UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIA E TECNOLOGIA DE ALIMENTOS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA E TECNOLOGIA DE ALIMENTOS

KARLA SUZANA MORESCO

POTENCIAL ANTIOXIDANTE, EFEITO DO PROCESSO DE SECAGEM E EXTRAÇÃO DE COMPOSTOS BIOATIVOS DE PIMENTAS *CAPSICUM*

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Dissertação apresentada ao Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos como requisito parcial para a obtenção de grau de Mestre em Ciência e Tecnologia de Alimentos.

Orientadora: Prof[a]. Dr[a]. Simone Hickmann Flores

Co-orientador: Prof Dr. Alessandro de Oliveira Rios

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Universidade Federal do Rio Grande do Sul – Porto Alegre, RS, Brasil.

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RESUMO

O Brasil, sendo o centro de diversidade de pimentas do gênero *Capsicum*, muito contribui para dispersão destas espécies em todo mundo. No entanto, apesar de existirem mais de 20 espécies do gênero e da importância econômica da exploração agrícola, as potencialidades de muitas espécies ainda não foram exploradas e caracterizadas. Este trabalho teve como objetivo caracterizar quanto ao teor de compostos bioativoas e citotoxicidade em células de neuroblastoma humano genótipos de pimentas *C. chinense* e *C. annum* provenientes do Banco Ativo de Germoplasma da Embrapa Amazônia Oriental, e do herbário da Universidade Federal do Rio Grande do Sul. Avaliou-se a eficiência do uso do solvente etanol 96% na extração de compostos bioativos (carotenoides, capsaicinoides e ácido ascórbico) em pimentas *Capsicum* além de identificar as variedades promissoras para serem utilizadas em futuros programas de melhoramento em relação à presença de quantidades expressivas de fitoquímicos e sua atividade antioxidante. Também foi avaliado efeito do processo de secagem em diferentes temperaturas (50 ^{o}C, 70 ^{o}C, 80 ^{o}C) no teor de carotenoides e proposto um modelo matemático que melhor representasse o processo de secagem. Capsaicinoides, carotenoides e ácido ascórbico foram quantificados por cromatografia liquida de alta eficiência - CLAE. A análise de atividade antioxidante foi realizada por meio do método de atividade antioxidante total - TRAP. Pode-se verificar que a concentração e a atividade dos compostos bioativos presentes em pimentas pode ser diretamente relacionado com as propriedades do solvente usado na extração. O etanol foi efetivo na extração de ácido ascórbico e capsaicinoides com destaque para extração de carotenóides. Dentre os cinco amostras analisadas, o acesso Guiana destacou-se em relação aos carotenoides e a citotoxicidade em células de neuroblastoma humano e o genótipo PCL-02 destacou-se com relação à atividade antioxidante, teor de compostos fenólicos e capsaicinoides. Obteve-se também, correlações positivas entre o conteúdo de capsaicinoides e atividade antioxidante ($r = 0.87$, $p < 0.05$) e entre o conteúdo de compostos fenólicos e carotenoides ($r = 0.76$, $p < 0.05$). O processo de secagem apresentou-se adequado e possível de ser modelado pelo modelo Dois Termos Exponencial. A secagem à 50 ºC possibilitou um aumento de carotenoides resultando em uma concentração total superior a encontrada na pimenta *in natura*. A secagem sob temperaturas mais elevadas (70 ºC e 80 ºC) mostrou-se prejudicial às propriedades bioativas em relação ao teor de carotenoides.

Palavras-chave: pimentas *Capsicum*, compostos bioativos, processo secagem, solvente etanol

ABSTRACT

Brazil, being the center of *Capsicum* diversity, greatly contributes to spread of these species worldwide. However, although there are 20 species of the genus and despite the economic importance of farming, the potential of many species have not yet been explored and characterized. This work proposes the characterization the content of compounds bioativoas and cytotoxicity in human neuroblastoma cells of genotypes of *Capsicum* peppers, *C. chinense* and *C. annum*, from the Active Germplasm Bank of Embrapa Eastern Amazon and the herbarium of the University Federal of Rio Grande do Sul to evaluate the efficiency of the use of 96% ethanol solvent in the extraction of bioactive compounds (carotenoids, capsaicinoids, and ascorbic acid) in *Capsicum* peppers. Additionally, work should be undertaken to identify promising varieties for use in future breeding programs to select for significant amounts of phytochemicals and high antioxidant activity as well as to evaluate the effect of the drying process at different temperatures (50 ºC, 70 ºC, 80 ºC) on carotenoid content and propose a mathematical model that best represents drying. Capsaicinoids, carotenoids, and ascorbic acid were quantified by high performance liquid chromatography (HPLC). The antioxidant activity analysis was performed using the method of total antioxidant activity (TRAP). It can be seen that the concentration and activity of bioactive compounds present in peppers can be directly related to the properties of the solvents. Ethanol was effective in the extraction of capsaicinoids and ascorbic acid and very effective in the extraction of carotenoids. Among the five genotypes, Guiana pepper stood out in terms of carotenoid contents and cytotoxicity in human neuroblastoma cells and genotype PCL-02 pepper stood out in terms of antioxidant activity, phenolic content, and capsaicinoids. Positive correlations were obtained between the capsaicinoid content and antioxidant activity ($r = 0.87$, $p < 0.05$) and between the contents of phenolics and carotenoids ($r = 0.76$, $p < 0.05$). The drying process proved to be appropriate and can be modeled by a two-term exponential model. The drying process at 50 ºC increased the carotenoid content, resulting in a total concentration higher than that found in fresh pepper. However, drying at higher temperatures (70 ºC and 80 ºC) proved to be detrimental to the bioactive properties in terms of the carotenoid contents.

Keywords: peppers *Capsicum*, bioactive compounds, drying process, ethanol solvent.

LISTA DE FIGURAS

INDICE

1 CAPÍTULO 1

INTRODUÇÃO

Os alimentos sofrem naturalmente constantes modificações, sejam elas tanto alterações físicas, enzimáticas, quanto microbiológicas ou mesmo alterações químicas, tais como as reações de oxidação de nutrientes susceptíveis à ação do oxigênio e radicais livres que causam efeitos indesejáveis no alimento como: degradação de lipídeos, vitaminas, pigmentos, perdas no valor nutricional, desenvolvimento de sabores e odores indesejáveis e a diminuição da vida de prateleira dos alimentos.

A fim de evitar estas alterações indesejáveis a indústria de alimentos faz uso de antioxidantes, no entanto os mais utilizados pela indústria de alimentos são artificiais e estudos toxicológicos em modelos com animais têm demonstrado a possibilidade de estes apresentarem efeitos carcinogênicos (RAMALHO; JORGE, 2006). Neste contexto, há grande interesse em encontrar nas substâncias naturais, princípios ativos que possuam ação antioxidante, que sua produção não gere danos ao ambiente e que possam contribuir para a conservação de alimentos, sem causar danos à saúde humana (COSTA, et al. 2009).

A importância também em explorar a ação antioxidante de substâncias naturais está em avaliar a capacidade de proteção celular frente ao estresse oxidativo e à formação de radicais livres, observada em algumas patologias como, por exemplo, o câncer, a doença de Alzheimer, arteriosclerose, artrite reumatóide, catarata, diabete, doença de Parkinson, entre outras (HALLIWELL; GUTTERIDGE, 1990; LIPINSKI, 2001).

Estima-se que existam de 250.000 a 500.000 espécies de plantas no mundo (BOSE, 1958), sendo que, uma pequena porcentagem (1-10%) é usada na alimentação humana e animal. Por outro lado, as populações indígenas empregam inúmeras plantas como fontes de agentes medicinais para o tratamento de doenças (COWAN, 1999). As pimentas e pimentões foram possivelmente os primeiros aditivos alimentares utilizados pelas civilizações antigas do México e da América do Sul (REIFSCHNEIDER 2000).

Atualmente as pimentas são amplamente valorizadas na culinária mundial como condimentos. Na indústria são amplamente utilizados os seus pigmentos, aromas e substâncias pungentes; elas são ricas em vitaminas, flavonóides, carotenoides e outros metabólitos secundários com propriedades antioxidantes que reduzem o risco de

desenvolvimento de câncer e de outras doenças crônico-degenerativas. (LUTZ; FREITAS, 2008).

Estima-se a existência de 20 a 25 espécies de pimentas silvestres e apenas 4 espécies domesticadas (MCLEOD; GUTTMAN; ESHBAUGH, 1983). Segundo Bianchetti (1996) o Brasil é o *habitat* de várias espécies de *Capsicum* desconhecidas, não utilizadas ou ainda não caracterizadas. Algumas destas espécies estão ameaçadas de extinção (BUSO, et al., 2001).

A importância do cultivo de pimenta no Brasil deve-se, tanto pelas características de rentabilidade, principalmente quando o produtor agrega valor ao produto e pela necessidade de poucos investimentos, quanto pela importância social, por empregar elevada mão de obra, principalmente na agricultura familiar (RUFINO; PENTEADO, 2006).

Este trabalho, desenvolvido em parceria com a Embrapa, através do projeto Amazônia Oriental objetivou aprofundar a caracterização de alguns genótipos de pimenta *Capsicum Chinense* e *Capsicum annum* de modo a estimular a exploração da biodiversidade nacional, esclarecendo as potencialidades destes frutos, sua utilização em prol dos benefícios à saúde, bem como proporcionar um avanço da economia através da comercialização.

1.1 Revisão

1. 1.2 Pimentas *Capsicum*

As pimentas do gênero *Capsicum*, (palavra grega Kapto = morder ou picar), pertencem à família *Solanacea* (REIFSCHNEIDER, 2000). Este gênero é composto de 20 a 25 espécies de pimentas silvestres e dentre estas quatro são domesticadas: *Capsicum annuum, Capsicum baccatum, Capsicum frutenscens e Capsicum chinense* (MCLEOD; GUTTMAN; ESHBAUGH, 1983). Grande parte das espécies brasileiras deste gênero ainda não foi estudada e domesticada (BIANCHETTI,1996).

Segundo Reifschneider (2000) a domesticação de uma espécie ocorre quando o homem interfere no processo natural de seleção, definindo de acordo com seus interesses de cultivo, produtividade e qualidade, quais as características que deseja

perpetuar, passando assim a selecionar apenas as sementes das plantas cujas características desejadas forem mais nítidas.

As espécies *Capsicum chinense* e *Capsicum annum* são consideradas as mais brasileiras das espécies, visto que foram cultivadas inicialmente pelos indígenas na Amazônia. Esta região apresenta uma área de maior diversidade, onde o cultivo de pimentas é um importante fator de geração de renda para as populações agrícolas. Sabe-se que atualmente as espécies apresentam mais de 40 variedades ou genótipos (REIFSCHNEIDER, 2000).

A designação *chinense* não é apropriada, pois não há nenhuma relação desta pimenta inegavelmente americana, com a China. Esta espécie destaca-se pela ampla adaptação às condições ambientais e por possuir diversidade de formas e cores dos frutos que são geralmente muito picantes e aromáticos (CARVALHO et al. 2006).

São genóticos da espécie *Capsicum chinense* e *Capsicum annum* as pimentas cujos nomes comuns são: pimenta “Biquinho”, pimenta “Guiana”, pimenta “Biquinho”, pimenta “Amarela olho de peixe”, pimenta “cheiro comum”, pimenta “Goiânia”, e pimenta “PCL - 02” (Figura 1).

FIGURA 1- Genótipos de pimentas das espécies *Capsicum chinense* e *Capsicum annum*.

Segundo Neto (2004) a tamanha diversidade das pimentas do gênero *Capsicum* é propiciada pela facilidade com que ocorrem cruzamentos entre uma mesma espécie ou de espécies diferentes. As abelhas melíferas são o principal agente de dispersão do pólen das flores. Uma característica da espécie é a autogamia onde as flores são hemafroditas e se autofecundam.

Embora o Brasil seja rico em diversidade e variabilidade para o gênero *Capsicum,* ainda pouco se sabe sobre muitas espécies e pouco se explora as potencialidades de diversas espécies silvestres e semi-cultivadas.

1.1.3 Dados de Produção

A pimenta é um dos produtos do agronegócio brasileiro que incentiva a agricultura familiar contribuindo para o aumento na geração de renda e emprego no país (KAPPEL, 2007).

Segundo dados da FAO, em 2010 foram cultivados cerca de 3,8 milhões halqueires de pimentas e pimentões por todo o mundo, totalizando uma produção de 30,6 milhões de toneladas. A área anual cultivada de pimenta no país possibilita produção de 249.000 toneladas de frutos por ano e produtividade média de 200 toneladas por alqueire, conforme o tipo de pimenta. No Brasil, a produção ocorre em praticamente todas as regiões tendo como principais estados produtores, Minas Gerais, Goiás, São Paulo, Ceará, e Rio Grande do Sul (FAO, 2012).

A produção de pimenta cresceu significativamente nos últimos anos. Estima-se que atualmente a demanda deste mercado seja de aproximadamente 80 milhões de reais, valores que reforçam a representatividade dessa cadeia do agronegócio. Segundo dados do Trade Information Brief (TIB, 2005), o aumento no consumo de pimentas em escala mundial é justificado pelo aumento da renda além da maior preocupação com uma dieta saudável em países desenvolvidos e é reflexo do aumento da produção e industrialização em países em desenvolvimento.

O faturamento anual proporcionado pelo aumento da demanda do mercado o tem impulsionado a um aumento da área cultivada e o estabelecimento de agroindústrias, classificando o agronegócio de pimentas (doces e picantes) como um dos mais importantes do país, (REIFSCHNEIDER; RIBEIRO, 2004a).

Segundo Reifschneider e Ribeiro (2004a), os produtos do processamento de pimentas doces e picantes podem ser divididos em três tipos, de acordo com sua utilização na indústria de alimentos:

- Flavorizantes: frutos processados ainda verdes ou maduros, que dão apenas sabor e aroma ao alimento, sendo a coloração uma característica secundária;
- Corantes: sua função principal é dar cor aos produtos, como a páprica vermelha, que pode ser suave ou picante;
- Condimentos: processadas na forma de pó, flocos, picles, escabeches, molhos líquidos, conservas de frutos inteiros e geléias.

Apesar da sua importância comercial as pimentas do gênero *Capsicum* têm sido pouco exploradas no Brasil em relação ao seu comportamento fisiológico (atividade respiratória, evolução de etileno, teores de pigmentos carotenoides e clorofílicos e potencial antioxidante) no período pós colheita (MATTOS et al. 2007), fato este que torna o fruto desconhecido por muitos.

Embora atualmente o mercado brasileiro de pimentas ainda seja pequeno comparado com o das demais hortaliças, em virtude do baixo consumo e da quantidade comercializada. Um dos problemas apontados pelas agroindústrias processadoras de pimenta é a baixa diversificação, ou seja, número limitado de cultivares disponíveis no mercado (REIFSCHNEIDER; RIBEIRO. 2008). A exploração de novos tipos de pimentas e o desenvolvimento de novos produtos com maior valor agregado, como conservas ornamentais, geléias especiais e outras formas processadas, apresenta boas perspectivas de mercado tanto para as pimentas *in natura* como para processadas (HENZ; RIBEIRO, 2008).

1.1.4 Radicais livres

Os radicais livres são átomos ou moléculas produzidas continuamente durante os processos metabólicos e atuam como mediadores para a transferência de elétrons em várias reações bioquímicas, desempenhando funções relevantes no metabolismo. São derivados do oxigênio, nitrogênio e moléculas de enxofre no sistema biológico e são altamente ativos para reagir com outras moléculas, devido seus elétrons desemparelhados. Estes radicais são parte importante dos grupos de moléculas

chamadas reativas de oxigênio e as espécies de nitrogênio (ROS / RNS), que são produzidos durante o metabolismo celular e atividades funcionais, tendo um papel importante na sinalização celular, apoptose, expressão gênica e do transporte de íons (LUE et al., 2010).

A presença de radicais livres tem sido correlacionada a um grande número de doenças. O excesso de ataque dos ROS em ácidos nucléicos, cadeias laterais de aminoácidos e em ligações duplas de ácidos graxos insaturados causa estresse oxidativo que pode danificar DNA, RNA, organelas celulares como mitocôndria e membranas, proteínas e lipídios, provocando alterações da estrutura e função celulares; (LUE et al., 2010), o que indica que estas espécies não têm um papel etiológico na grande maioria dos estados patológicos, mas que participam diretamente dos mecanismos fisiopatológicos que determinam a continuidade e as complicações presentes nestes processos (HALLIWEL, 2001; LIPINSKI, 2001).

O estresse oxidativo, portanto, é caracterizado por uma intensa sobrecarga de radicais livres resultantes do desequilíbrio entre a redox, a formação e a eliminação de ROS (VITVITSKY, 2006; FLORA, 2007), gerando o desequilíbrio entre moléculas oxidantes e antioxidantes (SIES, 1993). Portanto, a insuficiência dos sistemas biológicos em neutralizar a formação excessiva de radicais livres, que resulta na indução de danos celulares e doenças é o estresse oxidativo (FLORA, 2007)

O organismo possui mecanismos de proteção para evitar o acúmulo de radicais livres gerados por reações de oxidação, através de compostos antioxidantes. Antioxidantes são compostos que protegem sistemas biológicos contra os efeitos potencialmente danosos de processos ou reações que promovem a oxidação de macromoléculas ou estruturas celulares. O sistema de defesa antioxidante do organismo compreende um grande número de substâncias que atuam em diferentes níveis. O sistema de defesa primário é formado por substâncias que impedem a geração ou sequestram espécies reativas, de forma a evitar sua interação com alvos celulares bloqueando a etapa de iniciação radicalar (HALLIWELL; GUTTERIDGE, 1999).

