1.12	5.22	1.68
2-Methylcyclohexyl pentanoate, (E)-	1356	C	0.67	0.15	0.87	0.39	0.43	0.14	1.26	0.38
2-Methyltridecane	1366	A	1.88	1.79	2.33	2.19	1.60	1.12	3.19	1.58
3-Methyltridecane	1371	B	nd	0.03	nd	0.04	nd	0.02	nd	0.03
Ylangene	1374	B	0.16	0.05	0.12	0.06	0.14	0.05	0.16	0.05
α-Copaene	1378	C	0.96	0.42	0.92	0.62	0.79	0.35	1.13	0.43
2,3-Dimethylcyclohexanol	1383	B	3.51	0.74	2.93	1.09	4.19	0.93	4.71	1.07
3,3-Dimethylcyclohexanol	1391	B	13.67	3.45	15.68	5.27	15.79	3.87	25.28	5.37
β-Cubebene	1392	B	nd	0.20	nd	0.26	nd	0.15	nd	0.21

Volatile contents in 'Habanero' peppers from the second harvest (H2)[4]

Compounds[1]	**LRI_{exp}[2]**	**TI[3]**	***P11g***	***P11m***	***P12g***	***P12m***	***P21g***	***P21m***	***P22g***	***P22m***
Benzyl isovalerate	1396	B	1.48	0.07	1.76	0.10	1.55	0.08	2.01	0.09
Octyl 2-methylbutanoate	1398	B	1.53	0.16	0.95	0.21	1.08	0.18	1.18	0.26
Tetradecane	1400	A	0.35	0.39	0.43	0.34	0.34	0.21	0.67	0.35
Octyl isovalerate	1403	B	2.05	0.44	2.36	0.57	2.59	0.39	4.17	0.70
2,7-Octadien-1-ol	1417	C	0.36	0.04	0.28	0.06	0.35	0.04	0.45	0.07
Not identified	1420	-	1.48	0.28	1.46	0.49	1.53	0.26	2.24	0.50
2-Ethylcyclohexanol	1425	C	0.30	0.06	0.38	0.09	0.42	0.04	0.65	0.10
α-Ionone	1429	A	0.40	0.06	0.32	0.09	0.25	0.07	0.34	0.12
Cedrene	1433	B	0.63	0.12	0.56	0.12	0.54	0.10	0.71	0.14
cis-5-Octen-1-ol	1438	C	0.99	0.22	1.07	0.30	0.74	0.17	1.24	0.33
Octyl pentanoate	1440	C	1.74	0.38	1.95	0.48	2.21	0.28	3.24	0.45
2-Methyl-1-tetradecene	1450	B	3.30	2.47	3.19	2.84	2.44	1.60	5.11	2.20
α-Himachalene	1452	B	1.49	0.43	1.05	0.51	1.38	0.39	1.45	0.47
α-Humulene	1458	B	0.17	nd	0.13	nd	0.12	nd	0.16	nd
β-Farnesene (E)	1459	B	0.85	0.22	0.42	0.29	0.66	0.22	0.72	0.31
2-Methyltetradecane	1466	A	2.47	1.64	2.18	2.08	1.96	1.15	3.99	1.69
Caryophyllene	1471	A	0.25	0.07	0.13	0.07	0.20	0.06	0.22	0.07
β-Cadinene	1479	B	0.25	0.12	0.38	0.19	0.28	0.13	0.45	0.19

Volatile contents in 'Habanero' peppers from the second harvest (H2)[4]

Compounds[1]	**LRI_{exp}[2]**	**TI[3]**	***P11g***	***P11m***	***P12g***	***P12m***	***P21g***	***P21m***	***P22g***	***P22m***
Longifolene-(V4)	1483	C	13.37	3.78	8.39	4.38	11.84	3.21	12.15	3.87
trans-β-Ionone	1488	A	2.13	0.47	1.70	0.63	1.62	0.42	1.70	0.64
2,6,10-trimethyl-9-undecenal	1495	C	2.40	0.22	2.36	0.47	2.97	0.28	3.92	0.46
Pentadecane	1502	A	1.37	0.71	1.53	0.81	1.08	0.52	1.70	0.58
β-Himachalene	1505	B	0.55	nd	0.46	nd	0.47	nd	0.57	nd
γ-Cadinene	1517	B	0.40	0.13	0.30	0.17	0.29	0.10	0.37	0.14
δ-Cadinene	1527	B	1.71	0.47	1.37	0.81	1.30	0.45	1.69	0.59
Naphthalene, 1,2,3,4,6,8a-hexahydro-1-isopropyl-4,7-dimethyl-	1535	B	0.75	0.15	0.62	0.31	0.53	0.17	0.79	0.22
α-Muurolene	1541	B	0.51	0.12	0.41	0.23	0.47	0.10	0.62	0.23
2-Methylpentadecane	1564	B	0.28	0.20	0.31	0.27	0.21	0.12	0.36	0.20
3-Methylpentadecane	1571	B	0.10	0.04	0.11	0.06	0.06	0.03	0.10	0.05
Hexyl benzoate	1580	C	0.35	0.17	0.41	0.17	0.33	0.12	0.39	0.19
Butyl 9-decenoate	1592	C	0.71	0.31	0.83	0.55	1.10	0.20	1.73	0.44
Hexadecane	1599	A	0.16	0.10	0.13	0.12	0.10	0.05	0.15	0.09
Isoamyl decanoate	1608	B	0.04	0.02	0.05	0.05	0.07	0.02	0.08	0.04
Not identified	1630	-	0.12	0.05	0.10	0.07	0.13	0.05	0.16	0.07
2-Methyl-E-7-hexadecene	1646	B	0.04	0.03	0.04	0.05	0.04	0.02	0.04	0.03

Volatile contents in 'Habanero' peppers from the second harvest (H2)[4]

Compounds[1]	**LRI_{exp}[2]**	**TI[3]**	***P11g***	***P11m***	***P12g***	***P12m***	***P21g***	***P21m***	***P22g***	***P22m***
2-Methylhexadecane	1664	A	0.09	0.05	0.09	0.08	0.05	0.04	0.10	0.07
Not identified	1693	-	0.08	0.07	0.11	0.11	0.16	0.05	0.23	0.09
Heptadecane	1695	A	0.11	0.05	0.09	0.05	0.09	0.03	0.08	0.04
Hexyl decanoate	1703	C	0.03	0.02	0.03	0.02	0.03	0.01	0.03	0.02
Total			197.36	68.74	220.16	108.59	195.47	68.98	298.94	95.88

The samples are identified only with the code regarding to the availability water treatments (Fig. 4.1.). [1]Tentative identification. [2]Linear retention index on HP-5MS obtained experimentally. [3]Tentative identification, the reliability of the identification proposal is indicated by the following: A, mass spectrum and retention index agreed with standards; B, mass spectrum and retention index agreed with database or literature; C, mass spectrum agreed with mass spectral database. [4]The results are presented as the mean of triplicate analysis. nd, not detected.