Os antioxidantes, geralmente, catalisam a inativação de radicais livres no ambiente intracelular, previnem ligações com metais de transição, como ferro e cobre, interrompendo a interação desses com o peróxido de hidrogênio e com o radical ânion superóxido e, consequentemente, a formação de espécies altamente reativas, como o radical hidroxila. Os dois principais meios de defesa antioxidante são os meios enzimático.e não enzimático (HALLIWELL, 1991).

O sistema de defesa enzimático inclui as enzimas superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GSH-Px). A SOD elimina, por catalização, os radicais superóxidos convertendo-os em peróxido de hidrogênio e oxigênio. O peróxido de hidrogênio pode ser eliminado pela CAT e peroxidase. Entre as peroxidases está a GSH-Px que, junto com a glutationa redutase, possue um papel importante na eliminação do peróxido de hidrogênio, evitando assim o acúmulo de radicais hidroxilas (HALLIWELL; GUTTERIDGE, 1999).

O sistema de defesa antioxidante não enzimático é realizada por substâncias como as vitaminas E e C, os carotenoides, compostos fenólicos ou aminas aromáticas que atuam bloqueando a etapa de propagação da cadeia radicalar, sequestrando radicais intermediários (peroxila ou alcoxila e vários antioxidantes sintéticos (HALLIWELL; GUTTERIDGE, 1999).

1.1.5 Ação de compostos antioxidantes no organismo

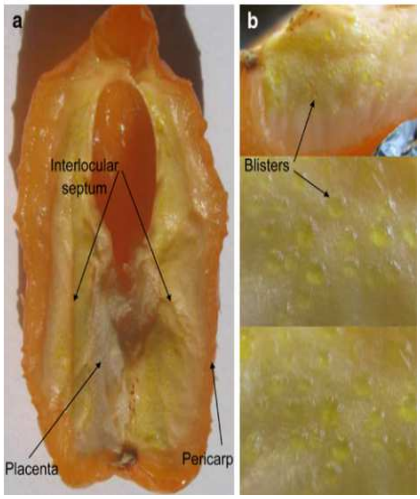
As pimentas além de micro e macronutrientes contêm uma série de substâncias com propriedades antioxidantes que podem intervir sobre o curso de doenças, contribuindo assim para a saúde. Dentre tais compostos pode-se destacar: ácido ascórbico, capsaicinoides, tocoferol e carotenoides, substâncias estas, que possuem efeitos importantes na proteção contra o dano oxidativo causado por radicais livres. (OGISO et al., 2008).

Melo et al. (2011) avaliaram a atividade antioxidante nas pimentas *Capsicum chinense* (bode), *Capsicum baccatum* variedade praetermissum (cumari) e *Capsicum frutescens* (malagueta) através do ensaio com DPPH (2,2-difenil-1-picril-hidrazil). Os autores verificaram que a capacidade antioxidante das pimentas não está apenas relacionada com o teor de compostos fenólicos. Dentre os principais compostos antioxidantes encontrados em pimentas do gênero *Capsicum* destacam-se os capsaicinoides, carotenoides, compostos fenólicos, ácido ascórbico e tocoferol (HENZ; RIBEIRO, 2008).

1.1.7 Capsaicinoides

Os capsaicinoides são compostos bioativos presentes nas pimentas do gênero *Capsicum*, responsáveis pela sensação de pungência e picância. Estes são sintetizados naturalmente na placenta da pimenta, e armazenados nas bolhas situadas na superfície externa da placenta conforme apresentado na Figura 2 (GONZALEZ; PALENIUS; ALEJO, 2010).

FIGURA 2 - Tecidos de frutos de pimenta dessecados e sem sementes (a). Septo interlocular mostrando as bolhas onde os capsaicinoides se acumulam (b).

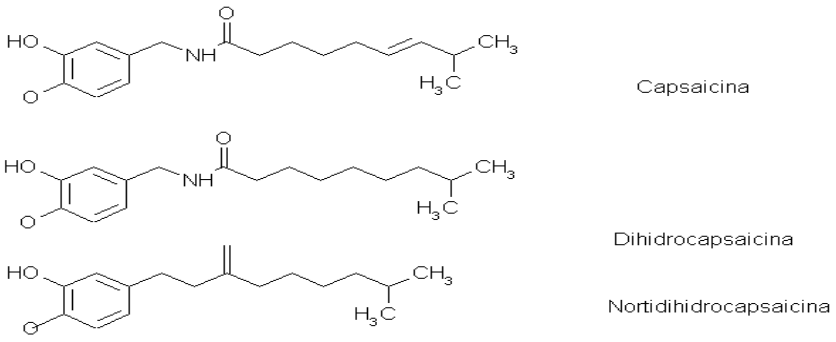


Fonte: GONZALEZ, PALENIUS , ALEJO, (2010).

A síntese dos capsaicinoides ocorre por meio da via dos fenilpropanóides através da condensação de uma molécula de vanililanida com um ácido graxo ramificado sintetizado (CURRY et al., 1999).

Segundo Mazourek et al. (2009), atualmente já têm-se identificadas mais de dez estruturas de capsaicinoides. A capsaicina e a dihidrocapsaicina correspondem a 90% destes (CHOI et al. 2006).

FIGURA 3 - Estruturas químicas dos principais capsaicinoides encontrados nas pimentas



Fonte: GONZALEZ; PALENIUS ; ALEJO, (2010).

Os principais capsaicinoides encontrados na pimenta são: capsaicina, dihidrocapsaicina e nortidihidrocapsaicina, cujas estruturas quimicas são expressas através da Figura 3 (DAVIS et al 2007).

O grau de pungência e o conteúdo de capsaicinoides do gênero *Capsicum* podem variar conforme a espécie e seus genótipos. Os fatores que influenciam na pungência são o estágio de desenvolvimento, crescimento e idade dos frutos, além das condições de manejo (BOSLAND, 1993). O inicio do acúmulo dos capsaicinoides em pimentas ocorre entre 14 e 40 dias após a antese (abertura das flores), atingindo os valores máximos entre os 25 e 70 dias. Por outro lado, o descrécimo do teor de capsaicinoides começa a ocorrer somente acima de 50 dias após o florecimento (ESTRADA et al., 2000) Kirschbaum-titze et al. (2002) afirmaram que pimentas cultivadas nas estações primavera e verão são mais pungentes que as cultivadas nas estações outono e inverno. Tal fato se deve ao estresse que ocorre nas estações mais quentes que influencia a via dos fenilpropanóides e afeta diretamente a síntese dos capsaicinoides. Siqueira et al. (1988), encontraram teores de 0,174% de capsaicina em frutos de *Capsicum annuum* na fase de maturação.

O primeiro método desenvolvido para avaliar o teor de capsaicinoides em pimentas foi o teste de Scoville, criado em 1992 pelo quimico Wilbur Scoville. O principio do teste consistia em um método de diluição e prova no qual um extrato de pimenta pura era diluida em solução de água com açúcar e submetida a análise sensorial. Quanto mais água fosse necessária para diluição maior seria o calor

proporcionado pela pimenta e maior a pungência quantificada em unidades de calor Scoville (Scoville Heat Units – SHU). A concentração de uma parte por milhão (ppm) de capsaicinoides corresponde a 15 SHU. Devido a certas limitações desse método, em virtude da subjetividade humana, atualmente a análise de capsaicinoides é realizada por meio de análises cromatográficas (BOSLAND, 1993).

A capsaicina e a dihidrocapsaicina possuem diferentes atividades biológicas das quais se destaca a ação antioxidante. Materska e Perucka (2005), ao avaliarem a atividade antioxidante pelo método DPPH verificaram que a ação antioxidante da capsaicina foi maior que da dihidrocapsaicina, mostrando que a dupla ligação da cadeia lipídica do primeiro composto influência nesta ação, ou mesmo outro fator que pode explicar tal diferença, seja a presença dos grupos metoxi e OH na posição orto do anel aromático.

Por meio de experimentos realizados *in vitro*, Ahuja et al. (2006) demostraram que extratos de pimenta contendo capsaicina, e dihidrocapsaicina proporcionam um aumento na resistência quanto à oxidação no LDL, tanto por meio do atraso do início da oxidação ou por retardar a taxa de oxidação.

Em experimentos *in vivo*, com ratos alimentados com dietas com elevado teor de gordura, o tratamento com capsaicina reduziu o colesterol total e o nível de peróxidos lipídicos, (MANJUNATHA; SRINIVASAN, 2006). Estes trabalhos demostraram a propriedade antioxidante de capsaicinoides indicando sua importância na prevenção de doenças cardiovasculares.

Apesar do potêncial antioxidante que os capsaicinoides apresentam, a aplicação destes, como antioxidantes em alimentos é limitada em virtude da forte pungência. No entanto recentemente, descobriu-se que no gênero *Capsicum* existem substâncias análogas aos capsaicinoides encontradas em frutos *Capsicum* não pungentes, o capsiate e dihidrocapsiate que também apresentam atividade antioxidante. (ROSA et al. 2002a)

1.1.8 Carotenoides

A cor vermelha intensa, característica dos frutos de *Capsicum* é devido aos carotenoides, pigmentos que são sintetizados principalmente durante a maturação (MENICHINI et al. 2009)

Os carotenoides são compostos bioativos presentes nas frutas e hortaliças que além de serem responsáveis pela cor apresentam excelentes propriedades antioxidantes. Eles tanto podem atuar na proteção de células contra radicais livres como sequestrar espécies reativas de oxigênio, (EDGE; MCGARVEY; TRUSCOTT, 1997). Segundo Young e Lowe (2001) a propriedade antioxidante dos carotenoides se deve a presença de um sistema de duplas ligações conjugadas, que confere a estes a capacidade de capturar os radicais livres.

O principio da atuação dos carotenoides na desativação do oxigênio singlete ocorre por meio da transferência de energia que tanto pode ser por meio de reações químicas (processo químico) como reações físicas (processo físico) (STRATTON; SCHAEFER; LIEBLER, 1993; RIOS; ANTUNES; BIANCHI, 2009). A desativação física envolve a transferência de energia de excitação do oxigênio singlete O_2 ($^1\Delta_g$), para o carotenoide (^{1}CAR), resultando em formação de oxigênio no seu estado fundamental O_2 ($^3\sum^-_g$), estado triplete excitado do carotenoide (^{3}CAR*). A energia do ^{3}CAR* é dissipada através de interações rotacionais e vibracionais entre o carotenoide e o solvente, para recuperar o estado fundamental do mesmo, sem ocorrer degradação Diferente da desativação física (*Kq*) (Equação 1), o processo químico (*Kr*) (Equação 2) resulta em destruição do cromóforo e na formação de produtos de oxidação com a possibilidade de reações de adição (STRATTON; SCHAEFER; LIEBLER, 1993; RIOS; ANTUNES; BIANCHI, 2009)

$$O_2(^1\Delta_g) + {}^1\text{CAR} \xrightarrow{Kq} O_2(^3\sum\nolimits_g^-) + {}^3\text{CAR}^* \quad \text{Eq. (1)}$$

$$O_2(^1\Delta_g) + {}^1\text{CAR} \xrightarrow{Kr} \text{produtos} \quad \text{Eq. (2)}$$

Radicais de oxigênio, como o ânion superóxido (O_2^-), pode ser reduzido pelo β-caroteno devido à capacidade do carotenoide em doar elétrons para espécies reativas de oxigênio (Equação 3). (STRATTON; SCHAEFER; LIEBLER, 1993; RIOS; ANTUNES; BIANCHI, 2009).

$$O^{\bullet -} + \text{CAR} + 2H^+ \longrightarrow \text{CAR}^{\bullet +} + H_2O_2 \quad \text{Eq. (3)}$$

Levy et al. (1995) classificaram os carotenoides em dois grupos: grupo com atividade de pró-vitamina A, do qual fazem parte: α-caroteno, β- caroteno e β-criptoxantina, e o grupo das xantofilas oxigenadas e sem atividade de pró-vitamina A,

mas que no entanto, são considerados potentes antioxidantes que auxiliam na prevenção da catarata e na degeneração macular relacionada com o envelhecimento (MATSUFUJI et al., 1998). Para Goddard e Mathews, (1979), os seis compostos mais importantes que conferem proteção aos estresses oxidativos são o α-caroteno e β-caroteno, luteína, zeaxantina e a β-criptoxantina.

Os principais carotenoides das pimentas *Capsicum* são a capsorubina e a capsantina cujas concentrações variam de 30 a 60% do total dos carotenoides em frutos plenamente maduros (MATSUFUJI et al. 1998). As pimentas contêm ainda, as pro-vitaminas: β-caroteno, α-caroteno, γ-caroteno e a β-criptoxantina, que são transformadas no fígado em vitamina A. O consumo diário de meia colher de sopa de pimenta vermelha desidratada em pó pode suprir as necessidades de vitamina A, que corresponde a 600 microgramas/dia. (LUTZ; FREITAS 2008). Topuz e Ozdemir (2007) detectaram em cultivares do *Capsicum annuum* através de cromatografia líquida de alta eficiência - CLAE a concentração de carotenoides variando de 2310-2390 mg/Kg de pimentas em base seca.

O acúmulo de carotenóides é dependente, além da espécie vegetal, de fatores ambientais externos, como características climáticas, que envolvem incidência de luz, temperatura e solo (ZANATTA; MERCADANTE, 2007). A estabilidade desses carotenóides depende de outros fatores como, o resíduo de oxigênio dissolvido na amostra, temperatura durante o armazenamento (VÁSQUEZ-CAICEDO, et al., 2007) e a matriz em que estão contidos.

O processamento pode afetar a qualidade do produto. Várias são as mudanças que um alimento pode sofrer durante o processamento, armazenamento e distribuição, desde alterações no aspecto físico, estrutura (KAREL et al., 1993) e reações bioquímicas que envolvem a degradação de substâncias nutritivas (ROOS e KAREL, 1991). A secagem é um dos processos, cujo principio consciste na retirada de umidade de um produto/substância, visando a preservação dos alimentos. No entanto, este processo é dependente de muitas váriáveis (RATTI; MUJUMDAR, 1995). Os produtos obtidos pela secagem podem ter vida de prateleira estendida por um ano ou mais, porém a qualidade inicial pode ser parcialmente ou drasticamente reduzida (RATTI, 2001).

O tratamento térmico incluindo o processo de secagem pode diminuir o conteúdo de carotenóides nos alimentos pela sua natureza propensa à degradação ou isomerização (MAIANI et al., 2009), ainda podem favorecer a inativação da enzima lipoxigenase que, nos tecidos vegetais, catalisa a peroxidação lipídica, dando origem a

hidroperóxidos que se descompõem nas formas radicais peroxil e alcoxil responsáveis pela degradação destes pigmentos (BALL, 2006).

1.1.9 Compostos Fenólicos

Os compostos fenólicos são estruturas que possuem anel aromático com um ou mais substituintes hidroxílico (LEE, et al. 2005). Dreosti (2000) afirma que existem mais de 8000 compostos fenólicos.

Segundo Li et al. (2009), os compostos fenólicos podem ser dividido em dois grandes grupos: os flavonóides, subdivididos em flavonas, flavanóis, flavonóis, flavanonas, isoflavonas e antocianidinas, e os não flavonóides, que compreendem os grupos dos ácidos fenólicos, lignanas e estilbenos.

Os compostos fenólicos se destacam pela sua excelente capacidade antioxidante (RICE-EVANS; MILLER; PAGANGA,1996). A efetividade destes compostos como antioxidantes depende do número e da localização dos radicais hidroxila na molécula (MELO; GUERRA, 2002; BURNS et al., 2001).

Estes compostos possuem a capacidade de bloquear a ação dos radicais livres, transformando-os em produtos estáveis por meio da doação de hidrogênio aos elétrons. Atuam também nas reações com radicais lipídicos, formando complexo antioxidante-lipídio (ARAÚJO, 2001). Segundo Atoui et al. (2000) eles também podem atuar como quelantes de metais.

As antocianinas são um grupo de compostos fenólicos que podem prevenir injúrias causadas pelos radicais livres por meio da estabilização das espécies reativas de oxigênio mediante sua reação com o componente reativo do radical. O alto poder de reação do grupo hidroxil das antocianinas com o radical faz com que o mesmo torne-se inativo (NIJVELDT et al., 2001).

Melo et al. (2011) avaliaram o teor de compostos fenólicos nas pimentas *Capsicum chinense* (bode), *Capsicum baccatum* variedade praetermissum (cumari) e *Capsicum frutescens* (malagueta). As concentrações de compostos fenólicos, expressas em ácido gálico foram: 29400 mg EAG /100g de matéria seco para pimenta “bode”, 34712 mg EAG/100g de matéria seca para pimenta “cumari” e de 1328,28 mg EAG/100g de matéria seca para pimenta malagueta.

Alvarez-Parrilla et al. (2011) avaliaram concentrações de compostos fenólicos em pimentas (*C. annuum*) frescas e processadas encontraram concentração fenólicos de 568 mg EAG/100g de matéria seca para pimentas processadas em concerva e valores de 1032 mg GAE/100g para pimentas frescas. Estes valores coincidem com trabalhos propostos por Chuah, et al (2008); Deepa, et al. (2007) que encontraram valores variando na faixa de 33 a 250mg g EAG/100g peso fresco para pimentas frescas e de 400-1200 mg EAG/100g peso seco para pimentas secas. A quantidade de compostos fenólicos em frutos depende da espécie, da variedade, do meio ambiente, do estado fisiológico e das condições climáticas (Scalbert e Williamson, 2000).

1.1.10 Ácido Ascórbico

O ácido ascórbico é uma vitamina hidrossolúvel e um importante antioxidante, ele reage diretamente com o oxigênio, radical hidroxila e radical superóxido, além de regenerar a vitamina E, esta vitamina mantém as enzimas sulfidrílicas em seus estados reduzidos e poupa a glutationa peroxidase, que é um importante antioxidante intracelular e cofator enzimático (CARR; FREI, 1999;)

O ácido ascórbico pode ser considerado também como um antioxidante sinergístico, ou seja, aquele que atua como removedor de oxigênio e complexante por agir na regeneração de radical fenoxil, doando hidrogênio e regenerando o antioxidante primário. Assim pode-se utilizar um antioxidante fenólico em menor quantidade quando for utilizado junto com um sinergístico (ARAÚJO, 2001). O ácido ascórbico é considerado um marcador para a determinação do grau de oxidação em frutas e hortaliças minimamente processadas (BARTH et al., 1993).