4.3.4. Principal component analysis (PCA)

A PCA was applied to the capsaicinoids and volatile profiles data, in order to verify if these compounds are able to classify the samples in their different treatments evaluated in this study.

The first attempt to perform the PCA analysis considered all the compounds detected in the samples as variables, with exception of those compounds that were detected just in few samples, like isovaleric acid 3-methylbutyl-2 ester, 2-methoxy-3-isobutylpyrazine, trans-2-hexenyl valerate, 3-methyltridecane, β-cubebene, α-humulene and β-himachalene. So, after excluding these seven compounds, the analysis was conducted using the 48 samples and 85 remaining variables (two capsaicinoids and 83 volatile compounds), resulting in a data matrix of *(48,85)*. The unity for capsaicinoids contents was $mg.g^{-1}$, while the unity for the volatile compounds was $mg.kg^{-1}$. The model for this PCA was constructed using 13 principal components, which explained 97.48% of the variance. The plots for the scores and loadings values are presented in Figure 4.4A and B, respectively. It is possible to see that the variance explained by using the two first principal components is 78.46%. In the scores graph (Fig. 4.4A), there are two major groups separated by the principal component 1 (Factor 1, axis). These groups correspond to the mature and green peppers. Thus, the effect of the fruits' maturity degree was so strong that covered any other effects who could also affect the samples classification. Only for the green peppers it was possible to see several minor groups arising from other effects, like the differences in the watered suspension treatments. Observing the loadings graph (Fig. 4.4B), the variables that most contributed to separate the

mature from the green peppers were the contents of the two capsaicinoids, capsaicin and dihydrocapsaicin, as well as the content of one volatile compound, isohexanol, which were higher in the mature samples. The other volatile compounds, in general, were detected in higher contents in the green samples in concentration levels far greater for the following compounds: hexyl 2-methylbutyrate, hexyl 3-methylbutanoate, heptyl isopentanoate, α-cubebene, 3,3-dimethylcyclohexanol, and longifolene.

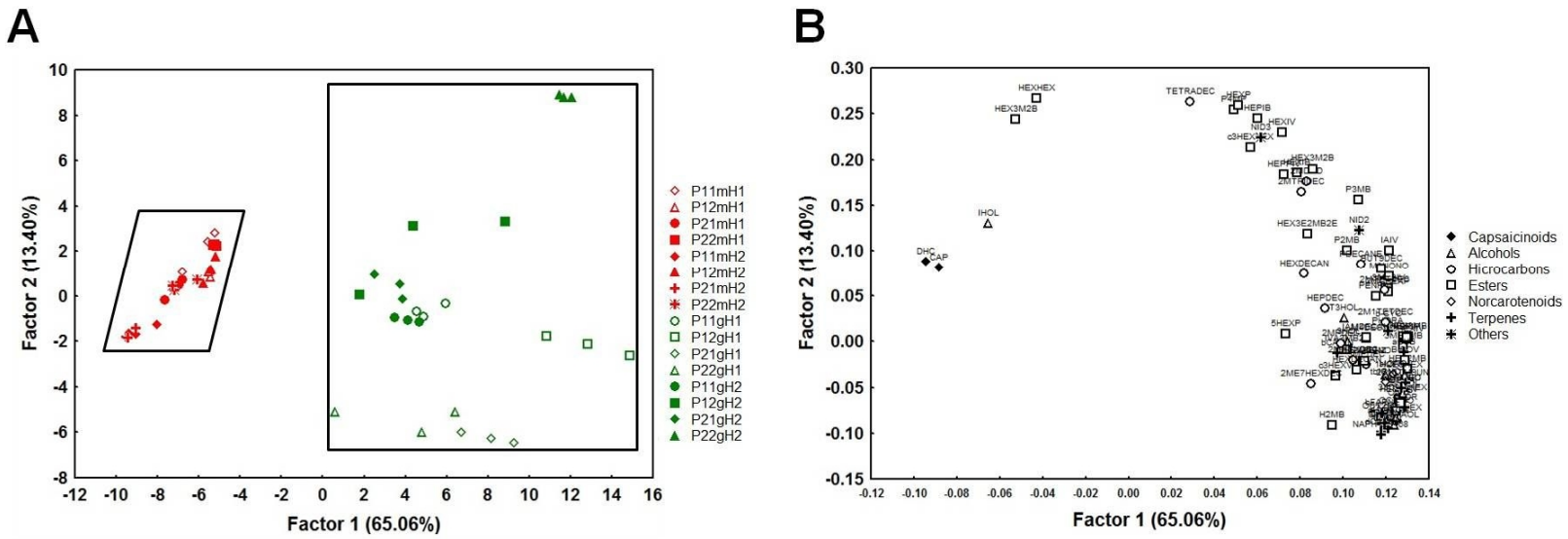

Figure 4.4. Results of the principal component analysis (PCA), showing the first and the second principalcomponents for the responses values. (A) scores graph, all the 'Habanero' samples; (B) loadings graph, all the 'Habanero' samples. The samples are identified only with the code regarding to the availability water treatments (Fig. 4.1.). CAP, capsaicin; DHC, dihydrocapsaicin; IHOL, isohexanol; 3HOL, 3-hexenol; T3HOL, trans-3-hexenol; 1HOL, 1-hexanol; IBIV, isobutyl isovalerate; IVA3MB2E, isoovaleric acid,3-methybuthyl-2-ester; 3MB2MB, 3-methylbuthyl 2-methylbutanoate; IAIV, iso-amil isovalerate; HEXIB, hexyl isobutyrate; P2MB, pentyl 2-methylbutyrate; P3MB, pentyl 3-methylbutyrate; HB, hexyl butanoate; 3M3BIV, 3-methyl-3-butenyl isovalerate; H2MB, hexyl 2-methylbutyrate; HEXIV, hexyl isovalerate; P4MP, pentyl 4-methylpentanoate; HEPIB, heptyl isobutyrate; b-CYC, β-cyclocitral; 5HEXP, 5-henenyl pentanoate; c3HEXV, cis-3-hexenyl valerate; c3HEXIV, cis-3-hexenyl isovalerate; HEX3MB, hexyl-3-methylbutanoate; HEXP, hexyl pentanoate; 2MDOD, 2-methyldodecane; M4DEC, methyl 4-decenoate; PHORA, phoracantolide; HEX3M2B, hexyl 3-methyl-2-butenoate; M8NONO, methyl 8-nonoate; OCT2M2ENO, octyl (E)-2-methylbut-2-enoate; TRIDEC, tridecane; HEPPEN, heptyl pentanoate; PENPIV, pentyl pivalate; HEPPIV, heptyl pivalate; HEXHEX, hexyl hexanoate; HEX3M2B, hexyl 3-methyl-2-butenoate; HEX3E2MB2E, (Z)-hex-3-enyl (E)-2-methylbut-2-enoate; HEP2MB, heptyl 2-methylbutanoate; HEPIP, heptyl isopentanoate; c3HEXHEX, cis-3-hexenyl hexanoate; aCUB, α-cubebene; 2MCHEXP, 2-methylcyclohexyl pentanoate (E); 2 MTRIDEC, 2-methyltridecame; YLAN, ylangene; aCOPA, α-copaene; 23DMCHEX, 2,3-dimethylcyclohexanol; 33DMCHEX, 3-3-dimethylcyclohexanol; BISOV, benzyl isovalerate; OCT2MB, octyl 2-methylbutanoate; TETRADEC, tetradecane; OCTISO, octylisovalerate; 27OCTAOL, 2,7-octadien-1-ol; NOTID1, first not identified compound; 2ECHEX, 2-ethylcyclohexanol; aIONO, α-ionone; CEDR, cedrene; c5O1OL, cis-5-octen-1-ol; OCTP, octyl pentanoate; 2M1TETDEC, 2-methyl-1-tetradecene; aHIMAC, α-himachalene; bFARNE, β-farnesene; 2MTETDEC, 2-methyltetradecane; CARY, caryophyllene, bCADIN, β-cadinene; LONGI, longifolene-(V4); tbIONO, trans-β-ionone; 2610TM9UND, 2,6,10-trimethyl-9-undecenal; PDECANE, pentadecane; gCADIN, γ-cadinene; d-cadin, δ-cadinene; NAPH123468, naphthalene, 123468a-hexahydro-1-isopropyl-4,7-dimethyl-; aMUURO, α-muurolene; 2MPDEC, 2-methylpentadecane; 3MPDEC, 3-methylpentadecane; HEXBENZ, hexyl benzoate; BUT9DEC, butyl-9-decenoate; HEXDECAN, hexadecane; IAMDECAN, isoamyl decanoate; NID2, second not identified compound; 2ME7HEXDEC, 2-methyl-E-7-hexadecene; 2MHEXDEC, 2-methylhexadecane; NID3, third not identified compound; HEPDEC, heptadecane; HEXDECAN, hexyl decanoate.

In order to search for another possibilities of sample classification, two new PCAs were performed, one taking into account only the green samples, while the other was calculated only for the mature samples.

The PCA model obtained for the green peppers consisted on 13 principal components, which explained 98.34% of the variance, for a data matrix of *(24,85)*. Considering only the two first principal components, 58.98% of the variance was explained. This value was lower than the variance explained by the two principal components in the PCA with all samples, but even though, it was possible to see clusters that correspond to the different watered suspension treatments. In Figure 4.5A, the scores graph for the green samples showed that the harvest had a significant effect, since the samples from both harvest periods were grouped separately. This separation was achieved due the differences in the capsaicinoids, hidrocarbons and some esters (pentyl 2-methylbutanoate, pentyl 4-methylbutanoate, cis-3-hexenyl isovalerate) contents, which were larger in the peppers from second harvest, while the alcohols (specially 3,3-dimethylcyclohexanol) and terpenes were found in major concentrations in the peppers from the first harvest. Also, the samples which were submitted to cycles of watered suspension during seedling phase grouped separately from those who were not, with more difficult visualization for P21gH2, located in the same cluster togheter with P11gH1 and P12gH2, but with a slight separation from other treatments due its higher content of 5-hexenyl pentanoate. The effect of the water refilled was more confusing, since for some samples it was significant, while for other samples it was not.