Apesar de ser amplamente conhecido por sua atividade antioxidante, o ácido ascórbico também pode atuar como pró-oxidante, pois ao doar os dois hidrogênios redutores fica susceptível a receber elétrons, devido ao radical ascorbila formado, que é um agente oxidante (HASSIMOTO et al., 2005). Assim, baixas concentrações de ascorbato aumentam a atividade dos radicais de oxigênio, enquanto que altas concentrações de ascorbato atuam sequestrando radicais hidroxila, oxigênio singlete e peróxidos (SAKAGAMI et al., 2000).

Segundo Reifschneider (2000) o gênero *Capsicum* é considerado uma boa fonte de ácido ascórbico. O conteúdo de ácido ascórbico encontrado nas pimentas brasileiras varia de 52 a 134 mg/100g nos frutos frescos. A pimenta *Capsicum chinense* (biquinho) contém 99mg de ácido ascórbico/100g de produto fresco, quantidade superior a necessidade diária de um indivíduo (60 mg/dia), (LUTZ; FREITAS 2008).

1.2 Objetivos

1.2.1 Objetivo Geral

Devido ao grande potencial, em relação aos compostos funcionais encontrados em outras espécies de *Capsicum*, este projeto propõe caracterizar genótipos de *C. chinense e C. annun* do Banco Ativo de Germoplasma da Embrapa Amazônia Oriental, visando identificar os genótipos promissores para futuros programas de melhoramento com relação à presença de quantidades expressivas de fitoquímicos e sua atividade antioxidante, bem como avaliar o efeito do processamento térmico na quantidade de compostos bioativos.

1.2.2 Objetivos específicos

1. Avaliar a atividade antioxidante dos genótipos de pimenta por meio da análise de potencial reativo total TRAP.

2. Identificar o perfil de compostos bioativos presentes nos genótipos de pimenta capsaicinoides, ácido ascórbico, carotenoides, e compostos fenólicos.

3. Verificar qual dos genótipos de pimenta destacou-se em relação ao potencial antioxidante total, compostos bioativos e nas análises de viabilidade em célula para uso em programas de melhoramento genético.

4. Verificar o teor de carotenóides dos genótipos *Capsicum chinense* Jacquin em relação ao processamento térmico em diferentes temperaturas, quanto ao teor de carotenoides.

2 CAPÍTULO 2

ARTIGOS CIÊNTIFICOS

2.1 ARTIGO 1: Extraction of bioactive compounds from peppers using a food grade solvent

EXTRACTION OF BIOACTIVE COMPOUNDS FROM PEPPERS USING A FOOD GRADE SOLVENT

ABSTRACT

The present study examined the efficacy of ethanol (96%) for the extraction of bioactive compounds (ascorbic acid, carotenoids and capsaicinoids) from several *Capsicum annum* pepper genotypes (Amarela olho de peixe, Guiana, Goiânia and PCL-02) and from one *Capsicum chinense* Jacquin pepper genotype (Cheiro comum) by high performance liquid chromatography (HPLC). Extractive solution prepared with ethanol 96% presented higher carotenoids concentration. The use of 96% ethanol was effective the preparation of extract with lower concentrations of capsaicinoids is a viable alternative and can be exploited in the food industry.

Keywords: ethanol, carotenoids, capsaicinoids, ascorbic acid, peppers.

1. INTRODUCTION

Bioactive compounds, such as ascorbic acid and carotenoids, are found naturally in many foods, including fruits and vegetables, such as peppers (WAHYUNI et al., 2011). The diversity and levels of each bioactive compound found in foods are determined by both genetic factors and environmental factors (BATTINO et al., 2009). According to Bae et al. (2012), another factor responsible for the variation in the levels of these compounds is the analytical method used to determine their concentration in foods.

Mithen et al. (2000) and Weiss et al. (2008) reported that one of the factors complicating the quantification of bioactive compound levels may be attributed to the synergy between the compounds of the food matrix that can bind and inactivate compounds of interest, thereby yielding inaccurate results.

The choice of extraction solvent can influence the effectiveness of bioactive compound concentration measurements (BETTAIEB; REBEY, et al., 2011; CHUNG et al. 2010). The concentration and activity of bioactive compounds naturally present in foods can be directly related to the properties of solvents, such as lipophilic and hydrophilic solvents, and their respective polarity (MENICHINI et al., 2009; SUN et al., 2007). Carotenoids, which are lipophilic compounds, are easily extracted with nonpolar

solvents, but flavonoids, which are hydrophilic, are extracted in higher quantity with polar solvents.

Although the use of organic solvents in the production of plant extracts is conventional, questions remain regarding the pharmacological and environmental toxicity of these extracts as well as the danger resulting from manipulating these extracts. Thus, substitution of other solvents by 96% ethanol is of great interest for decreasing operational costs, environmental impacts and toxicity.

Considering, the presence of the high content of the bioactive compounds in extracts de pepper *Capsicum*, the influence of solvent of the extracts, the present work was designed to investigate the efficiency of extraction of bioactive compounds from pepper genotypes using ethanol (96%).

2 MATERIALS AND METHODS

2. 1 Chemical Reagents

The following standards were purchased from Sigma (St. Louis, Missouri, USA): capsaicin (purity > 98%), dihydrocapsaicina (purity > 90%), β-cryptoxanthin (purity > 97%), β-carotene (purity > 93%), α-carotene (purity > 95%), zeaxanthin (purity > 95%), ascorbic acid (purity $\underline{\sim}$ 98%) and supra pure sulfuric acid.

The lutein standard (purity > 95%) was purchased from Indofine (Hillsborough, New Jersey, USA). The HPLC-grade solvents, including acetonitrile, methyl-tert-butyl, and methanol, were purchased from Scientific Hexis (Jundiaí, São Paulo, Brazil). Acetic acid, 96% ethanol, and glycine were purchased from Vetec (Duque de Caxias, Rio de Janeiro, Brazil).

2.2 Plant material

Fruits from four genotypes of *Capsicum annun* L. *var*. *annuum* peppers identified in the Embrapa Amazonia Oriental as Goiânia (voucher number IAN 186314), Guiana (voucher number IAN 186303), PCL-02 (voucher number IAN 186306), and Amarela olho de peixe (voucher number IAN 186308) as well as one genotype of *Capsicum chinense* Jacquin pepper identified as Cheiro comum (voucher

number IAN 186307) were used in the present study. These plants were harvested between the months of August and September 2011 in the region of Belém, Pará, Brazil.

Fruits were selected and packed under vacuum (Vacuum Sealer F200 Flash, Brand Fastvac, Ansan-City, South Korea) in polyethylene bags with aluminum foil to avoid contact with light, and the packaged fruit was frozen (-10 °C) until analysis.

2.3 Preparation of extracts

Extracts prepared with food-grade ethanol result in extracts rich in bioactive compounds that are likely used in foods and are free toxic compounds. To compare the content of bioactive compounds in the alcoholic extracts, quantitative extractions were performed with each pepper extracted by methods well established in the literature.

2.3.1 Alcoholic extraction

The peppers were weighed, macerated with an ethanol solvent (96%) until complete removal of pigments and filtered using a vacuum system. The extract was concentrated under reduced pressure in a rotary evaporator at ambient temperature (Fisatom, Model 801, St. Paul), diluted in 5 mL of ethanol, fractionated into 2 mL aliquots and placed in amber vials. The solvent was removed under nitrogen flow, and the extracts were stored at -10 °C until the quantification of carotenoids, ascorbic acid and capsaicinoids by high performance liquid chromatography (HPLC). The results were compared with those obtained by other methods described below.

2.3.2 Extracts for quantification of total bioactive compounds

The quantification of total carotenoids was performed as previously described by Mercadante and Rodriguez-Amaya, (1998). This methodology consists of pigment extraction with acetone and methanol, saponification with 10% KOH for 24 hours at room temperature, removal of alkali and concentration under reduced pressure in a rotary evaporator (Fisatom, Model 801) (T<25 °C). Immediately after being concentrated, the sample was rediluted with a small aliquot of solvent, dried with nitrogen flow and stored in the freezer (-18 °C) for later quantification by high performance liquid chromatography.

The quantification of total capsaicinoids was performed as previously described by Collins, Wasmund and Bosland, (1995). Was collected 2 g of sample was added to 12 mL of acetonitrile, and the mixture was incubated in a water bath for 4 hours at 80 °C. Subsequently, a 1 mL aliquot was collected and filtered (0.45 μm Millipore filter).

The quantification of ascorbic acid was carried out according to Rose et al. (2007) with modifications proposed by Pereira et al. (2012). Briefly, 5 g of sample was homogenized in an Ultra-Turrax (Ultra-digital loggerheads T25) with 20 mL of 0.05 M supra pure sulfuric acid for 1 minute and centrifuged under cooling at 4°C for 30 minutes at 3500xg (Walita-Philips®, São Paulo, Brazil). A 1 mL aliquot was used for quantification using a high performance liquid chromatography system.

2.5 Chromatographic conditions

The analyses were performed using an HPLC system (Agilent series 1100, Santa Clara, CA, USA) that was equipped with an online degasser, a quaternary pump, and an automatic injector.

2.5.1 Carotenoids

For the chromatographic analysis, the concentrated extract was diluted in methyl tert-butyl ether (MTBE), placed in an ultrasound (Unique, Model USC 1400) for 15 minutes and filtered (0.45 μm Millipore filter) for subsequent injection into the chromatograph.

The carotenoids were separated on a polymeric reverse phase column (YMC C_{30} 250 micrometer x 4.6 micrometer; particle size of 3 mM) with a mobile phase gradient elution beginning with water/methanol/MTBE at 5:90:5 and reaching 0:95:5 after 12 minutes, 0:89:11 after 25 minutes, 0:75:25 after 40 minutes and 00:50:50 after 60 minutes with a flow rate of 1 mL/minutes at 33 °C Zanatta and Mercadante, (2007). The spectra were conducted between 250 and 600 nm, and the chromatograms were processed at a fixed wavelength of 450 nm for carotenoids. Identification was performed by comparison of peak retention times obtained in each sample with the retention times of standards analyzed under the same conditions.

The quantification was performed constructing standard curves for the carotenoids in the following concentration ranges: 5-50 μg/mL for β-carotene, 2-25

µg/mL for α-carotene, 1-65 µg/mL for lutein, 4-100 µg/mL for cryptoxanthin and 1-40 µg/mL for zeaxanthin.

The limits of detection (LOD) and limits of quantification (LOQ) were determined as previously described by Long and Winefordner, (1983). The following LOD and LOQ scores were, respectively obtained: $6.5x10^{-2}$ and $10.9x10^{-2}$ mg/kg for β-carotene; $6.9x10^{-3}$ and $1.2x10^{-2}$ mg/kg for lutein; $2.1x10^{-2}$ and $3.5x10^{-2}$ mg/kg for cryptoxanthin; $9.6x10^{-2}$ and $1.6x10^{-2}$ mg/kg for zeaxanthin; and $2.0x10^{-2}$ and $3.3x10^{-2}$ mg/kg for α-carotene.

2.5.2 Capsaicinoids

For the analysis of capsaicinoids, the concentrated extract was diluted in 1 mL of HPLC grade acetonitrile, placed in an ultrasound (Unique, model USC 1400, São Paulo, Brazil) for 5 minutes and filtered (0.45 µm Millipore filter) before injection into the chromatograph. The capsaicinoids were separated on a polymeric Vydac C_{18} column (218TP54; length of 250 mm length, internal diameter of 4.6 mm and particle size of 5 mm) with an acetonitrile/water/acetic acid mobile phase (100:100:1) for 23 minutes with a flow rate of 1.2 mL/min at 20° C as previously described by Peusch et al. (1997).

The spectra were conducted between 250 and 600 nm, and the chromatograms were processed at a fixed wavelength of 280 nm. The identification was performed by comparing the peak retention times obtained for the standard and for samples analyzed under the same conditions.

The quantification was performed by constructing standard curves for the carotenoid in the following concentration ranges: 0.03-0.08 µg/mL for capsaicin and 0.04-0.22 µg/mL for dihydrocapsaicin.

The limits of detection (LOD) and limits of quantification (LOQ) were determined as previously described by Long and Winefordner, (1983). The following LOD and LOQ values were, respectively obtained: $6.62x10^{-2}$ and $1.10x10^{-1}$ mg/kg for capsaicin and $2.90x10^{-2}$ and $4.84x10^{-1}$ mg/kg for dihydrocapsaicin.

2.5.3 Ascorbic acid

The ascorbic acid was eluted isocratically using 0.05 M supra pure sulfuric acid as the mobile phase with a flow rate of 1 mL/min and an injection volume of 10 µL. The chromatograms were processed at 254 nm. Quantification was carried out by constructing standard curves according to Rosa et al., (2007) with concentrations ranging from 0.001 to 1 mg/mL.

2.3 Statistical analysis

The results were evaluated by analysis of variance (ANOVA). Tukey's test was used to compare the mean differences using Statistica 10 software.

3 RESULTS AND DISCUSSION

3.1 Extraction of bioactive compounds

3.1.1 Carotenoids

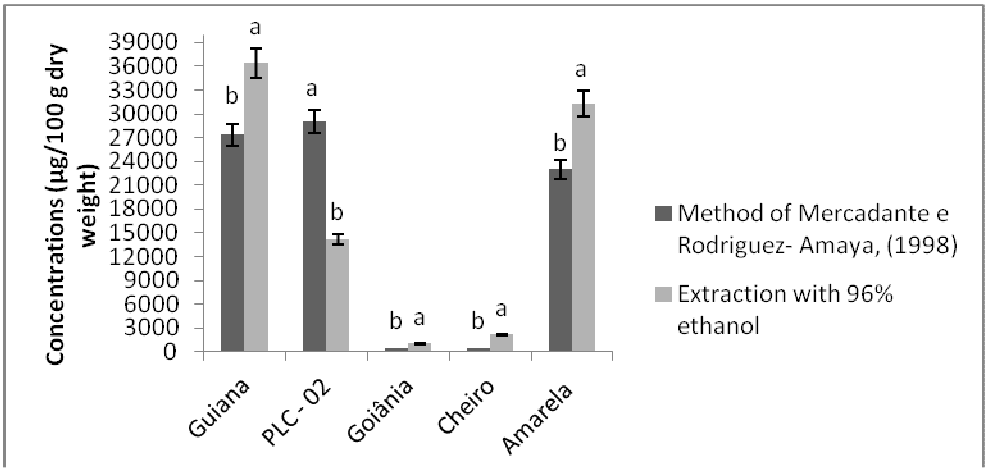
The measured content of carotenoids depends on the type of solvent used to extract peppers Bae et al., (2012). To obtain antioxidant products or dyes for the food industry, extractions are usually performed using water, alcohol or an alcohol/water mixture Costa et al., (2000).

Figure 1 shows the total content of carotenoids in the 96% ethanol extract. The extraction method with 96% ethanol allowed greater extraction of carotenoids in all genotypes, except for the PCL-02 genotype, compared to the method of Mercadante and Rodriguez- Amaya, (1998).

The total amount of carotenoids extracted with 96% ethanol from the Guiana, Cheiro Comum, Amarela olho de peixe, PCL-02 and Goiânia peppers was 36417, 2144, 31274, 14162 and 1039 µg of carotenoids/100 g of dry pepper, respectively (Figure 1). Extraction with 96% ethanol allowed higher extraction of total carotenoids compared to the methodology proposed by Mercadante and Rodriguez-Amaya (1998), who obtained values of 27382, 462, 2298, 29099 and 460 µg of carotenoids/100 g of dry pepper for

the Guiana, Cheiro Comum, Amarela olho de peixe PCL-02 and Goiânia peppers, respectively.

Figure 1: Comparison of carotenoids in *Capsicum* pepper extracts prepared as previously reported by Mercadante and Rodriguez-Amaya, (1998) to carotenoids extracted with 96% ethanol (concentrations expressed as µg/100 g dry weight). Different letters for the same pepper indicate significant differences between treatments ($p < 0.05$).



According to Mercadante and Rodriguez Amaya (1988), carotenoids are lipophilic, insoluble in water and soluble in organic solvents, such as acetone, alcohol, ethyl ether, chloroform, and ethyl acetate. Carotenes are readily soluble in petroleum ether, hexane, and toluene, but xanthophylls dissolve better in methanol and ethanol, which is the solvent used in the present work.

According Rodriguez-Amaya et al. (1988), saponification is an effective mean of removing chlorophylls and unwanted lipids, which may interfere with the chromatographic separation and shorten the life of the column in HPLC.

However, saponification extends the analysis time, and it may provoke degradation of carotenoids. The extent of degradation depends on the conditions used with more degradation occurring with higher alkali concentrations and hot saponification (KIMURA et al., 1990). According to Rodriguez-Amaya et al. (1988), lutein, violaxanthin, and other carotenoids are reduced considerably during saponification and the subsequent washing step (RISO; PORRINI, 1997).

Bae et al. (2012) evaluated the influence of the use of solvents, including hexane, ethyl acetate, acetone, methanol, and methanol: water (80:20; v/v), on samples without saponification. The gradient mobile phase was composed of (A) MeOH and (B)

tert-butyl methyl ether with a flow rate of 0.8 mL/min, and the samples were eluted as follows: 0–80% B (0–15 min), 100% B (15–20 min), and 100–0% B (20–25 min). For the extraction of bioactive compounds from peppers, the highest levels of carotenoids were found in hexane extracts followed by ethyl acetate and acetone extracts. The highest amount of carotenoids was found in hexane extracts (72200 µg/100 g dry weight) from the *C. annuum* cv. 'Tuxtlas' cultivar, and the lowest amount of carotenoids (3100 µg/100 g dry weight) was found in hexane extracts from the *Capsicum annuum* cv. 'CA408' and 'Mesilla' cultivars. The differences in the carotenoid concentrations may be attributed to the different cultivars.