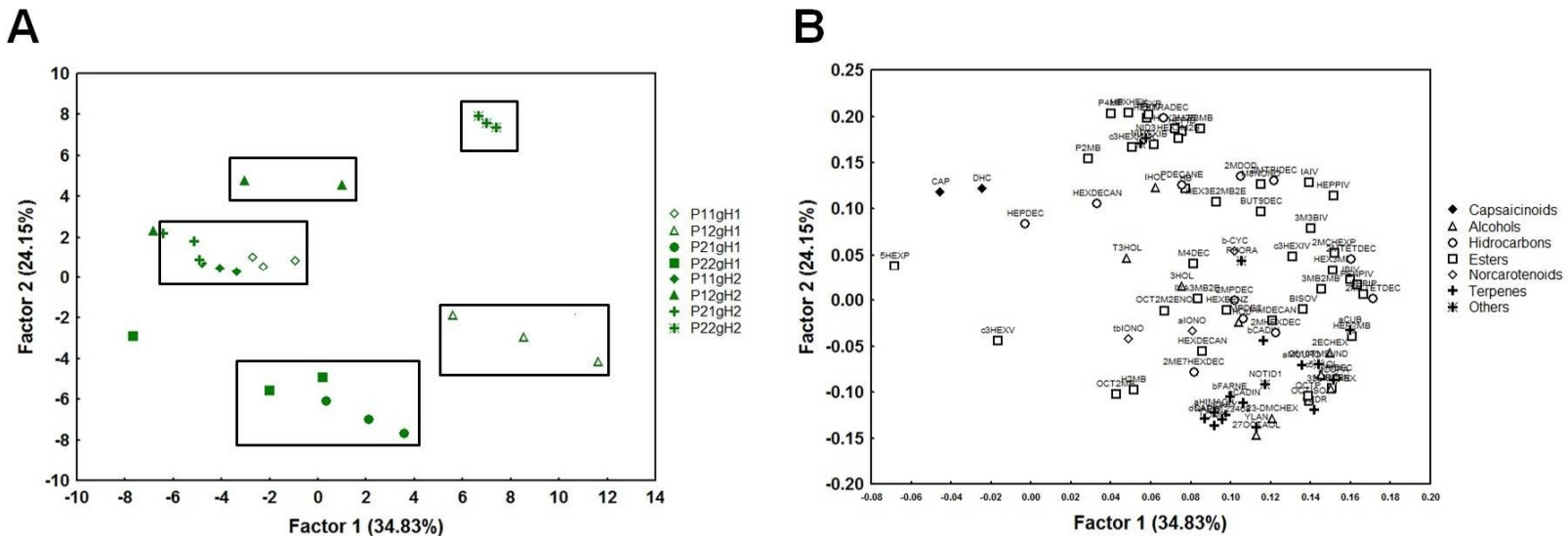

Figure 4.5. Results of the principal component analysis (PCA), showing the first and the second principalcomponents for the responses values. (A) scores graph, green stage 'Habanero' samples; (B) loadings graph, green stage 'Habanero' samples. The samples are identified only with the code regarding to the availability water treatments (Fig. 4.1.). CAP, capsaicin; DHC, dihydrocapsaicin; IHOL, isohexanol; 3HOL, 3-hexenol; T3HOL, trans-3-hexenol; 1HOL, 1-hexanol; IBIV, isobutyl isovalerate; IVA3MB2E, isoovaleric acid,3-methybuthyl-2-ester; 3MB2MB, 3-methylbuthyl 2-methylbutanoate; IAIV, iso-amil isovalerate; HEXIB, hexyl isobutyrate; P2MB, pentyl 2-methylbutyrate; P3MB, pentyl 3-methylbutyrate; HB, hexyl butanoate; 3M3BIV, 3-methyl-3-butenyl isovalerate; H2MB, hexyl 2-methylbutyrate; HEXIV, hexyl isovalerate; P4MP, pentyl 4-methylpentanoate; HEPIB, heptyl isobutyrate; b-CYC, β-cyclocitral; 5HEXP, 5-henenyl pentanoate; c3HEXV, cis-3-hexenyl valerate; c3HEXIV, cis-3-hexenyl isovalerate; HEX3MB, hexyl-3-methylbutanoate; HEXP, hexyl pentanoate; 2MDOD, 2-methyldodecane; M4DEC, methyl 4-decenoate; PHORA, phoracantolide; HEX3M2B, hexyl 3-methyl-2-butenoate; M8NONO, methyl 8-nonoate; OCT2M2ENO, octyl (E)-2-methylbut-2-enoate; TRIDEC, tridecane; HEPPEN, heptyl pentanoate; PENPIV, pentyl pivalate; HEPPIV, heptyl pivalate; HEXHEX, hexyl hexanoate; HEX3M2B, hexyl 3-methyl-2-butenoate; HEX3E2MB2E, (Z)-hex-3-enyl (E)-2-methylbut-2-enoate; HEP2MB, heptyl 2-methylbutanoate; HEPIP, heptyl isopentanoate; c3HEXHEX, cis-3-hexenyl hexanoate; aCUB, α-cubebene; 2MCHEXP, 2-methylcyclohexyl pentanoate (E); 2 MTRIDEC, 2-methyltridecame; YLAN, ylangene; aCOPA, α-copaene; 23DMCHEX, 2,3-dimethylcyclohexanol; 33DMCHEX, 3-3-dimethylcyclohexanol; BISOV, benzyl isovalerate; OCT2MB, octyl 2-methylbutanoate; TETRADEC, tetradecane; OCTISO, octylisovalerate; 27OCTAOL, 2,7-octadien-1-ol; NOTID1, first not identified compound; 2ECHEX, 2-ethylcyclohexanol; aIONO, α-ionone; CEDR, cedrene; c5O1OL, cis-5-octen-1-ol; OCTP, octyl pentanoate; 2M1TETDEC, 2-methyl-1-tetradecene; aHIMAC, α-himachalene; bFARNE, β-farnesene; 2MTETDEC, 2-methyltetradecane; CARY, caryophyllene, bCADIN, β-cadinene; LONGI, longifolene-(V4); tbIONO, trans-β-ionone; 2610TM9UND, 2,6,10-trimethyl-9-undecenal; PDECANE, pentadecane; gCADIN, γ-cadinene; d-cadin, δ-cadinene; NAPH123468, naphthalene, 123468a-hexahydro-1-isopropyl-4,7-dimethyl-; aMUURO, α-muurolene; 2MPDEC, 2-methylpentadecane; 3MPDEC, 3-methylpentadecane; HEXBENZ, hexyl benzoate; BUT9DEC, butyl-9-decenoate; HEXDECAN, hexadecane; IAMDECAN, isoamyl decanoate; NID2, second not identified compound; 2ME7HEXDEC, 2-methyl-E-7-hexadecene; 2MHEXDEC, 2-methylhexadecane; NID3, third not identified compound; HEPDEC, heptadecane; HEXDECAN, hexyl decanoate.