The concentrations obtained in this study with 96% ethanol ranged from 1039 to 36417 µg/100 g dry weight, which were lower than the values reported by Bae et al. (2012). Although the use of organic solvents in the production of plant extracts is conventional, questions regarding the pharmacological and environmental toxicity as well as the danger in manipulating the plant extracts must be considered.

Santamaria et al. (2000) obtained of extracting with industrial ethanol (96% purity from ADYDSA, Mexico) both directly from dried flour and from the paste resulting after an aqueous enzymatic treatment, 73% carotenoids from Chili Guajillo Puya (*Capsicum annuum* L.). Extraction with 96% ethanol in the present study allowed greater extraction of carotenoids in all genotypes, except for the PCL-02 genotype.

Finding a single method that is suitable for the extraction of a carotenoid group is important, but it is not an easy task due to the diversity of structures, chemical variations and sensitivity of compounds to extraction conditions. The polarity of the solvent can affect the transfer of electrons and hydrogen atoms, which are key aspects in the measurement of antioxidant activity.

The large variance in carotenoid content indicated that the Guiana pepper contained more carotenoids than the PCL–02 pepper, Amarela olho de peixe pepper and other cultivars, thereby suggesting that the levels of carotenoids were influenced by the genetic variation of the peppers Wahyuni, et al., (2011).

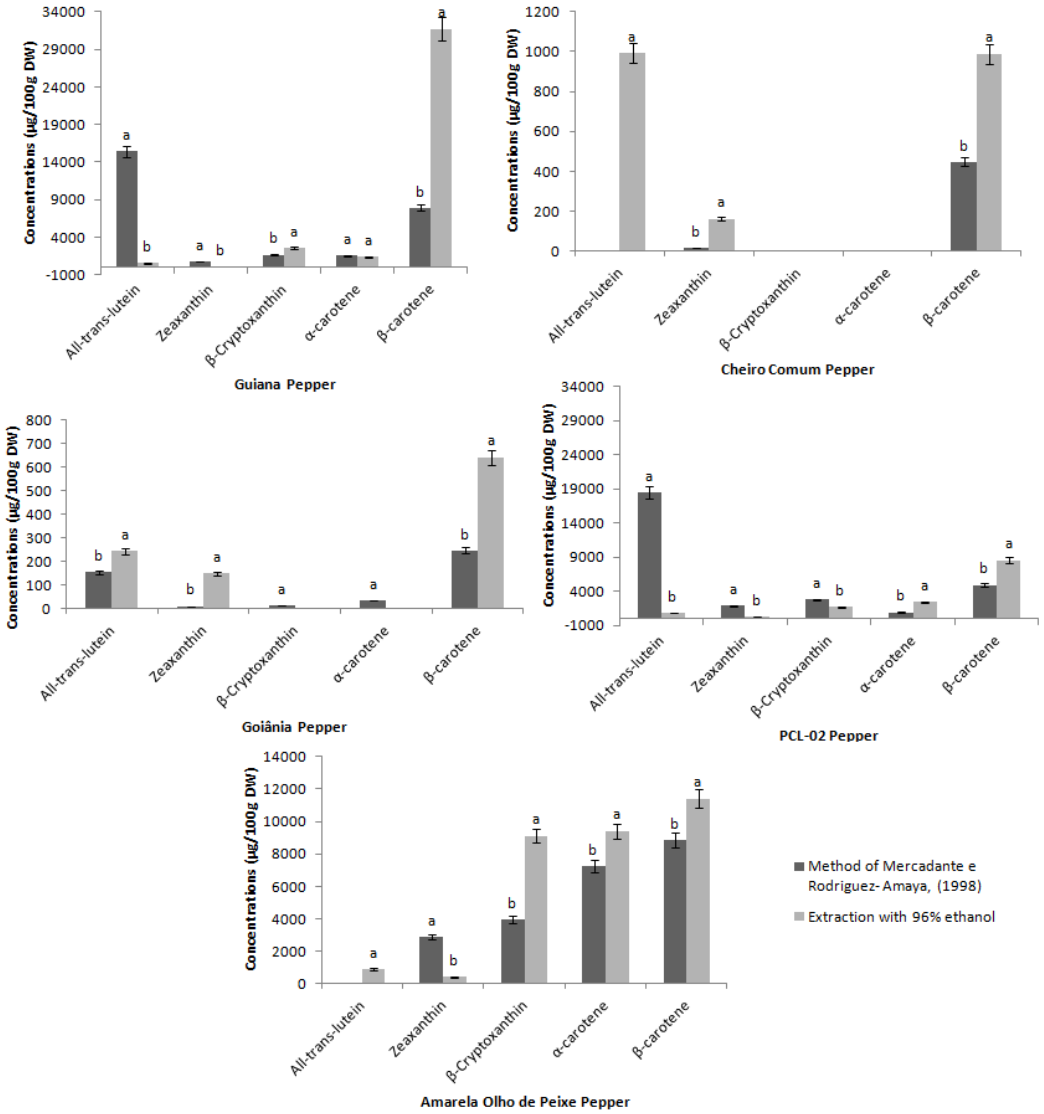
In some pepper genotypes, the extract prepared with 96% ethanol allowed a greater preservation of the original content of carotenoids compared with method reported by Mercadante and Rodriguez-Amaya, (1998).

The carotenoid profiles found in peppers vary due to the differences in the matrices analyzed (Chili Guajillo Puya peppers and peppers in nature). In addition to plant species, the accumulation of carotenoids is dependent on external environmental

factors, such as climate characteristics, which involve the incidence of light, temperature and soil (ZANATTA; MERCADANTE, 2007).

The carotenoid, all-*trans*-lutein, in the Cheiro-comum, Amarela Olho de Peixe and Goiânia peppers exhibited higher concentrations when extracted with 96% ethanol than when extracted by the saponification method proposed by Mercadante and Rodriguez-Amaya (1998) (Figure 2).

Figure 2: Comparison of each carotenoid concentration in *Capsicum* pepper genotypes obtained according to the method proposed by Mercadante and Rodriguez-Amaya (1998) and obtained by extracting with 96% ethanol (concentrations expressed as µg/100 g dry weight). Different letters for the same pepper indicate significant differences between treatments ($p < 0.05$).



In the present study, the use of 96% ethanol increased the extraction of β-carotene in all genotypes. Some carotenoids, such as β-carotene, α-carotene and β-cryptoxanthin, are capable of being converted to vitamin A, which indicates that they have an important nutritional role (OLSON, 1989). Street et al. (1994) reported that β-carotene is the carotenoid with the greatest activity of provitamin A, and they suggested that there is a significant association between low levels of β-carotene and increased incidence of myocardial infarction and that a diet rich in β-carotene is associated with a lower risk of premature death from coronary heart disease.

With regard to the profile of carotenoids, the Guiana and Goiânia genotypes had higher levels of β-carotene compared to the other genotypes. In addition to containing high levels of β-carotene, Cheiro comum peppers had high levels of lutein. Moreover, Amarela olho de peixe peppers contained high contents of β-carotene, α-carotene and cryptoxanthin. The PCL-02 genotype did not have higher levels of β-carotene but instead had a considerable amount of lutein and lower amounts of cryptoxanthin, zeaxanthin and α-carotene.

Studies have shown that carotenoids are responsible for reducing the risk of degenerative diseases, preventing cataracts, reducing macular degeneration caused by aging and reducing coronary heart disease Krinsky, (1994). In the present study, the identification of five carotenoids (*all-trans*-lutein, zeaxanthin, β-cryptoxanthin, β-carotene, and α-carotene) was possible through chromatographic separation.

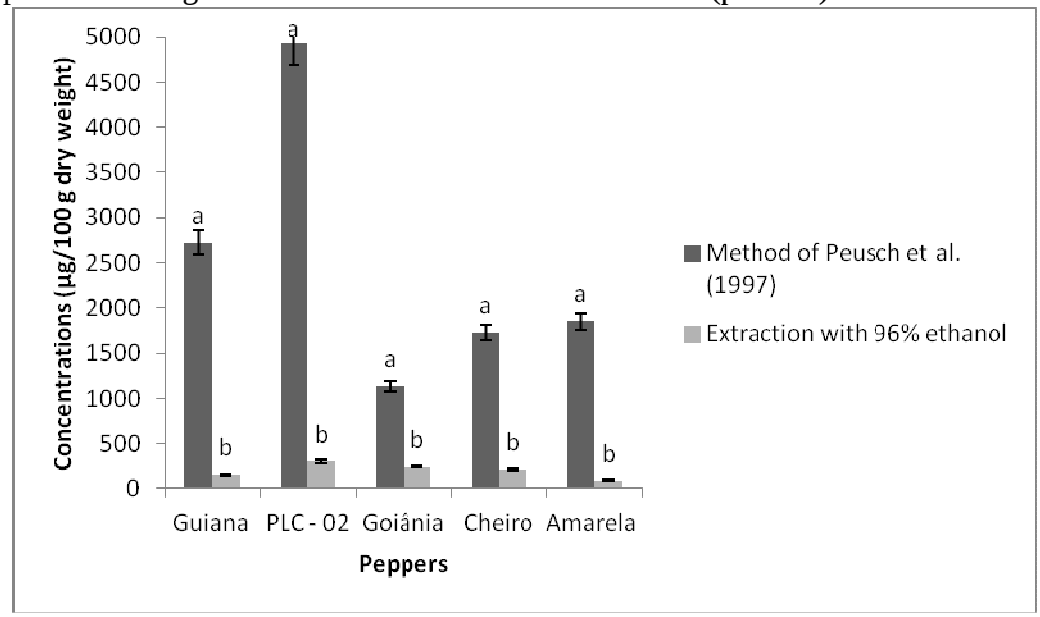
The results obtained with the 96% ethanol extraction demonstrated that the concentrations of zeaxanthin exhibited the lowest variation among the genotypes. In the *Capsicum annuum* cultivars, β-cryptoxanthin showed less variation indicating that the synthesis of β-cryptoxanthin in *Capsicum annuum* fruits is independent of the cultivar Topuz and Ozdemir, (2007).

3.1.2 Capsaicinoids

Capsaicinoids (capsaicin and dihydrocapsaicin) are regarded as compounds responsible for the pungency in peppers (BENNETT; KIRBY, 1968). The fractions obtained by chromatographic analysis revealed the presence of capsaicinoids, including capsaicin and dihydrocapsaicin, in all genotypes indicating that *Capsicum annun* L. *var. annuum* and *C. chinense* Jacquin can be considered as pungent peppers.

The use of an ethanol solvent allowed an average capsaicinoid extraction of 6.16% in the pepper genotypes as compared to the method of Peusch et al. (1997). The capsaicinoid concentrations ranged from 89 µg/100 g dry weight for the Amarela Olho de Peixe pepper to 304 µg/100 g dry weight for the PCL-02 pepper (Figure 4). A similar range of values (78.65 to 386.38 µg/g) has been found for *Capsicum annun* peppers (Jalapeño and Serrano) using an extract prepared from methanol and utilizing HPLC-MS for the identification and quantification of hot pepper capsaicinoids Alvarez-Parrilla et al. (2011).

Figure 4: Comparison of the amount of capsaicinoids by the well-established method of Peusch et al. (1997) and by extraction with 96% ethanol in *Capsicum* pepper genotypes (concentrations are expressed as µg/100 g dry weight). Different letters for the same pepper indicate significant differences between treatments ($p < 0.05$).



Despite the antioxidant potential that capsaicinoids present, the application of these as antioxidants in foods is limited due to the strong pungency Rose et al. (2002). Because the ethanol extract contained a low concentration of capsaicinoids, it is an excellent option for use as antioxidants in food. In addition to the low cost of ethanol, the use of ethanol does not provide major environmental impacts.

Govindarajan et al. (1987) determined the concentration of capsaicinoids in various species of peppers, including *C. annuum* and *C. chinense*, using a spectrophotometric method. These authors found that different solvents extract different concentrations of capsaicinoids. For the *C. annuum* var. L. variety, extraction with

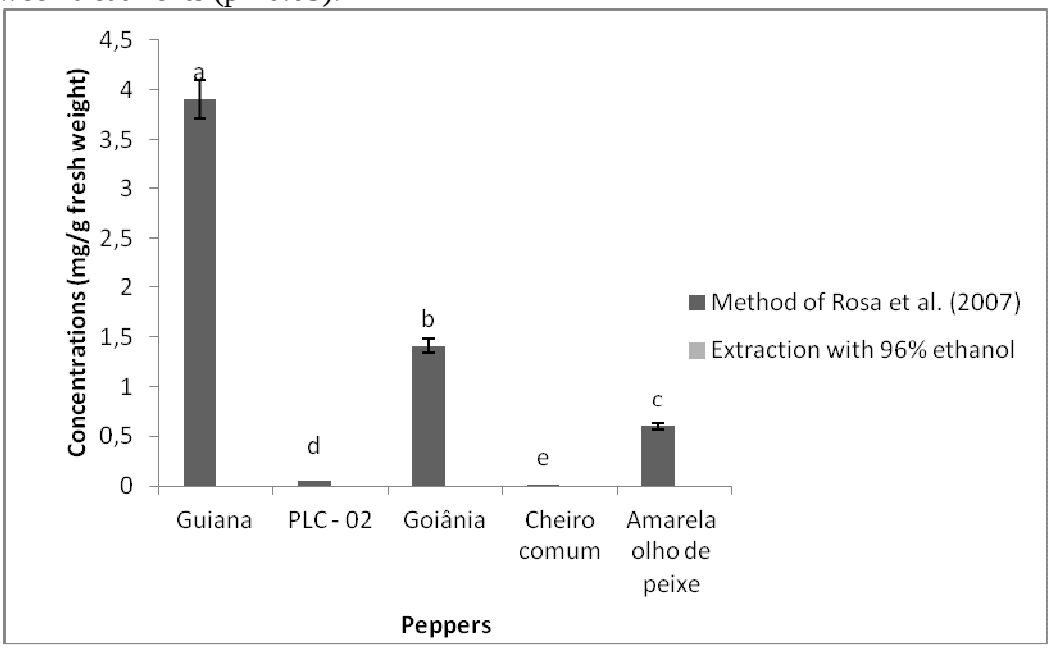
alcohol, acetone, chloroform, ether and hexane resulted in capasaicinoid concentrations of 0.523, 0.515, 0.587, 0.701 and 0.605%, respectively. In their research, the results of extraction with nonpolar solvents were better than those with polar solvents, which is consistent with the present study.

Zhuang et al. (2012) obtained ethanol extracts from nine varieties of peppers, including varieties of *Capsicum annuum* L. and *Capsium frutescens* L., and they found total capsaicinoid concentrations ranging between 58.77 and 1242.88 μg/g fresh weight.

3.1.3 Ascorbic acid

The ascorbic acid contents of the five genotypes of fresh peppers in the present study are shown in Figure 4.

Figure 4: Concentrations (mg/g fresh weight) of ascorbic acid found in the genotypes of *Capsicum* peppers. Different letters for the same pepper indicate significant differences between treatments ($p < 0.05$).



Not allow the quantification of ascorbic acid by method of Rosa et al. (2007) Figure 4. The chromatogram obtained by analysis of the extract prepared from 96% ethanol showed two peaks in all samples in the even retention time of ascorbic acid,

which may represent isomers that were not separated, thereby preventing the quantification of ascorbic acid.

The extraction with 96% ethanol may have contributed to the formation of isomers because the method proposed by Rose et al. (2007) allowed the identification and quantification of total ascorbic acid in the peppers analyzed.

Zhuang et al. (2012) analyzed the ascorbic acid content in 80% ethanol extracts of nine peppers, including *Capsium* frutescens L. and *Capsium annuum* L. varieties, and reported low concentrations of ascorbic acid ranging from 0.93 to 3.35 mg/g fresh weight, but these values were higher than the values reported by Castro et al. (2008) and Ghasemnezhad et al. (2011), who reported *Capsicum annuum* L. pepper ascorbic acid contents of 0.12–2.77 mg/g fresh weight. Topuz and Odemir (2007) studied ascorbic acid contents in five different *Capsicum annuum* varieties, and they reported lower values ranging between 0.15 and 0.57 mg/g fresh weight.

The total quantification method resulted in ascorbic acid values ranging from 0.03 mg/g fresh weight for the Cheiro comum pepper to 3.9 mg/g fresh weight for the Guiana pepper. The ascorbic acid content of the Cheiro comum pepper was lower than the values of the other genotypes with a value of 0.03 mg/g fresh weight. These differences can be explained by genetic diversity among genotypes.

The variability of the ascorbic acid results among genotypes can also be explained in terms of differences in cultivars, soil, growing conditions and maturation (ZHUANG et al. 2012). For green peppers, Yahia et al. (2001) found values of ascorbic acid between 0.12 and 1.80 mg/g fresh weight. For red peppers, Howard et al. (1994) reported ascorbic acid contents between 0.75 and 2.77 mg/100 g fresh weight demonstrating a decrease in ascorbic acid content with fruit ripening.

Therefore, optimization of mixed solvents to be applied in the extraction of each specific food results in a significant recovery of bioactive compounds.

4 CONCLUSIONS

Using 96% ethanol to prepare extracts of peppers rich in carotenoids is a viable alternative and can be widely exploited in the food industry. The use of 96% ethanol was effective in the preparation of extracts with low levels of capsaicinoids. Moreover, the concentration and activity of these bioactive compounds naturally present in foods can be directly related to the properties of solvents, such as lipophilic and hydrophilic solvents, and their respective polarity. For future work based on the present data obtained, mixtures of other nontoxic solvents that are permitted in food should be tested for use in extracting bioactive compounds of peppers.