Considering only the mature peppers, the PCA model consisted also on 13 principal components, explaining 98.40% of the variance. The two first principal components explained 65.14% of the variance. The resulting scores graph is showed in the Figure 4.6A, where different clusters of samples can be visualized. Once again, the harvest had a significant effect, with the samples belonging to each harvest date being grouped separately due the higher concentration levels of capasaicinoids, terpenes, hidrocarbons and isohexanol in the peppers from the second harvest. On the other hand, the predominance of esters, norcarotenoids and the major part of alcohols in the composition of the peppers allowed the separation of the fruits from the first harvest. Samples submitted or not to the watered suspension also generated separated groups. For the mature peppers, the water refilled effect was significant for almost all the samples.

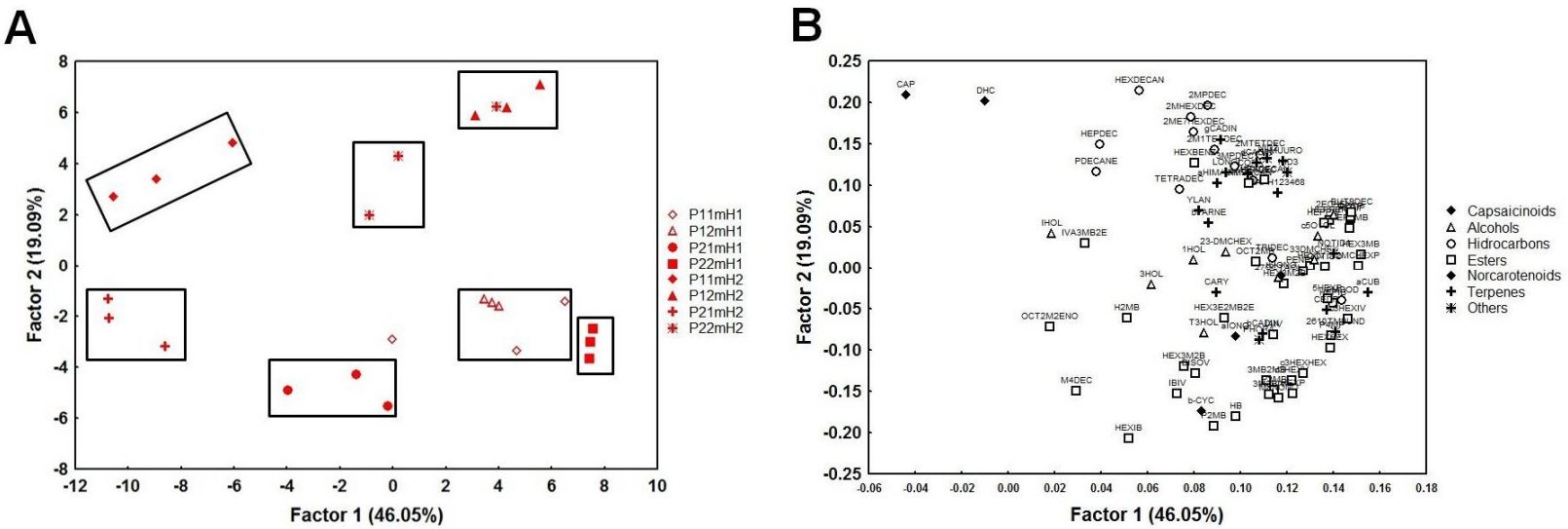

Figure 4.6. Results of the principal component analysis (PCA), showing the first and the second principalcomponents for the responses values. (A) scores graph, mature stage 'Habanero' samples; (B) loadings graph, green stage 'Habanero' samples. The samples are identified only with the code regarding to the availability water treatments (Fig. 4.1.). CAP, capsaicin; DHC, dihydrocapsaicin; IHOL, isohexanol; 3HOL, 3-hexenol; T3HOL, trans-3-hexenol; 1HOL, 1-hexanol; IBIV, isobutyl isovalerate; IVA3MB2E, isoovaleric acid,3-methybuthyl-2-ester; 3MB2MB, 3-methylbuthyl 2-methylbutanoate; IAIV, iso-amil isovalerate; HEXIB, hexyl isobutyrate; P2MB, pentyl 2-methylbutyrate; P3MB, pentyl 3-methylbutyrate; HB, hexyl butanoate; 3M3BIV, 3-methyl-3-butenyl isovalerate; H2MB, hexyl 2-methylbutyrate; HEXIV, hexyl isovalerate; P4MP, pentyl 4-methylpentanoate; HEPIB, heptyl isobutyrate; b-CYC, β-cyclocitral; 5HEXP, 5-henenyl pentanoate; c3HEXV, cis-3-hexenyl valerate; c3HEXIV, cis-3-hexenyl isovalerate; HEX3MB, hexyl-3-methylbutanoate; HEXP, hexyl pentanoate; 2MDOD, 2-methyldodecane; M4DEC, methyl 4-decenoate; PHORA, phoracantolide; HEX3M2B, hexyl 3-methyl-2-butenoate; M8NONO, methyl 8-nonoate; OCT2M2ENO, octyl (E)-2-methylbut-2-enoate; TRIDEC, tridecane; HEPPEN, heptyl pentanoate; PENPIV, pentyl pivalate; HEPPIV, heptyl pivalate; HEXHEX, hexyl hexanoate; HEX3M2B, hexyl 3-methyl-2-butenoate; HEX3E2MB2E, (Z)-hex-3-enyl (E)-2-methylbut-2-enoate; HEP2MB, heptyl 2-methylbutanoate; HEPIP, heptyl isopentanoate; c3HEXHEX, cis-3-hexenyl hexanoate; aCUB, α-cubebene; 2MCHEXP, 2-methylcyclohexyl pentanoate (E); 2 MTRIDEC, 2-methyltridecame; YLAN, ylangene; aCOPA, α-copaene; 23DMCHEX, 2,3-dimethylcyclohexanol; 33DMCHEX, 3-3-dimethylcyclohexanol; BISOV, benzyl isovalerate; OCT2MB, octyl 2-methylbutanoate; TETRADEC, tetradecane; OCTISO, octylisovalerate; 27OCTAOL, 2,7-octadien-1-ol; NOTID1, first not identified compound; 2ECHEX, 2-ethylcyclohexanol; aIONO, α-ionone; CEDR, cedrene; c5O1OL, cis-5-octen-1-ol; OCTP, octyl pentanoate; 2M1TETDEC, 2-methyl-1-tetradecene; aHIMAC, α-himachalene; bFARNE, β-farnesene; 2MTETDEC, 2-methyltetradecane; CARY, caryophyllene, bCADIN, β-cadinene; LONGI, longifolene-(V4); tbIONO, trans-β-ionone; 2610TM9UND, 2,6,10-trimethyl-9-undecenal; PDECANE, pentadecane; gCADIN, γ-cadinene; d-cadin, δ-cadinene; NAPH123468, naphthalene, 123468a-hexahydro-1-isopropyl-4,7-dimethyl-; aMUURO, α-muurolene; 2MPDEC, 2-methylpentadecane; 3MPDEC, 3-methylpentadecane; HEXBENZ, hexyl benzoate; BUT9DEC, butyl-9-decenoate; HEXDECAN, hexadecane; IAMDECAN, isoamyl decanoate; NID2, second not identified compound; 2ME7HEXDEC, 2-methyl-E-7-hexadecene; 2MHEXDEC, 2-methylhexadecane; NID3, third not identified compound; HEPDEC, heptadecane; HEXDECAN, hexyl decanoate.

4.4. Conclusions

The ‘Habanero’ peppers obtained in this study presented elongated shape pointed at the end. The contents of capsaicinoids in the ‘Habanero’ peppers showed varied pungency levels in the fruits from different conditions of water availability. The major values of capsaicin and dihydrocapsaicin were found in the mature peppers and was observed a tendency of higher content of capasaicinoids in the peppers obtained from the second harvest, in both maturity degree of the peppers, and also, considering all different water availability treatments.

The mainly volatile compounds were esters, terpenes and alcohols. Total volatile compounds presented higher values for the peppers in green stage of maturity with ranges from 195.47–298.94 mg kg^{-1}, whereas in the mature samples the range was from 68.74–118.50 mg kg^{-1}.

According the results obtained and the PCA performed, firstly it was possible achieve the separation of the ‘Habanero’ peppers in function of the degree of maturity that had a strong effect on the composition of the samples, mainly for higher content of capsaicinoids and isohexanol in mature peppers and the other volatile compounds in the green peppers. Other possibilities of sample classification were performed through the PCA apllied, separately, for green and mature peppers and in this step was observed the clear separation in function of the harvest date. In addition, the peppers were clustered in function of the water availability treatments received, but to better understand the effects of water availability on the composition

of the peppers, another strategy of evaluation, with more frequent monitoring would be interesting.

4.5. Acknowledgments

This work was supported by grants from CAPES – Coordenação de Aperfeiçoamento de Pessoal de Nível Superior and FAPESP – Fundação de Amparo à Pesquisa do Estado de São Paulo (Project number 2011/17094-2). The authors also acknowledge the Agronomic Institute of Campinas for supplying the samples. The author M. Sganzerla would like to thank CAPES by the exchange fellowship (Institutional program PDSE, BEX process number 1262/12-2) and the author C. A. Ballus would like to thank FAPESP by the post-doctoral fellowship (process no. 13/25242-7).

4.6. References

Alexandratos, N.; J. Bruinsma. (2012). World agriculture towards 2030/2050: the 2012 revision. ESA Working paper No. 12-03. Rome, FAO.

Antony, E.; Singandhupe R. B. (2004). Impact of drip and surface irrigation on growth, yield and WUE of capsicum (*Capsicum annum* L.). Agricultural Water Management .65: 121 – 132.

Barbero, G. F.; Liazid, A.; Palma, M.; Barroso, C. G. (2008). Ultrasound-assisted extraction of capsaicinoids from peppers. Talanta. 75(5), 1332 – 1337.

Bennett, D. J.; Kirby, G. W. (1968). Constitution and biosynthesis of capsaicin. Journal Chemical Society C. 90, 442 – 446.

Bianchi, F.; Careri, M.; Conti, C.; Musci, M.; Vreuls, R. (2007). Comparison of comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry and gas chromatography-mass spectrometry for the qualitative characterisation of roasted barley by solid-phase microextraction. Journal of Separation Science, 30(4), 527 – 533.

Bogusz Junior, S.; Tavares de Melo, A; Alcaraz, C. Z., Teixeira, H. G. (2011). Optimization of the extraction conditions of the volatile compounds from chili pepper by headspace solid phase micro-extraction. Journal of Chromatography A. 1218, 3345 – 3350.

Bogusz Junior, Stanislau; Março, Paulo Henrique; Valderrama, Patrícia; Damasceno, Flaviana Cardoso; Aranda, Maria Silvana; Zini, Cláudia Alcaraz; Caramão, Elina Bastos; Melo, Arlete Marchi Tavares; Teixeira Filho, José; Godoy, Helena Teixeira. (2014). Analysis of volatile compounds in *Capsicum spp.* by headspace solid-phase microextraction and GCxGC-TOFMS. Analytical Methods, 1-9.

Bouwer, H (2000). Integrated water management emerging issues and challenges. Agricultural Water Management. 45: 217 – 228.

Canto-Flick, Adriana; Balam-Uc, Eduardo; Bello-Bello, Jericó Jabín; Lecona-Guzmán, Carlos; Solís-Marroquín, Daniela; Avilés-Viñas, Susana; Gómez-Uc, Eunice; López-Puc, Guadalupe; Santana-Buzzy, Nancy. (2008). Capsaicinoids

Content in Habanero Pepper (*Capsicum chinense* Jacq.): Hottest Known Cultivars. Hortscience, 43(5), 1344 – 1349.

Chinn, Mari S.; Sharma-Shivappa, Ratna R.; Cotter, Jacqueline L. (2011). Solvent extraction and quantification of capsaicinoids from *Capsicum chinense*. Food and bioproducts processing, 89, 340 – 345.