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2.2 ARTIGO 2: Bioactive compounds, and its cytotoxic effect of peppers (*Capsicum*) on human neuron-like cells (SH-SY5Y)

BIOACTIVE COMPOUNDS AND ITS CYTOTOXIC EFFECT OF PEPPERS (*CAPSICUM*) ON HUMAN NEURON LIKE CELLS (SH-SY5Y)

ABSTRACT

Four genotypes of peppers *Capsicum annun* L. *var. annuum* (Amarela olho de peixe, Guiana, Goiânia, PCL-02) and one genotype of pepper *Capsicum chinense* (Cheiro comum) were analysed for concentrations of total phenolic compounds, capsaicinoids, carotenoids and total antioxidant activity to evaluate the potential of these compounds for bioactivity, and obtain subsidies that allow for the advancement of the breeding program of pepper, with emphasis on the content of antioxidants. The anticancer activity of the extracts were tested by methods like SRB, LDH assay for cell viability, morphological analysis and cytotoxicity. The genotypes that stood out in relation to total antioxidant activity were the PCL-02 and Goiânia peppers, there was a positive correlation ($r = 0.87$, $p < 0.05$) of the total antioxidant reactivity – (TAR) with the content of capsaicinoids. At 72hours in concentraction of 200 µg/mL the genotype Guiana pepper there was about 48.6% decrease in human neuroblastoma cell viability. The genotype Guiana stood out on the carotenoid content, and the genotype PCL-02 stood out in the phenolic. These genotypes of pepper stood out as candidates for use in breeding programs, given the market demand for new sources of bioactive compounds

Keywords: Antioxidants, carotenoids, capsaicinoids, phenolic, peppers *Capsicum*.

1. INTRODUCTION

Peppers *Capsicum* ssp. are important spices used in cooking worldwide, and are considered excellent sources of antioxidants (HOWARD et al., 2000). This antioxidant property of the fruit is justified by the presence of bioactive compounds, such as capsaicinoids, carotenoids, flavonoids, phenolic acids, tocopherol and ascorbic acid (ZHUANG et al., 2012). These components provide a number of health benefits such as the prevention in the treatment of neurodegenerative diseases (DAIRAM et al. 2008), protection against cisplatin nephrotoxicity (SHIMEDA et al., 2005) and anticancer properties (MIN et al. 2004).

Although the species (*Capsicum annun* L.) and (*Capsicum chinense*) are the most cultivated pepper in South America (DEWITT and BOSLAND, 1996), many of its varieties are yet unknown about chemical composition and bioactive compounds,

which can be a factor affecting both the consumption and in the selection of genotypes of this species for breeding programs.

Agribusiness pepper has a promising future with great prospects and potential given its versatility for culinary purposes, industrial, medicinal and ornamental. In addition, there is a growing demand for new cultivars of pepper that combine agronomic characteristics of interest such as higher nutritional quality, productivity and disease resistance (BUSO et al., 2001). However there will only be increasing production, higher nutritional quality and reducing environmental impacts the genetic diversity available is used efficiently. The challenges of agribusiness pepper can be overcome through genetic improvement and obtaining commercial materials with high standards of quality and productivity

Considering, the presence of the high content of the bioactive compounds in extracts of peppers, and the therapeutic potential of the extracts, the present work was designed to investigate to select the best among four genotypes of *Capsicum annun* L. *var. annuum* and one of *C. Chinense* Jacquin, belonging to the Active Germplasm Bank (AGB) of Embrapa Amazônia Oriental, to based on the total antioxidant activity and the concentrations of capsaicinoids, carotenoids and phenolic compounds, for use in breeding programs and obtain subsidies that allow for the advancement of the breeding program of pepper, with emphasis on the content of antioxidants from fruits.

2 MATERIALS AND METHODS

2. 1 Chemicals

Luminol, capsaicin (purity > 98%), dihydrocapsaicina (purity > 90%), β-cryptoxanthin (purity ≥ 97%), β-carotene (purity > 93%), α-carotene (purity ≥ 95%), zeaxanthin (purity > 95%) were purchased from Sigma Chemical St. Louis, MO, USA. 2,2'-Azobis(2-methylpropionamidine)dihydrochloride (AAPH) was purchased from Aldrich Chemical, Milwaukee, WI, USA. The carotenoids lutein standard (purity > 95%), was purchased from Indofine Chermical Company Inc. Hillsborough, USA. Acetonitrile HPLC grade, methyl-tert-butyl HPLC grade and methanol HPLC grade, were purchased from Scientific HEXIS the S/A (Mexico). Acetic acid, ethanol 96%, and glycine, were purchased from Vetec (Duque de Caxias, RJ, Brazil).

2.2 Plant material

Fruits from four genotypes of peppers *Capsicum annun* L. *var*. *annuum* identified in the AGB - Embrapa Amazonia Oriental as: Goiânia (voucher number IAN 186314), Guiana (voucher number IAN 186303); PCL-02 (voucher number IAN 186306); Amarela olho de peixe (voucher number IAN 186308) and one genotype of pepper *Capsicum chinense* Jacquin identified Cheiro comum (voucher number IAN 186307), were harvested between the months of August and September 2011.

Fruit, was selected vacuum packaged (Vacuum Sealer Sealer F 200 Flash, Brand Fastvac) wrapped in polythene bags with aluminum foil to avoid contact with light and frozen (-10 °C) until the time of analysis.

2.3 Preparation of extracts

For the analysis of the antioxidant activity, carotenoids and capsaicinoids the extract were prepared with solvent 96% ethanol. The fruit was ground in a microprocessor (Arno Performa Magiclean Duetto) for 5 minutes. Five grams of the genotypes of (Goiânia) pepper and (Cheiro comum) pepper and 2.5 g of the genotype (Guiana) (PCL-02) and (Amarela olho de peixe) were soaked with 96% ethanol solvent, until all of pigments were removed, following which the samples were vacuum filtered. The extracts were prepared in duplicate.

The extract was concentrated on a rotary evaporator at ambient temperature (Fisatom, Model 801 - St. Paul), diluted in 5 mL of 96% ethanol and fractionated into aliquots of 2 mL in amber vials. The the solvent was evaporated under nitrogen flow and the concentrated extracts were stored in a freezer (-10 ^{o}C) until the time of analysis.

For the analysis of phenolic compounds, the solvent was methanol and the extract was prepared as described by Swain and Hillis, (1959), 5 g of sample was homogenized in an Ultra-Turrax (Ultra-digital odds T25) with 20 mL of methanol and centrifuged (Walita-Philips ®) under cooling to 4 °C for 30 minutes at 3.500g. An aliquot of 1 mL of supernatant was collected and diluted in 1 mL of water.

2.4 Total reactive antioxidant potential (TRAP) and total antioxidant reactivity TAR)

TRAP and TAR were measured and calculated as previously described by Dresch et al. (2009). The ethanol extract concentrate was diluted with water. Briefly, TRAP represents the non-enzymatic antioxidant capacity of the cells once they are treated (in this study, with extracts of peppers). This is determined by measuring the luminol chemiluminescence intensity of emission induced by thermolysis of 2,2′-azobis (2-amidinopropane) hydrochloride (AAPH) as free radical source.

The system was left to stabilize for 2 hours at room temperature (22 ^{o}C). Then, samples (final concentration 16µg/mL) were added and the readings monitored for 2 hours. Results were transformed into a percentile rank and the area under the curve (AUC) was calculated by utilizing the GraphPad software (San Diego, CA, USA), as previously described. The smaller the AUC is (in comparison to the system), the higher is the total reactive antioxidant potential of the sample. TAR represents the total antioxidant properties of all antioxidants. This parameter is closely related to the quality of the antioxidants within the sample. In our study, TAR was calculated as the ratio of light in the absence of samples (I_o)/light intensity right after sample addition (I). The higher these values are, the higher is the total antioxidant reactivity of the sample.

2.5 Total phenolic content

The extract of peppers was diluted with water, subsequently either 20 µL of sample or 20 µL of the control (methanol), was combined with 1.58 mL of water, 100 mL of Folin-Ciocateau and 300 mL of saturated sodium carbonate and then incubated for 2 hours at room temperature before the absorbance was read at 734 nm on a spectrophotometer (Amersham, Model 3100 UV-Vis Ultrospec Pro Amersham Bioscience). The standard curve was constructed to quantify using gallic acid in concentrations ranging from 0.0020 µg/mL to 0.005 µg/mL. The results were expressed as gallic acid equivalents GAE/100 g dry weight (DW).

2.6 Profile of carotenoids and capsaicinoids by High-performance liquid chromatography (HPLC)

The analyses **w**ere carried out using an HPLC system (Agilent series 1100**,** Santa Clara, CA, USA) that was equipped with an online degasser, a quaternary pump, an automatic injector.

For the analysis of carotenoids, the concentrated extract was diluted in 2 mL of methyl tert-butyl ether (MTBE) HPLC grade, placed on an ultrasound (Unique model USC 1400, Sao Paulo, Brazil) for 5 minutes and filtered through a Millipore filter (0.45 μm) as described by Zanatta and Mercadante, (2007). The sample was then run on a YMC C_{30} reverse phase polymer column (250 mm length x 4.6 mm internal diameter and 3 mm particle size), with an elution gradient of mobile phase was composed of: water/methanol/methyl tert-butyl ether (MTBE) that started at a ratio of 5:90:5, reached 0:95:5 within 12 minutes, 0:89:11 at 25 minutes, 0:75:25 at 40 minutes and finally, 00:50:50 after 60 minutes with a flow rate of 1 mL/min at 33 °C.

For the analysis of the capsaicinoids, the concentrated extract was diluted in 1 mL of acetonitrile HPLC grade, placed in an ultrasound (Unique, model USC 1400, Sao Paulo, Brazil) for 5 minutes and filtered through a Millipore filter (0.45 mM) for injection into the chromatograph. A Vydac C_{18} column polymer (218TP54), 250 mm long, 4.6 mm internal diameter and 5 μm in particle size was used with a mobile phase of acetonitrile/water/acetic acid in the ratio 100:100: 1 for 23 minutes with a flow rate of 1.2 mL / min at 20 °C as described by Peusch et al., (1997)

The spectra were conducted between 250 and 600 nm and the chromatograms processed in fixed wavelength of 450 nm to 280 nm for carotenoids and capsaicinoids. Identification was performed by comparing the retention times of peaks obtained for the standard with those of the samples, analyzed under the same conditions.

Quantification was performed by constructing standard curves for the carotenoid for the β-carotene 5-50 μg/mL, α-carotene 2-25 μg/mL, of a lutein 65 μg/mL, cryptoxanthin 4-100 μg/mL zeaxanthin and 1-40 μg/mL, and for the capsaicinoids, capsaicin 0.03 to 0.08 μg/mL and dihydrocapsaicina 0.04 to 0.22 μg/mL.

The limits of detection (LOD) and of quantification (LOQ) determined according to the methodology described by Long and Winefordner, (1983) and were,

respectively, for the following: β-carotene: $6.5x10^{-2}$ and $10.9x10^{-2}$ mg/kg; luteín: $6.9x10^{-3}$ and $1.2x10^{-2}$ mg/kg; cryptoxanthin: $2.1x10^{-2}$ and $3.5x10^{-2}$ mg/kg; zeaxanthin: $9.6x10^{-2}$ and $1.6x10^{-2}$ mg/kg; α-carotene: $2.0x10^{-2}$ and $3.3x10^{-2}$ mg/kg; capsaicin $6{,}6x10^{-2}$ and $1{,}1x10^{-1}$ mg/kg; dihydrocapsaicina $2{,}9x10^{-2}$ and $4{,}8x10^{-1}$ mg/kg.

Cell culture

Human neuroblastoma derived SH-SY5Y cells were purchased from Rio de Janeiro Cell Bank (BCRJ, Rio de Janeiro, Brazil). The cells were cultured in a 1:1 mixture of Ham's F12 and Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), were maintained in a humidified atmosphere of 5% CO_2. Preliminary MTT viability assay indicated that cell viability was not affect during incubations.

Sulforhodamine B (SRB) incorporation-based viability assay

The sulforhodamine B (SRB) has been widely used for the assessment of cytotoxicity and cell proliferation assays on microtiter plates (LIN et al. 1999). This colorimetric assay estimates cell number by staining total cellular protein with SRB (SKEHAN et al., 1990). Briefly, cells were fixed by layering 100 μL of ice-cold 40% trichloroacetic acid (TCA) on top of the culture medium and incubated at 4°C for 1 h. Plates were then washed five times with cold water. The excess water was then decanted and the plates left to dry in air. SRB stain (50 μl; 0.4% in 1% acetic acid) was added to each well and incubated for 30 min. The cells were then washed with 1% acetic acid and rinsed 4 times until only the dye adhering to the cells was left. The plates were then air-dried and 100 μL of 10 mM Tris base (pH 10.5) were added to each well to dilute the dye. The plates were gently shaken for 20 min on an orbital shaker and the absorbance of each well was read in a microplate reader (Perkin Elmer, Massachusetts, USA) at 492 nm. Cell survival was measured as the fold absorbance compared to the absorbance of control (non-treated cells).

Quantification of activity of Lactate Dehydrogenase (LDH)

Activity of LDH released to the cell culture medium was monitored following the formation of formazan by coupled enzymatic reaction at 500 nm according to the manufacture kit. Cell membrane rupture was defined as the ratio of LDH activity in the supernatant of treated cells to the LDH activity released in the control cells. When in cultured cells, the analysis of LDH activity in the culture medium provides a good index of cell disruption due to necrotic or apoptotic processes late. Cells (60 cells/mL) were seeded to 96-well plates and incubated for 24h. Thereafter, the cells were exposed to extrac of pepper at concentrations the 200µg/mL, 100µg/mL and 50µg/mL for 24h and 48h. The optical density was measured in a microplate reader (Perkin Elmer, Massachusetts, USA) at 500nm. The effect of the peppers extracts on cell viability was expressed using the following formula: Percent of viability = (Absorbance of peppers extract treated sample at 500nm wave length / Absorbance of peppers extract non treated sample at 500nm wave length) * 100%.

2.3 Statistical analysis

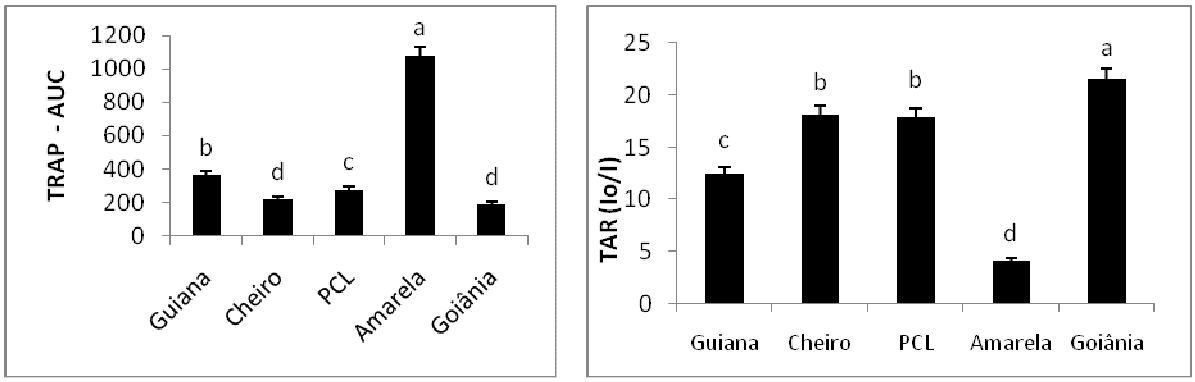
The results were evaluated by analysis of variance (ANOVA). Pearson's correlation coefficient and the Tukey test for mean differences using Software Statistic 10.

3 RESULTS AND DISCUSSION

3.1 Antioxidant activity

Through the analysis of TAR and TRAP the *in vitro* uptake of free radicals can be assessed. According to Dresch et al. (2009), the TRAP is an accurate method, and its main advantage is that it applies to antiradicals with different kinetic behaviours. In other words, TRAP applies equally well for antioxidants that exhibit distinct lag phases and or do not exhibit phase lags, and may thus be used for any antioxidant compound (or complex mixture) independent of the compound's kinetic emission profile. The results of TRAP expressed by the area under the curve (AUC) (Fig. 1A).

Figure. 1. A) Area under the curve – AUC. The smaller the AUC is (in comparison to the system), the higher is the total reactive antioxidant potential of the sample. B) TAR light in the absence of samples (I_o)/light intensity right after sample addition (I) profile five fruit genotypes *Capsicum chinense* Jacquin. The values are expressed as the mean ± deviation of the mean. Different letters in columns differ significantly between samples ($p < 0.05$).



According Dresch et al. (2009), loss in chemiluminescence can be assessed by measuring the AUC. When the extent of the decay of the luminescence is large, the expected AUC value is low, i e the sudden loss of chemiluminescence (TAR) and the AUC value are inversely proportional. The smaller the value of the AUC increased antioxidant activity of extracts.

The genotypes (*Capsicum annun* L. *var*. *annuum*), (Cheiro) and (Goiânia) did not differ significantly ($p<0.05$) between itself the AUC. When compared with other genotypes that were grown in the same environment at the same time and collected and analyzed at the same concentrations, these two extracts had a greater ability to neutralize free radicals. According to Dresch et al. (2009), AUC has the advantage of incorporating both the inhibition and the duration of inhibition of the free radical generator.

The results of the TAR analysis also revealed that the extracts of the five genotypes of pepper *Capsicum annun* L. *var. annuum* and one of *C. Chinense* Jacquin, (Fig. 1B) were reactive, provides a sudden reduction in the chemiluminescence of luminal. There were significant differences ($p<0,05$) between the genotypes according to TAR.

In addition to the AUC, the TAR differentiated the genotypes (Goiânia), (Cheiro) and (PCL-02) from the other genotypes of peppers, because of their higher

quality in relation to the antioxidants evaluated by means of a sudden decrease in the initial luminescence of luminol after addition of the extract to system generating free radicals.