Cisneros-Pineda, Olga; Torres-Tapia, Luis W.; Gutiérrez-Pacheco, Luis Carlos; Contreras-Martín Fernando; González-Estrada, Tomás, Peraza-Sánchez, Sergio R. (2007). Capsaicinoids quantification in chili peppers cultivated in the state of Yucatan, Mexico. Food Chemistry, 104, 1755 – 1760.

Contreras-Padilla, M. and E.M. Yahia. (1998). Changes in capsaicinoids during development, maturation and senescence of chili pepper and relation with peroxidase activity. Journal of Agricultural and Food Chemistry, 46, 2075 – 2079.

Cuevas-Glory, L. F.; Sosa-Moguel, O.; Pino, J.; Sauri-Duch, E. (2014). GC-MS Characterization of volatile compounds in Habanero pepper (*Capsicum chinense* Jacq.) by optimization of headspace solid-phase microextraction conditions. Food Analytical Methods, 1 – 9.

Deng, Xiang-Yuan; Gao, Kun; Huang, Xin; Liu, John. (2012). Optimization of ultrasonic-assisted extraction procedure of capsaicinoids from Chili peppers using orthogonal array experimental design. African Journal of Biotechnology, 11(67), 13153 – 13161.

Estrada, A.B.; Pomar, F.; Díaz, J.; Merino, F.; Bernal, A. (1997). Evolution of capsaicinoids in *Capsicum annuum* L. var. *annuum* cv. Padrón fruit at different growth stages. Capsicum and Eggplant Newsletter , 16:60 – 63.

Estrada, A.B., Pomar F.; Díaz, J.; Merino, F.; Bernal, A. (1999). Pungency level in fruits of Padrón pepper with different water supply. Science Horticultural, 81, 385 – 396.

Garcés-Claver, A.; Arnedo-André, M. S.; Avier Abadía, R.; Gil-Ortega, S. J.; Álvarez-Fernández, A. (2006). Determination of capsaicin and dihydrocapsaicin in *Capsicum* fruits by liquid chromatography-electrospray/time-of-flight mass spectrometry. Journal of Agricultural and Food Chemistry. 54(25), 9303 – 9311.

Howell T. (2001). Enhancing water use efficiency in irrigated agriculture. Agron. Journal, 93, 281 – 289.

Iwai, K.; Suzuki, T.; Fujiwake, H. (1979). Formation and accumulation of pungent principle of hot pepper fruits, capsaicin and its analogues, in *Capsicum annuum* L. var. *annuum* cv. Karayatsubusa at different growth stages after flowering. Agr. Biol. Chem. 43, 2493 – 2498.

Jarret, R. L.; Perkinsb, B.; Fanc, T.; Princec, A.; Guthrieb, K.; Skoczenski, B. (2003). Using EIA to screen *Capsicum spp.* germplasm for capsaicinoid content. Journal of Food Composition and Analysis. 16(2), 189 – 194.

Jin, R.; Pan, J.; Xie, H.; Zhou, B.; Xia, X. (2009). Separation and quantitative analysis of capsaicinoids in chili peppers by reversed-phase argentation LC. Chromatographia. 70(5-6), 1011 – 1013.

Kollmannsberger, Hubert; Rodríguez-Burruezo, Adrián; Nitz, Siegfried; Nuez, Fernando. (2011). Volatile and capsaicinoid composition of ají (*Capsicum baccatum*) and rocoto (*Capsicum pubescens*), two Andean species of chile peppers. Journal of the Science of Food and Agriculture, 91, 1598 – 1611.

Kurian, A.L.; Starks, A.N. (2002). HPLC analysis of capsaicinoids extracted from whole orange habanero chili peppers. Journal of Food Science, 67(3), 956 – 962.

Luo, X. J.; Peng, J.; Li, Y. J. (2011). Recent advances in the study on capsaicinoids and capsinoids. European Journal of Pharmacology, 650(1), 1 – 7.

Mazida, M.M.; Salleh, M.M.; Osman, H. (2005). Analysis of volatile aroma compounds of fresh chilli (*Capsicum annuum*) during stages of maturity using solid phase microextraction (SPME). Journal of Food Composition and Analysis, 18, 427 – 437.

Mondello, Luigi; Casilli, Alessandro; Tranchida, Peter Quinto; Dugo, Paola; Costa, Rosaria; Festa, Saverio; Dugo, Giovanni. (2004). Comprehensive multidimensional GC for the characterization of roasted coffee beans. Journal of Separation Science, 27, 442 – 450.

Perkins, B.; Bushway, R.; Guthrie, K.; Fan, T.; Stewart, B.; Prince, A.; Williams, M. (2002). Determination of capsaicinoids in salsa by liquid chromatography and enzyme immunoassay. Journal of AOAC International. 85(1), 82 – 85.

Pino, J.; González, M.; Ceballos, L.; Centurión-Yah, A. R.; Trujillo-Aguirre, J.; Latournerie-Moreno, L.; et al. (2007). Characterization of total capsaicinoids, Colour and volatile compounds of Habanero chile pepper (Capsicum chinense Jacq.) cultivars grown in Yucatan. Food Chemistry, 104, 1682 – 1686.

Pino, J.; Sauri, E.; Marbot, R. (2006). Changes in volatile compounds of Habanero chilli pepper (*Capsicum chinense* Jacq. Cv. Habanero) at two ripening stages. Food Chemistry, 94, 394 – 398.

Ribeiro, C. S. C.; Lopes, C. A.; Carvalho, S. I. C.; Henz, G. P.; Reifschneider, F. J. B. (2008). Pimentas *Capsicum*. (1st Ed.). Brasília-DF, Embrapa Hortaliças, Brazil, (Chapter 1) p. 1 – 14.

Rodríguez-Burruezo, A.; Kollmannsberger, H.; González-Mas, M. C.; Nitz, S.; Nuez, F. (2010). HS-SPME comparative analysis of genotypic diversity in volatile fraction and aroma contributing compounds of *Capsicum* fruits from the annuum-chinense-frutescens complex. Journal of Agricultural and Food Chemistry, 58, 4388 – 4400

Rosa, A.; Deiana, M.; Casu, V.; Paccagnini, S.; Appendino, G.; Ballero, M.; Dessi, M.A. (2002). Antioxidant activity of capsinoids. Journal of Agricultural and Food Chemistry, 50, 7396 – 7401.