Corroborating these results Kappel et al. (2008) also observed that extracts prepared with peppers (*Capsicum baccatum* L. var. *Pendulum*) were also active in reducing the chemiluminescence of luminol.

The antioxidant capacity of fruits and vegetables is directly related to the presence of bioactive compounds. There are several factors that influence the variability of these compounds in peppers, including: cultivar, growing conditions, maturity, post harvest handling (MATERSKA and PERUCKA, 2005) and country of origin (ANTONIOUS et al. 2009). In addition, the extraction of such compounds of the fruit is directly influenced by the solvent used (BAE et al., 2012)

The solvent used in the preparation of extracts for total antioxidant activity of the, carotenoids and capsaicinoids was 96% ethanol which besides being a solvent permitted for use in food. The extract was diluted with water and because capsaicin, the major component responsible for the pungency of pepper (GALANO; MARTINEZ, 2011), tends to react more quickly as an antioxidant against oxygenated free radicals in aqueous solutions than nonpolar solutions according to (CORDELL; ALLEN, 1993)

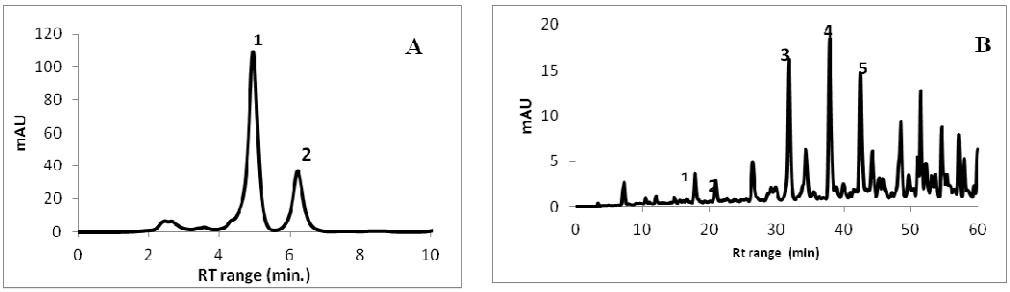
3.2 Capsaicinoids

Capsaicinoids are regarded as compounds responsible for pungency in peppers (KRAJEWSKA and POWERS, 1988) provide a range of health benefits, especially for the treatment of rheumatic diseases, neural diseases, headaches (TSUCHIYA, 2001) besides having antimutagenic and anticancer properties (MIN et al., 2004).

Capsaicin and dihydrocapsaicin are found in the major capsaicinoids peppers and correspond to 90% of the total pungent compounds (AZA-GONZALEZ; NUNEZ-PALENIUS; OCHOA-ALEJO, 2010).

The fractions obtained by chromatographic analysis (Fig. 2A) revealed the presence of capsaicinoids, capsaicin and dihydrocapsaicina in all genotypes indicating that the *Capsicum annun* L. *var. annuum* and *C. chinense* Jacquin are considered pungent, this feature makes it suitable both for use as seasoning in cooking but also for use as parents in breeding programs.

Figure 2. A) Chromatogram of capsaicinoids in genotype PCL-02 pepper (1 – Capsaicin; 2 – Dihydrocapsaicin. B) Chromatogram of carotenoids found in genotype amarela-olho de peixe (1 - A*ll-trans*-lutein 2 - Zeaxanthin 3 - β-Cryptoxanthin 4 - α-carotene,5-β-carotene)



Extraction of the peppers with ethanol to obtain concentrations of capsaicinoids total (the sum content of the capsaicin with dihydrocapsaicin), yielded a range of 87 µg/g DW to the genotype (*Capsicum annun* L. *var. annuum*), (Amarela olho de peixe) and 304 µg/g DW for genotype (PCL-02), (Table 1).

Table 1
Capsaicinoids found in the genotypes of peppers (*Capsicum annun* L. *var. annuum*) and (*Capsicum chinense* Jacquin) with their respective concentrations (µg/100g DW).

			Concentrations (µg/100g DW).				
N° Peak	Capsaicinoid	Rt range (min)	Guiana	PCL - 02	Goiânia	Cheiro comum	Amarela olho de peixe
1	Capsaicin	4.93-4.95	141 ± 0.25[a]	142 ± 1.32[a]	74 ± 0.02[b]	76 ± 0.03[b]	76 ± 1.50[b]
2	Dihydrocapsaicin	6.20-6.22	13 ± 0.42[d]	162 ± 0.12[b]	173 ± 0,14[a]	136 ± 0.02[c]	11 ± 0.98[d]
Total			154	304	247	212	87

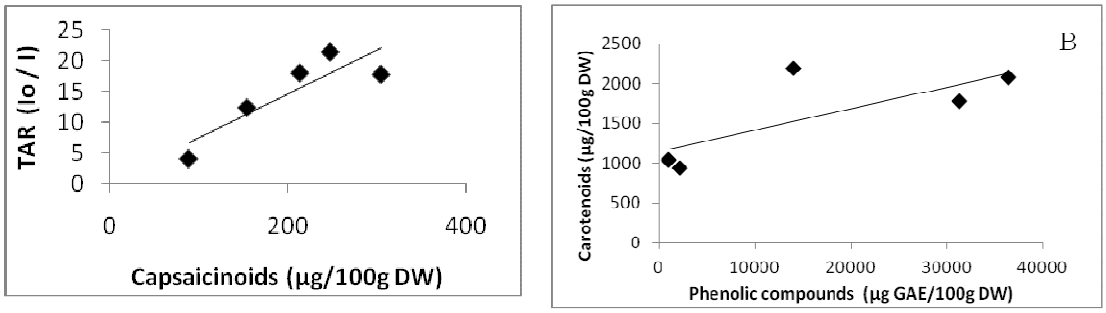
The values are expressed as the mean ± deviation of the mean. Means with same letters in the vertical do not differ significantly between samples ($p < 0.05$).

This study's results coincide with those found by Zhuang, et al., (2012) who obtained extracts with ethanol solvent seven varieties of peppers (*Capsicum annuum* L.) and (*Capsium frutescens* L.) found total capsaicinoid concentrations between 58.77 and 1242.88 µg/g DW. Alvarez-Parrilla et al., (2011) found similar values 78.65 to 386.38 µg/g to were peppers (*Capsicum annun*) (Jalapeño) and (Serrano).

A positive correlation ($r = 0.87$, $p<0.05$) content of capsaicinoids with TAR (Fig. 3A) was observed, supposedly the capsacinoid concentration this is a major contributor

to the antioxidant activity of these extracts. Materska and Perucka, (2005) also found a positive correlation between antioxidant activity and contents of capsaicinoids among peppers of the species (*Capsicum annum*).

Figure 3. A) Correlation between TAR (Io/I) and capsaicinoids (µg/100g DW) in genotypes of *Capsicum chinense*. Jacquin. B) Correlation between carotenoids (µg/100g DW) and phenolics (µg GAE/g DW) in five genotypes of *Capsicum chinense*. Jacquin.



Kappel et al. (2008) observed that extracts prepared with shares of fruit as the placenta and the seed of (*Capsicum baccatum* L. var. *Pendulum*), presented a higher antioxidant activity than pericarp. According to the Aza-Gonzalez, Nunez-Palenius, and Ochoa-Alejo, (2010) it is justified because it is in the placental region of the fruit that the synthesis of these compounds.

However, it is important to note that the extracts consist of a complex matrix with a number of bioactive compounds, including: carotenoids, phenolics, capsaicinoids, as revealed by the analysis of these extract thus the antioxidant activity can be explained by the synergism of these compounds.

3.3 Phenolic compounds

Phenolic compounds also stand out because they have antioxidant activity, associated with the redox property of the hydroxyl groups and the ability to establish structural relationships between its chemical structure components (MATERSKA; PERUCKA, 2005). Because these antioxidant properties, phenolic compound, are considered important compounds in the prevention of diseases such as cancer, cardiovascular and neurodegenerative diseases (MATERSKA; PERUCKA, 2005)

The analysis of phenolic compounds (Table 2), demonstrated a significant difference ($p < 0.05$) between the extracts and the results ranged from 934 μg GAE/g DW for the genotype pepper (*Capsicum chinense* Jacquin), (Cheiro comum) pepper GAE 2187 μg/g matters dried for genotype (*Capsicum annun* L. *var*. *annuum*), (Guiana), Zhuang, et al. (2012) obtained similar results when analyzed nine species peppers (*Capsicum frutescens* L.) and (*Capsicum annuum* L.) from seven cultivars in China in a range of 1078.26 to 4992.40 μg GAE/g DW.

Table 2
Phenolic compounds found in the genotypes of peppers (*Capsicum annun* L. *var. annuum*) and *Capsicum chinense* Jacquin with their respective concentrations (μg GAE /g DW)

Genotypes of peppers *Capsicum chinense* Jacquin	Phenolic compounds (μg GAE / g DW)
Guiana	2079 ± 0.67^{d}
PCL-02	2187 ± 0.78^{a}
Goiânia	1040 ± 0.64^{c}
Cheiro comum	934 ± 1.04^{e}
Amarela	1782 ± 0.69^{b}

The values are expressed as the mean ± deviation of the mean. Different letters in the same row indicate significant differences between treatments ($p < 0.05$).

The amount of phenolic compounds in fruits also depends on the species, variety, environmental, physiological status and climatic conditions (SCALBERT; WILLIAMSON, 2000), factors that may explain the variability in the phenolic content of the varieties of peppers.

The method of determination of the total phenolic content by the Folin-Ciocalteu method consists of a simple and widely used method of Howard et al. (2000) and the use of methanol as a solvent is justified because, according to Bae et al. (2012) is a solvent that has higher throughput and thus allows for a greater extraction of flavonoids.

The analysis of Person correlation showed a positive correlation ($r = 0.76$, $p<0.05$) between phenolic compounds and carotenoids (Fig. 3B). Although there are studies reporting that the amount of total phenolic compounds decreases with fruit ripening. Conforti, Statti and Menichini, (2007) the opposite effect was observed for genotypes in this study, possibly indicating that the genotypes with a higher content of carotenoids may also have a higher total phenolic content.

This result consistent with those found by Howard et al. (2000) and Deepa et al. (2007) who observed a trend of increase in total phenolics during the maturation stage in cultivars of (*Capsicum annuum* L).

3.4 Carotenoids

Studies show that carotenoids offer protection against macular degeneration Mares-Perlman et al. (2012) and coronary heart disease Osganian et al. (2003), have chemopreventive activity Mayne, (1996) and such protection does not depend on the consumption of high doses of carotenoids. According to the Institute of Medicine (2000) a suitable dietary intake for carotenoids for chemopreventive effects is greater than 2.5 to 5.9 μg/day for β-carotene, 1- 5μg/day for α-carotene and 4.2 μg/day for lutein and zeaxanthin.

Identification of five carotenoids (*all-trans*-lutein, zeaxanthin, β-cryptoxanthin, β-carotene, α-carotene) was possible through chromatographic separation (Fig. 2B). High concentrations of carotenoids were observed in pepper genotypes genotypes (*Capsicum annun* L. *var. annuum*) (Guiana) and (Amarela olho de peixe), (Table 3).

Table 3 Carotenoids in order of elution found in the genotypes of peppers (*Capsicum annun* L. *var. annuum*) and (*Capsicum chinense* Jacquin) with their respective concentrations (μg/100g DW).

			Concentrations (μg/100g DW).				
Nº Peak	Carotenoid	Rt range (min)	Guiana	PCL - 02	Goiânia	Cheiro comum	Amarela olho de peixe
1	*All-trans*-lutein	18.03-18.10	570 ± 0.25 [d]	863 ± 1.32 [c]	244 ± 0.25 [e]	1981 ± 1.32 [a]	904± 1.12 [b]
2	Zeaxanthin	21.07-21.18	127 ± 0.60 [d]	360 ± 0.80 [b]	150 ± 0.40 [c]	163 ± 0.24 [c]	421 ± 0.76 [a]
3	β - Cryptoxanthin	31.89-32.04	2599 ± 0.85 [c]	1766 ± 0.42 [b]	ND	ND	9125 ± 1.1 [a]
4	α-carotene	38.4-38.53	1389 ± 0.70 [c]	2457 ± 0.50 [b]	ND	ND	9416 ± 0.5 [a]
5	β-carotene	42.95-43.43	31730 ± 1.0 [a]	8576 ± 4.3 [c]	643 ± 0.2 [d]	ND	11407± 1.6 [b]
	Total		**36417**	**14162**	**1039**	**2144**	**31274**

ND: No detect. The values are expressed as the mean ± deviation of the mean. Different letters in the same row indicate significant differences between treatments ($p < 0.05$).

A significant difference ($p<0.05$) was observed among the five genotypes in analysis of carotenoids. Those genotypes with color varying from red to orange (Guiana) and (Amarela olho de peixe), had a greater content of carotenoids, 36417 and 31274 μg/100 g DW respectively. Have Topuz and Ozdemir, (2007) reported that the red color represents more than 60% of total carotenoids for all cultivars of the species (*Capsicum annuum* L). According Conforti, Statti and Menichini, (2007), the variability of carotenoids in fruits may be explained by genetic factors as well as in the stages of maturation.

Deepa et al. (2007) found values of carotenoids ranging from 5700 to 43200 μg / 100 g of dry matter varieties of *Capsicum annuum* green and a 11400 and 134500 μg/100g dry matter varieties to mature. Have Topuz and Ozdemir, (2007) reported that in mature cultivars of (*Capsicum annuum*) carotenoid concentrations ranging from 231000 to 239000 μg /100g WD.

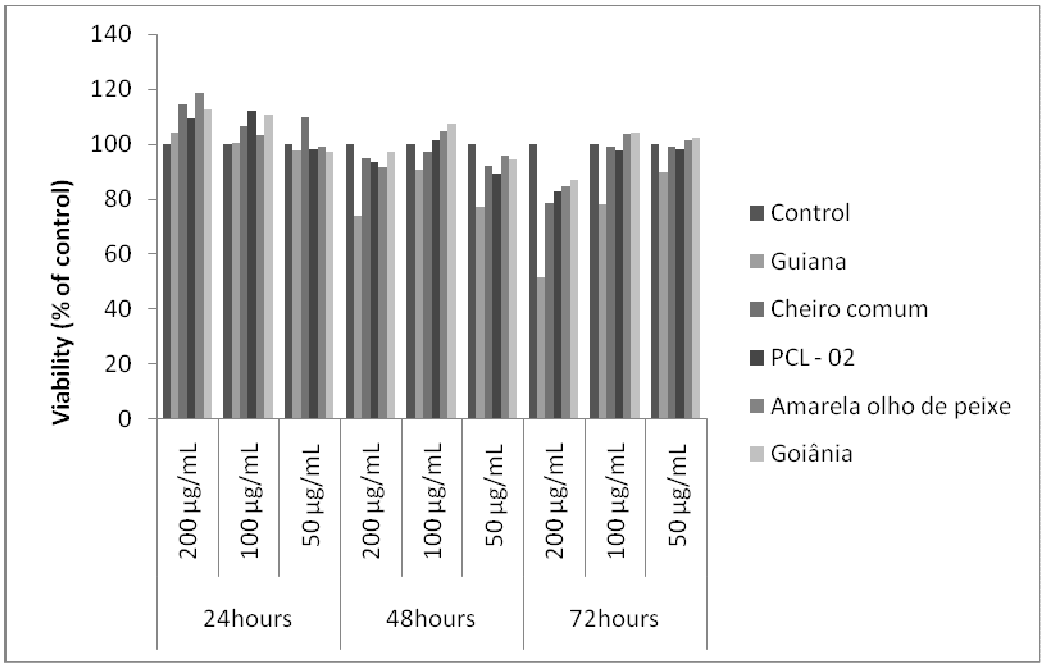
The concentrations of the carotenoid zeaxanthin exhibited the least variation among the genotypes. Topuz and Ozdemir (2007) reported that in cultivars of (*Capsicum annuum*) the carotenoid β-cryptoxanthin showed less variation, indicating that the synthesis of β-cryptoxanthin in fruits (*Capsicum annuum*) is independent of cultivar.

Increasing concentrations of extrats of peppers decrease the number of viable SH-SY5Y cells by elevating the percentage of late apoptotic cells for SRB

The obtained results indicate that tested peppers extract has an anticancer activity against human neuroblastoma cell line with notable effect even at low concentration (Fig. 3). At 72hours in concentraction of 200 μg/mL the genotype Guiana pepper there was about 48.6% decrease in cell viability and at 50 μg/mL concentration cell viability decreased to less than 10%. The results were evaluated statistically (T-TEST) with significance set at a p value of < 0.02.

The anti cancer effectiveness of peppers in low concentrations could be due to targeting one or more of the signaling pathways related more to cancer cells than to normal cells.

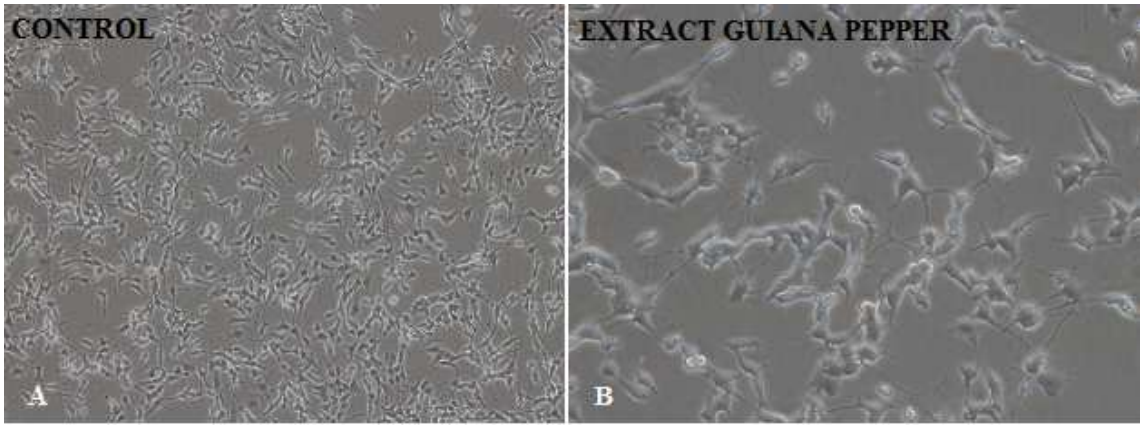
Figure 3. Percentage of viable of human neuroblastoma cells SH-SY5Y were then treated with extract of peppers (200 µg/mL, 100 µg/mL and 50 µg/mL) for 72, 48 and 24 hours.