Ruiz-Lau, Nancy; Medina-Lara, Fátima; Minero-García, Yereni; Zamudio-Moreno, Enid, Guzmán-Antonio, Adolfo; Echevarría-Machado, Ileana; Martínez-Estévez, Manuel. (2011). Water deficit affects the accumulation of capsaicinoids in fruits of *Capsicum chinense* Jacq. Hortscience, 46(3), 487 – 492.

Salgado-Garciglia, R.; Ochoa-Alejo, N. (1990). Increased capsaicin content in PFP resistant cells of chili peppers (*Capsicum annuum* L.). Plant Cell Rep. 8, 617–620.

Sezen S. M.; Yazar A.; Eker S. (2006). Effect of drip irrigation regimes on yield and quality of field grown bell pepper. Agricultural Water Management, 81 (2006):115 – 131

Sezen, S. Metin; Yazar, Attila; Tekin, Servet; Eker, Salim; Kapur, Burçak. (2011). Yield and quality response of drip-irrigated pepper under Mediterranean climatic

conditions to various water regimes. African Journal of Biotechnology, 10(8), 1329 – 1339.

Sganzerla, M.; Coutinho, J. P.; Melo, A. M. T.; Godoy, H. T. (2014). Fast method for capsaicinoids analysis from *Capsicum chinense* fruits. Food Research International, 64, 718 – 725.

Souza, E. T.; Rodrigues, F. M.; Martins, C. C.; Oliveira, F. S.; Pereira, P. A. P.; Andrade, J. B. (2006). Multivariate optimization and HS-SPME/GC-MS analysis of VOCs in red, yellow and purple varieties of *Capsicum chinense* sp. peppers. Microchemical Journal, 82, 142 – 149.

Yildirim M. (2010). Water management in coastal areas with low quality irrigation water for pepper growth. Journal of Coastal Research, 26(5), 869 – 898.

Yildirim, Murat; Demirel, Kursad; Bahar, Erdem. (2012). Effect of restricted water supply and stress development on growth of Bell pepper (*Capsicum Annuum* L.) under drought conditions. Journal of Agro Crop Sci J, 3(1), 1 – 9.

Zewdie, Y.; Bosland, P. W. (2001). Capsaicinoid profiles are not good chemotaxonomic indicators for *Capsicum* species. Biochemical Systematics and Ecology. 29(2), 161 – 169.

CONCLUSÃO GERAL

Os métodos desenvolvidos para a extração e separação dos capsaicinoides apresentaram como principais vantagens a rapidez, eficiência e confiabilidade. Além disso, ambos podem ser largamente empregados para a quantificação de capsaicina e dihidrocapsaicina em diferentes níveis de concentração. O tempo total de preparação foi de 15 min, com reduzido consumo de amostra, solventes e instrumentação, o que também resultou em menor interferência cromatográfica. A extração assistida por ultrassom, com base no método desenvolvido, permitiu a extração quantitativa e reprodutível dos capsaicinoides presentes nas pimentas, usando metanol como solvente extrator. Devido à sua simplicidade e capacidade analíticas, o método desenvolvido pode ser aplicado para análise de rotina dos capsaicinoides em pimentas *Capsicum* e seria particularmente adequado para a análise rotineira de capsaicinoides em programas de melhoramento genético, bem como pode ser usado para determinar as concentrações de outros capsaicinoides minoritários, quando padrões estiverem disponíveis.

O uso de estratégias experimentais multivariadas permitiu identificar a combinação ótima de temperatura e tempo de extração, com reduzido número de experimentos, para aplicação da HS-SPME na análise da fração volátil de pimentas *C. chinense* 'Habanero'. As condições ótimas de HS-SPME para a pimenta 'Habanero', utilizando a fibra de PDMS/Car/DVB, foram tempo de extração de 30 min e temperatura de extração de 40 ºC. Oitenta e dois compostos foram identificados por tentativa na fração volátil da pimenta 'Habanero'. Os compostos

voláteis mais abundantes na pimenta 'Habanero' foram hexil isovalerato, cis-hexenil isovalerato, hexil 3-metilbutanoato, 3,3-dimetilciclohexanol, longifoleno e 2-metil-1-tetradeceno. Os compostos 2,3-dimetilciclohexanol e longifoleno foram reportados pela primeira vez em pimentas 'Habanero' (*C. chinense*).

As pimentas 'Habanero' cultivadas sob diferentes condições de disponibilidade de água foram caracterizadas quanto a composição de capsaicinoides e voláteis.

Os conteúdos de capsaicinoides mostraram variações na pungência dos frutos obtidos em diferentes tratamentos de estresse hídrico. Os maiores teores de capsaicina e dihidrocapsaicina foram encontrados nas pimentas maduras, com valores de 2.85 – 3.33 mg g^{-1} para capsaicina e 1.06 – 1.71 mg g^{-1} para a dihidrocapsaicina.

Os principais compostos voláteis identificados para todas as pimentas foram os ésteres, terpenoides e álcoois. O teor total de compostos voláteis foi maior para as pimentas verdes, com valores de 195.47–298.94 mg kg^{-1}, enquanto que para as pimentas maduras os valores ficaram entre 68.74–118.50 mg kg^{-1}.

Os resultados obtidos a partir da aplicação da PCA possibilitaram, inicialmente, obter a separação das pimentas 'Habanero' em função do grau de maturação. Posteriormente, as PCAs aplicadas separadamente nas pimentas verdes e maduras demonstraram uma separação evidente em função da data de colheita. Isso se deve ao fato de que influências do grau de maturação e época da colheita se sobressaíram no estudo. Foi obtido o agrupamento das pimentas em função do tratamento de irrigação recebido porém, para uma melhor compreensão

dos efeitos dos tratamentos de estresse hídrico sobre a composição das amostras, seria necessário uma estratégia de estudo com monitoramento mais frequente dos frutos formados bem como o emprego de outras investigações sensoriais, como o uso de olfatometria.