Currently there are already several studies demonstrate that the capacity of capsaicina to inhibit in vivo and in vitro the growth of tumor cells such as leukemia, adenocarcinoma and glioblastoma cells (ITO ET AL. 2004; QIAO et al., 2005; MORI et al., 2006) by inducing apoptosis. As well as for capsaicin there are already several papers showing that carotenoids too are very important in cancer prevention.

Milder signs of the morphological events were also observed in cells treated with 200 µg/mL of extract of pepper Guiana (Fig. 4). In these cultures, cells seem to have lost the typical neuronal phenotype and characteristic neuritic processes of SH-SY5Y cells, became rounded, and thereafter, lost. The anticancer activity against both cell lines increased by increasing the extract concentrations from 50 to 200µg/mL for all three extracts.

Figure 4. Extract pepper altered cellular morphology and colony forming potential of human neuroblastoma cells. SH-SY5Y cells were then treated with extract (200 μg/mL) for 48 hours.

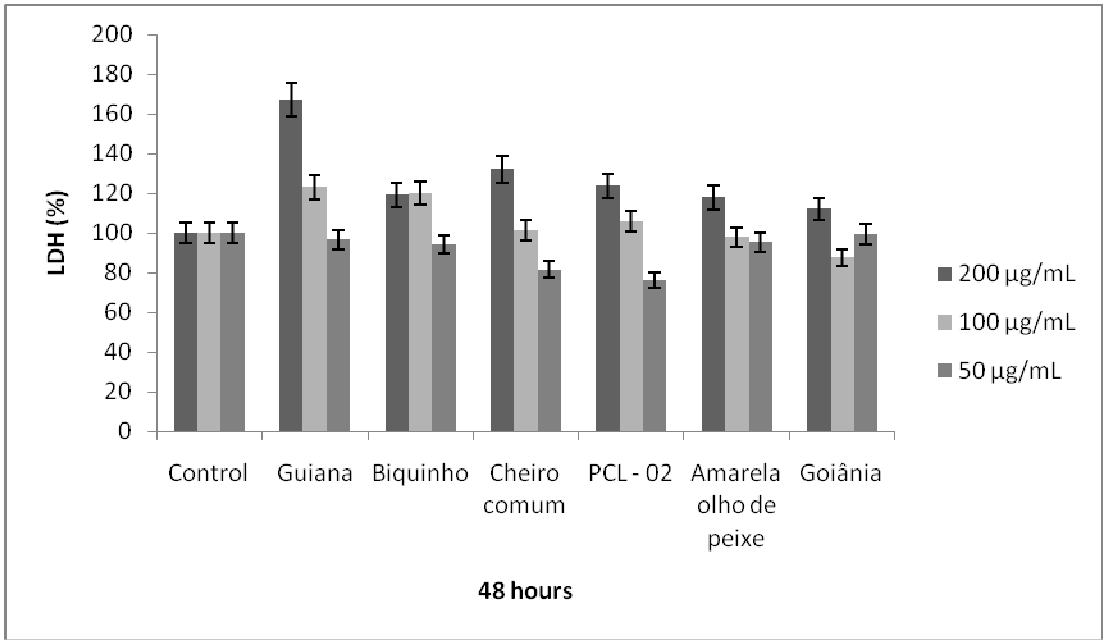


Quantification of activity of Lactate Dehydrogenase – LDH

The effect of extracts was studied in the human neuroblastoma cell culture (SH-SY5Y) through the cell viability by measuring the cell membrane integrity using the lactate dehydrogenase released method (LDH) (Fig 5).

The assay principle is based on consideration that tumor cells possess high concentration of intracellular LDH. In the presence of the drugs, cytokines or extracts that trigger cell death receptors superfamily tumor cells undergo apoptosis or necrosis. After cell membrane damage, LDH can be released and thus we detect death cells. (HAGGINS, 1999).

Figure 5. The released of LDH from human neuroblastoma derived (SH-SY5Y) cells after 48 h of treatment with extract of peppers (200µg/mL, l00µg/mL, and 50µg/mL). Data are presented as mean values ± standard deviation from experiments made in triplicate.



For calculation of percentage of dead cells, it is needed to calculate the intracellular LDH amount in respect to the released LDH amount. In the present work all extract of peppers tested increased the release of LDH from the cells into the surrounding culture medium significantly and were higher than control

4 CONCLUSIONS

Analyses of four genotypes of peppers *Capsicum annun* L. *var. annuum* and *C. chinense* Jacquin revealed that all extracts are redox active. This antioxidant property of the fruits can be attributed supposedly the content of capsaicinoids. The genotypes that had higher content and antioxidant activity of capsaicinoids and phenolic compounds were *Capsicum annun* L. *var. annuum* (PCL-02). Regarding the content of carotenoides, phenolic compounds and cytotoxic effect in human neuroblastoma cells the genotype *Capsicum annun* L. *var. annuum* (Guiana) presented itself as a candidate for use in breeding programs.

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2.3 ARTIGO 3: Effect of drying on carotenoid content of *Capsicum chinense* Jacquin peppers

EFFECT OF DRYING ON CAROTENOID CONTENT OF *CAPSICUM CHINENSE* JACQUIN PEPPERS

ABSTRACT

The processing of fresh food by removing water is increasingly being used for both preservation and providing seasonal products all year round. Furthermore, there is great interest in processes that retain bioactive compounds present in foods, such as carotenoids, which help to protect against and prevent disease. This study evaluated the effects of the temperature of the drying process (50 ºC, 70 ºC, and 80 ºC) on the carotenoid levels in *C. chinense* Jacquin peppers (Biquinho). The peppers proved to be good sources of carotenoids, and the fruits dried at 50 ºC showed total carotenoid levels twelve times higher than those of fruit in nature.

Keywords: drying, carotenoids, (*Capsicum chinense* Jacquin)

1. INTRODUCTION

Peppers *Capsicum* ssp.are important spices in cooking worldwide and are considered excellent sources of antioxidants (HOWARD et al., 2000). Peppers are commonly consumed fresh or processed, such as paprika, dehydrated peppers, sauces and other products.

Taking into account the variations in the consumption of different peppers regions of the country and the pepper *C. chinense* Jacquin peppers Biquinho can achieve high commercial value, it has shown an interesting alternative for families of small producers. You can grow it in small areas with a small number of plants and still get a satisfactory income. Its consumption is popular, especially in its processed form as a function of pickled aroma, crispness and lack of spiciness

To prevent fungal proliferation and improve the quality of pepper products, different drying methods have been employed (TOPUZ et al., 2011). The control of temperature, humidity, and water activity is essential for obtaining a quality product.

Processing can affect the stability of bioactive compounds present in peppers that play an important role in the prevention in the treatment of neurodegenerative diseases (DAIRAM et al., 2008), protection against cisplatin nephrotoxicity (Shimeda et al., 2005), and protection against cancer (MIN et al., 2004) due to the bioactive compounds, including carotenoids.

The heating and drying processes break up the food matrix, which can increase

the bioaccessibility of many phytochemicals and improve the nutritional quality of plants (PELLEGRINI et al., 2010). These processes may also decrease the phytochemical content (BALL, 2006) depending on the temperature used.

Various physical, chemical, and/or biological changes occur during processing and storage. The color of ripe pepper fruits, which originates from carotenoids, changes over time. The quality of some dyes including paprika prepared with peppers is evaluated using the intensity of the red color as a primary criterion; the intense red color of paprika originate mainly from carotenoids, which form in the fruit during ripening (KIM; PARK; HWANG, 2004).

Before the color of pepper fruit changes, carotenoid biosynthesis occurs, increasing the content of the carotenoid precursors β-carotene, β-cryptoxanthin, and zeaxanthin. These compounds are further transformed into antheraxanthin and violaxanthin, which are substrates for capsanthin-capsorubin synthase, which produces capsanthin and capsorubin, red carotenoids that are found exclusively in *Capsicum* spp. (HIRSCHBERG, 2001). These processes also occur after harvest and at the beginning of the drying process (HORNERO-MÉNDEZ; MÍNGUEZ-MOSQUERA, 2000).

The yellow carotenoids of peppers are mainly comprised of zeaxanthin, violaxanthin, antheraxanthin, β-cryptoxanthin, β-carotene, and capsolutein. All of these compounds are present in the chloroplast of red pepper and partially esterified with fatty acids (MINGUEZ-MOSQUERA; HORNERO-MENDEZ, 1994).

The two main roles of carotenoids in photosynthetically active plant tissue are trapping light energy at wavelengths poorly adsorbed by chlorophyll and protecting photosynthetic apparatus, dissipating energy, and “quenching” harmful reactive species, such as singlet oxygen and excited chlorophyll (FRANK; COGDELL, 1993).

Considering, the presence of the high content of the carotenoids in extracts of pepper the therapeutic potential of the dry extracts and the carotenoids are vulnerable to heat the objective of this study was to evaluate the effect of different drying temperatures (50 ºC, 70 ºC, and 80 ºC) in the profile of carotenoids in *C. chinense* Jacquin peppers.

2 MATERIALS AND METHODS

2.1 Chemicals

β-Cryptoxanthin (purity ≥ 97%), β-carotene (purity > 93%), α-carotene (purity ≥ 95%), and zeaxanthin (purity > 95%) were purchased from Sigma Chemical (St. Louis, MO, USA). The lutein standard (purity > 95%) was purchased from Indofine Chemical Company Inc. (Hillsborough, USA). HPLC-grade acetonitrile, methyl-tert-butyl, and methanol were purchased from HEXIS S/A (Mexico).

2.2 Plant material

One genotype of pepper *Capsicum chinense* Jacquin (Biquinho pepper) was identified in the herbarium of the Federal University of Rio Grande do Sul (voucher number ICN 173142). The peppers were harvested between the months of August and September 2011.

The fruit was vacuum packaged (Vacuum Sealer Sealer F 200 Flash, Brand Fastvac), wrapped in polythene bags with aluminum foil to avoid contact with light, and frozen (-10 ºC) until analysis.

2.3 Drying process

The peppers were dried in a tray dryer under constant-temperature convection. The samples 15g were distributed evenly on a tray, and the hot air was passed perpendicularly to the tray with a velocity of 0.72 m/s.

The drying curve was obtained by weighing the tray at intervals of 15 min until the weight variation was less than 1 g. The experiments were performed in duplicate for temperatures of 50 ºC, 70 ºC, and 80 ºC. The variation in the weight of the samples was measured using a balance accurate to 0.01 g.

The dryer temperature was monitored throughout the process using a manual thermometer with a maximum variation of ± 2 ºC.

To obtain the water content and water activity (a_w) curves, the samples were removed from the dryer and immediately analyzed. To determine the carotenoid profile, dried pepper was then vacuum packed and kept frozen (-18 ºC) until analysis.

2.4 Water content and water activity

Water content and water activity curves were obtained in duplicate for each of the three temperatures. The water content was determined in duplicate by weight loss in a 105 ºC oven to constant weight (AOAC, 2005) on an analytical balance with a precision of 0.0001 g. To determine water activity, samples were cut into 4 pieces and measured using a portable meter (Rotronic Hygropalm, model Hygropalm AW1, Crawley, UK) after calibration with supersaturated solutions of potassium sulfate and sodium chloride. Water activity measurements were performed in duplicate.

2.5 Mathematical modeling and coefficient diffusivity

The modeling of the drying process was performed by adjusting the experimental data to the equations of Table 1 using Statistica ® software version 10.

Table 1
Five commonly used drying curve models

No.	Model name	Model	References
1	Newton	$MR = \exp(-kt)$	Ayensu (1997)
2	Page	$MR = \exp(-kt^n)$	Sharma and Prasad (2001)
3	Modified Page	$MR = \exp(-kt)^n$	Diamante and Munro (1993)
4	Henderson and Pabis	$MR = a \exp(-kt)$	Westerman et al. (1973)
5	Two-term exponential	$MR = a \exp(-kt) + (1 - a) \exp(-kat)$	Sharaf-Eldeen et al. (1980)

To evaluate the quality of fit of each model, the coefficient of determination (R^2), root mean square error (RMSE), and reduced chi-square (χ^2) were used. These parameters can be calculated according to equations (1), (2), and (3):

1. $$RMSE = \frac{\sqrt{\sum_{i=1}^{N}\left(MR_{i,pre} - MR_{i,\exp}\right)^2}}{N}$$

2. $$\chi^2 = \frac{\sum_{i=1}^{N}\left(MR_{i,\exp} - MR_{i,pre}\right)^2}{N - p}$$

3. $$MR = \frac{X - X_E}{X_0 - X_E}$$

where *MR* is moisture dimensionless, MR_{pre} and MR_{exp} are the predicted and experimental values for moisture ratio (*MR*) respectively; *N* is the number of experimental points; *p* is the number of constants in the drying model; and *X*, X_E, and X_0 are the water content (expressed in kilograms of water per kilogram of dry solid, kg/kg) at any given moment, the equilibrium water content, and initial water content of the pepper, respectively. The equilibrium humidity content was determined by the extrapolation of the curve *dX/dt* in the graph *dX/dt* versus *X* to *dX/dt* equal to zero.

The best model for describing the drying behavior of peppers was chosen as the model with the highest coeficient of determination the least mean relative percent error, and the least root mean square error. In addition, reduced chi-square was used to determine the goodness of fit, with a lower value indicating a better goodness of fit.

The difusivities was calculated by general series solution of Fick's second law in spherical coordinates with the assumptions of constant moisture diffusivity and temperature, negligible shrinkage during drying is given as follows (CRANK, 1975).

$$MR = \frac{X - X_E}{X_0 - X_E} = \frac{6}{\pi^2}\sum_{n=1}^{\infty}\frac{1}{n^2}\exp\left(-\frac{n^2\pi^2 D_{eff}\,t}{r^2}\right) \quad (4)$$

Where D_{eff} is the moisture effective diffusivity (m^2/s) and *r* is the radius of the sphere (m). For long drying times (*MR* < 0.6) the solution of Fickian equation can be reduced by considering only the first term in its series expansion solution (CRANK, 1975):

$$MR = \frac{X - X_E}{X_0 - X_E} = \frac{6}{\pi^2}\exp\left(-\frac{\pi^2 D_{eff}\,t}{r^2}\right) \quad (5)$$

The difusivities are typically calculated by plotting experimental drying data in terms of ln(*MR*) versus drying time, which give a straight line with slope of $-\pi^2 D_{eff}/r^{2.}$

2.6 Color analysis

The color analysis dried product was performed by a MINOLTA CR 310 colorimeter using the *L* a* b**, illuminant D65, and a factor observer angle of 10º. The readings were performed in triplicate.

2.7 Preparation of extracts

The carotenoid extract was prepared according to Mercadante & Rodriguez-Amaya, 1998. The pigments were extracted with acetone and methanol saponification with 10 % KOH overnight at room temperature. After the removal of alkali, the extract was concentrated on a rotary evaporator (Fisatom, Model 801) (T < 25 ºC), dried in a stream of nitrogen and stored in the freezer (-18 ºC) for quantitation by high performance liquid chromatography (HPLC).

2.8 Carotenoid profiling by high-performance liquid chromatography (HPLC)

The analyses were carried out using an HPLC system (Agilent series 1100, Santa Clara, CA, USA) equipped with an online degasser, a quaternary pump, and an automatic injector coupled to a C_{30}.

For the analysis of carotenoids, the concentrated extract was diluted in 3 mL of HPLC-grade methyl tert-butyl ether (MTBE), placed on an ultrasound (Unique model USC 1400, Sao Paulo, Brazil) for 5 min and filtered through a Millipore filter (0.45 μm) for subsequent injection into the chromatograph.

The sample was then run on a YMC C_{30} reverse phase polymer column (250 mm length × 4.6 mm internal diameter and 3 μm particle size) with the following water/methanol/methyl tert-butyl ether (MTBE) mobile phase elution gradient: initially 5:90:5, 0:95:5 at 12 min, 0:89:11 at 25 min, 0:75:25 at 40 min, and 00:50:50 at 60 min with a flow rate of 1 mL/min at 33 ºC (ZANATTA; MERCADANTE, 2007). The spectra were recorded between 250 and 600 nm, and the chromatograms were processed at fixed wavelengths from 450 nm to 280 nm for carotenoids and capsaicinoids. Identification was performed by comparing the retention times of the peaks obtained for the standard with those of the samples analyzed under the same conditions.

Quantification was performed by constructing a standard curve for the carotenoids in the following concentration ranges: β-carotene, 5-50 μg/mL; α-carotene, 2-25 μg/mL; lutein, 1-65 μg/mL; cryptoxanthin, 4-100 μg/mL; zeaxanthin, 1-40 μg/mL. The limits of detection (LOD) and quantification (LOQ) (Long & Winefordner, 1983) were, respectively, as follows: β-carotene, 6.5×10^{-2} and 10.9×10^{-2} mg/kg; lutein, 6.9

$\times 10^{-3}$ and 1.2×10^{-2} mg/kg; cryptoxanthin, 2.1×10^{-2} and 3.5×10^{-2} mg/kg; zeaxanthin, 9.6×10^{-2} and 1.6×10^{-2} mg/kg; α-carotene, 2.0×10^{-2} and 3.3×10^{-2} mg/kg.

2.9 Statistical analysis

The results were evaluated by analysis of variance (ANOVA) and the Tukey test for mean differences using Statistic ® software v. 10.

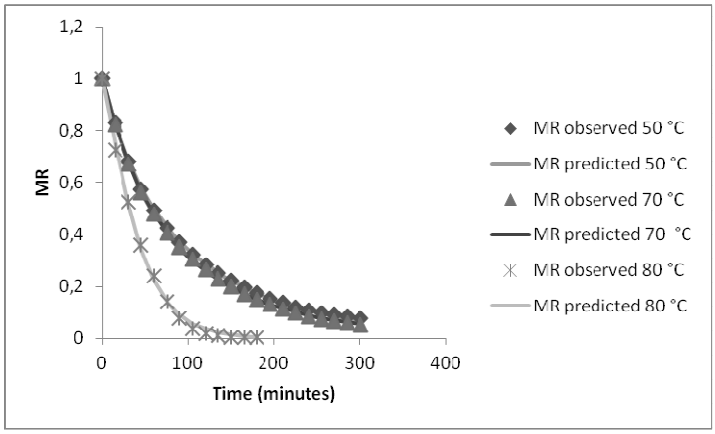
3 RESULTS AND DISCUSSION

3.1 Water activity, water content, and mathematical modeling

The average initial water content of the pepper was 86 %. The 50 ºC, 70 ºC, and 80 ºC drying processes corresponded to total dehydration durations of 300 min, 210 min, and 180 min and final water contents of 38 %, 15 %, and 7.0%, respectively.

The drying rates as a function of moisture ratio (*MR*) are shown in Figure 1, where it can be seen that the higher the temperature, the higher the drying rate.

Figure. 1. Drying curves of pepper *C. Chinense* Jacquin as a function of time at 50 ºC, 70 ºC, and 80 ºC.



The drying curves were modeled using the Newton, Page, Modified Page, Henderson and Pabis, and Two-term exponential models. The values calculated from the regression coefficient (R^2), root mean square error (RMSE), and reduced chi-square (χ^2), shown in Table 2, indicate that although all models can express the drying process the curves for each temperature were best described by the Two-term exponential

model.

This model showed the highest R^2 values, 0.9998, 0.9999, and 0.9989 for drying temperatures of 50 ºC, 70 ºC, and 80 ºC respectively and the lowest RMSE and χ^2 values when compared to the other models, and was thus the best representation of the drying behavior of peppers.

Table 2
Statistics of *C. chinense* pepper drying curves at 50 ºC, 70 ºC, and 80 ºC

Temperature	Model	R^2	RMSE	χ^2
50 ºC	Newton	0.9951	6.18×10^{-3}	8.42×10^{-4}
	Page	0.9951	1.55×10^{-3}	5.30×10^{-5}
	Modified Page	0.9951	6.18×10^{-3}	8.42×10^{-4}
	Henderson and Pabis	09967	5.08×10^{-3}	5.98×10^{-4}
	Two-term exponential	0.9998	1.10×10^{-3}	2.83×10^{-5}
70 ºC	Newton	0.9973	4.46×10^{-3}	4.39×10^{-4}
	Page	0.9973	4.46×10^{-3}	4.39×10^{-4}
	Modified Page	0.9973	4.46×10^{-3}	4.39×10^{-4}
	Henderson and Pabis	0.9982	3.65×10^{-3}	3.09×10^{-4}
	Two-term exponential	0.9999	7.89×10^{-4}	1.44×10^{-5}
80 ºC	Newton	0.9952	7.54×10^{-3}	8.00×10^{-4}
	Page	0.9952	7.54×10^{-3}	8.00×10^{-4}
	Modified Page	0.9952	7.54×10^{-3}	8.00×10^{-4}
	Henderson and Pabis	0.9957	7.10×10^{-3}	7.74×10^{-4}
	Two-term exponential	0.9989	3.58×10^{-3}	1.97×10^{-4}

The values of humidity and Aw, was 0.9 for peppers in nature and for 0.34 dried peppers, with humidity between 7% having aw between 0.34. The moisture content is an important parameter for food preservation, referring to the quality and stability.

Table 3
Water content, water activity (a_w) and effective diffusivity (D_{eff}) of dried *C. chinense* pepper at drying temperatures of 50 ºC, 70 ºC, and 80 ºC.

Temperature	Water content (%)	a_w	Effective diffusivity (m^2/s)
50 ºC	38.9	0.36	8.48×10^{-10}
70 ºC	15.5	0.33	1.01×10^{-9}
80 ºC	7.4	0.31	4.00×10^{-9}

Fick's second diffusion law has been widely used to describe the drying process during the falling rate period for biological materials (DOYMAZ, 2008). Moisture diffusion in solids during drying is a complex process that may involve molecular

diffusion, capillary flow, Knudsen flow, hydrodynamic flow, surface diffusion and all other factors which affect drying characteristics. Since it is difficult to separate individual mechanism, combination of all these phenomena into one, the effective or apparent diffusivity (a lumped value) can be defined from Fick's second law (ABBASI; MOWLA, 2008).

The effective diffusivities determined for temperatures of 50 ºC, 70 ºC, and 80 ºC where 8.48×10^{-10} m^2/s, 1.01×10^{-9} m^2/s, and 4.00 10^{-9} m^2/s, respectively (Table 3).

These values are comparable with the reported values of 1.5×10^{-9} m^2/s for raisin (LOMAURO, et al., 1985), 2.02×10^{-9} m^2/s for 60 ºC hot-air drying of paprika (RAMESH, et al., 2001), and 1.79×10^{-9} m^2/s to 4.45×10^{-9} m^2/s for apple slices at 60 ºC (VELIC, et al. 2004).

It can be also observed an increase of effective diffusivity with temperature, indicating that the increase of the temperature will increase the drying rate (as observed in Figure 1). Similar behavior was observed for several food drying process as fresh green beansin a fluidized bed dryer (ABBASI; MOWLA, 2008), strawberries (DOYMAZ, 2008), grape by-products (CELMA et al., 2009) and seedless grapes (ESMAIILI et al., 2007).

3.2 Carotenoids

The color of *Capsicum* fruit is determined in large part by the quantity and composition of carotenoids deposited in the chromoplasts. Previous studies indicated that red fruits tend to accumulate a larger amount of fruit carotenoids than non-red fruits (Ha et al., 2007).

Identification of five carotenoids (all-*trans*-lutein, zeaxanthin, β-cryptoxanthin, β-carotene, α-carotene) was possible through chromatographic separation. High concentrations of carotenoids were observed in *C. chinense* Jacquin pepper.

Table 4

Carotenoids in order of elution found in *C. chinense* Jacquin pepper dried at 50 ºC, 70 ºC, and 80 ºC with their respective concentrations (µg/100 g DW).

			Concentrations (µg/100 g DW)			
Peak No.	Carotenoid	Rt range (min)	Pepper *in nature*	50 ºC	70 ºC	80 ºC
1	*All-trans*-lutein	17.4-18.53	7 976 ± 3.1^{a}	2 580 ± 0.5^{c}	625 ± 0.1^{d}	752 ± 0.2^{d}
2	Zeaxanthin	20.8- 21.53	1 084 ± 0.8^{b}	28 425 ± 0.4^{b}	5 947 ± 4.7^{b}	8 594 ± 0.2^{a}
3	β-Cryptoxanthin	31.01-32.87	350 ± 0.5^{e}	23 834 ± 0.5^{b}	3 798 ± 0.2^{c}	3 408 ± 0.1^{c}
4	α-Carotene	38.4-38.53	578 ± 0.2^{d}	2 328 ± 0.0^{c}	ND	ND
5	β-Carotene	42.95- 43.43	677 ± 0.5^{d}	70 402 ± 1.6^{a}	13 833 ± 1.0^{b}	7 173 ± 0.1^{c}
Total			**10 667**	**127 571**	**24 203**	**19 928**

ND: Not detected. The values are expressed as the mean ± standard deviation. Different letters in the same row indicate significant differences between treatments ($p \leq 0.05$).

Peppers in nature had a greater content of lutein (7976 µg/100 g DW) than peppers dried at 50 ºC, 70 ºC, or 80 ºC. Lutein is the primary carotenoid pigment in chlorophyllous tissues (HA et. al., 2007; RODRIGUEZ-AMAYA, 2001) and plays an important role in photosynthesis (SANDMANN et. al., 2006). Food sources of lutein and zeaxanthin are of interest because they are essential in protecting the macula of the eye (BOTELLA-PAIVA; RODRÍGUEZ-CONCEPCIÓN, 2006).

According Minguez-Mosquera and Hornero-Mendez (1994), with increasing temperature, there is decrease in lutein content (yellow color) and an increase in the contents of carotenoids, such as zeaxanthin, β-cryptoxanthin, and α- and β-carotene (red color). Their study postulated that a slow drying process, depending on drying time and temperature, would increase the production of red carotenoids from their yellow precursors and that the newly formed pigments would be associated with incomplete maturation (MINGUEZ-MOSQUERA; HORNERO-MENDEZ, 1994).

Topuz et al., (2011) demonstrated that the quantification method also affects the carotenoid content in paprika. The drying method that maintains the carotenoid content is natural convective drying, which provides a 90 % increase in the β-carotene content relative to fresh sample. In the drying method used in this work, the β-carotene content in the sample dried at 50 ºC was approximately over 100 times greater than that found in fresh peppers.

Kevrešan et al. (2009) dried *Capsicum annum* peppers at 60 ± 5 ºC in the same manner as used in this study and observed a decrease in the number of carotenoids present in the samples. The total yellow carotenoids decreased by a factor of 2.48 and the total red carotenoids increased by a factor of 1.46 after the drying process, demonstrating yeloow carotenoid degradation and increased bioaccessibilidade of red carotenoids.

Carotenoid degradation occurs at temperatures near or above 40 ºC (THAKKAR et al., 2009). In this work, 50 ºC yielded an increase of carotenoids this can be justified by the fact that the heating causes the rupture of the food matrix, which can provide for increased bioaccessibilidade many phytochemicals and improve the nutritional quality of plants (PELLEGRINI et al. 2010). Thus for foods containing high amounts of bioactive compounds, choosing the right method for the preservation becomes very important to successful operation, since processing can partially or completely affect the quality of a product.

The peppers dried at 50 ºC exhibited total carotenoid contents twelve times higher than that found in fresh peppers. Relative to fresh pepper, the pigment concentrations were approximately 26 times higher for zeaxanthin, 68 times higher for β-cryptoxanthin, 4 times higher for α-carotene, and 104 times higher for β-carotene. Relative to the sample dried at 50 ºC, samples dried at 70 ºC and 80 ºC exhibited lower total carotenoids content by factors of 5.27 and 6.40, respectively, but still higher when compared to in nature pepper because the rupture of the food matrix, which can provide for increased bioaccessibilidade of carotenoids. At 70 ºC and 80 ºC, the carotene contents were undetectable due to degradation at elevated temperature.

3.3 Color analysis

The decrease of color in dried peppers may be explained by degradation and synthesis of carotenoids with increasing temperature (Table 5).

Table 5 Color analysis parameters for *C. chinense* Jacquin pepper dried at 50 ºC, 70 ºC, and 80 ºC.

	L^*	a^*	b^*	C^*
In nature	35.8 ± 1.0[a]	22.3 ± 1.0[b]	20.1 ± 1.0[a]	30.0 ± 1.1[a]
50 ºC	30.8 ± 1.0[a]	25.7 ± 1.0[b]	16.9 ± 0.7[b]	30.8 ± 1.1[a]
70 ºC	30.4 ± 2.4[a]	31.2 ± 4.7[a]	16.6 ± 4.5[b]	34.9 ± 1.2[a]
80 ºC	28.2 ± 1.4[b]	33.2 ± 1.8[a]	15.2 ± 1.5[b]	36.5 ± 2.0[a]

Different letters in the same row indicate significant differences between treatments ($p \leq 0.05$).

The achromatic component L^* (lightness) decreased with increasing temperature, which is in agreement with previous studies indicating that L^* decreases with the appearance of red color, causing a loss of brightness due the synthesis of carotenoids and decreased chlorophyll (LÓPEZ CAMELO; GÓMEZ, 2004).

The chromatic component (a^*), which express the degree of red ($+a^*$) and green ($-a^*$), increases with increasing temperature, corresponding to the increase of some carotenoids (zeaxanthin, β-cryptoxanthin, β-carotene, and α-carotene) and degradation of lutein (yellow). The chromatic component values of b^* ($+b^*$, grade of yellow; $-b^*$, degree of blue) indicated a decrease in yellow color with increasing temperature.

The chroma (C^*) parameter incorporates both a^* and b^* parameters and is directly related to color stability. Even for a single variety of red pepper, the color stability could differ by the area of cultivation and environmental factors. The value of red pepper powders could be estimated by the difference in the C^* value (KIM et al,. 2002). However, in this case, there were no significant differences in the chroma of fresh and dried peppers, indicating that the color remained stable for drying at 50 ºC, 70 ºC, and 80 ºC.

4 CONCLUSION

The drying of peppers to produce dehydrated peppers at 50 ºC, 70 ºC, and 80 ºC was adequate to create safe products for human health, with values of a_w below the limit of growth by pathological microorganisms. Additionally, the drying of peppers at 50 ºC presented the highest total content of carotenoids by rupture of the food matrix, providing for increased bioaccessibilidade of carotenoids. The two-term exponential model best simulated the drying of pepper. The results show that the proposed model can be used to optimize the drying process to obtain higher-quality dried red pepper from a nutritional perspective with respect to carotenoids.

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3. CAPITULO 3

DISCUSÃO GERAL

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O presente trabalho foi desenvolvido com o intuito de obter e difundir informações a respeito da pimenta, um fruto com enorme potencial de produção e elevada capacidade antioxidante, a fim de incentivar seu cultivo e sua comercialização no país, especialmente em prol dos benefícios à saúde que lhe são alegados, bem como do favorecimento da economia regional e nacional, visto que os fitoquímicos presentes são capazes de produzir efeitos protetores ao organismo e que tais frutos podem oferecer uma fonte de renda adicional ao pequeno agricultor, fortalecendo o sistema produtivo.

Nesse sentido, no primeiro capítulo, foram abordados os principais aspectos referentes à cultura da pimenta, perspectivas mercadológicas e propriedades bioativas e antioxidantes. Além disso, tratou-se dos compostos presentes neste fruto, em especial, os capsaicinoides, carotenoides, compostos fenólicos e ácido ascórbico. Apresentou-se uma revisão a respeito da estrutura química, e ações biológicas dessas substâncias na prevenção e tratamento de doenças degenerativas.

O capítulo 2 foi elaborado com os artigos científicos desenvolvidos, correspondendo o artigo 1: “Extração de compostos bioativos em pimentas *Capsicum* usando solvente de grau alimentício”. Pode-se verificar que a concentração e a atividade destes compostos bioativos naturalmente presentes em alimentos pode ser diretamente relacionado com as propriedades dos solventes tais como solventes lipofílicos e hidrofílicos e a sua respectiva polaridade. O etanol foi efetivo na extração de ácido ascórbico e capsaicinoides com destaque para a extração de carotenoides, apresentando-se muito efetivo pois segundo método proposto por Mercadante e Rodriguez-Amaya (1998) durante a saponificação alguns carotenoides como a all-*trans*-luteína são degradados. Embora o uso de solventes orgânicos para a produção de extratos de plantas seja convencional, questões relativas à toxicidade farmacológica e ambientais, bem como o perigo da manipulação permanecem. Assim, a substituição de outros solventes orgânicos por etanol a 96% é de grande interesse para a redução de custos operacionais, impactos ambientais e de toxicidade. As pimentas possuem vários compostos bioativos alguns apolares e outros polares, sendo assim, todos estes compostos não podem ser extraídos apenas por um solvente. Sugere-se que em trabalhos futuros, com base nos dados obtidos, a mistura de outros solventes não

tóxicos, cuja utilização seja permitida nos alimentos para a extracção de compostos bioactivos seja explorada.

No artigo 2: “Compostos bioativos e efeito citotóxico de extratos de pimentas (*Capsicum*) em células de neuroblastoma humanos (SH-SY5Y) ”, onde o objetivo foi selecionar o genótipo que se destacasse em relação ao potencial antioxidante, viabilidade em células e compostos bioativos para uso em programas de melhoramento genético. Foi possível observar que, os cinco acessos podem ser considerados antioxidantes e fonte de compostos bioativos. Dentre os cinco o acesso Guiana destacou-se em relação aos carotenoides e proporcionou redução de 52% na células de neuroblastoma humano após 72horas e o genótipo PCL-02 destacou-se com relação a atividade antioxidante, teor de compostos fenólicos e capsaicinoides. Obtiveram-se também, correlações positivas entre o conteúdo de capsaicinoides e atividade antioxidante ($r = 0.87$, $p < 0.05$) e entre o conteúdo de compostos fenólicos e carotenoides ($r = 0.76$, $p < 0.05$). No entanto, é importante destacar que os extratos consistem numa matriz complexa, com um número de compostos bioativos, incluindo: carotenoides, fenólicos, capsaicinoides, como revelado pela análise destes extratos, assim, a actividade antioxidante pode ser explicada pelo sinergismo dos compostos.

O artigo 3: “Efeito da secagem no conteúdo de carotenóides em pimentas *Capsicum Chinense* Jacquin”. A secagem dos frutos de pimenta biquinho apresentou-se adequada e possível de ser modelada, com valores de a_w abaixo do limite de contaminação por microrganismos patológicos (nas temperaturas de 50 ºC, 70 ºC e 80 ºC). Dentre os cinco modelos, o de Dois Termos Exponencial foi que mais representou secagem da pimenta biquinho, por apresentar maior valores de R^2 e menores *RMSE* e χ^2 que outros modelos nas três temperaturas testadas. Observou-se por meio do estudo que o processo de secagem a 50 ºC possibilitou um aumento de carotenoides resultando em uma concentração total superior a encontrada na pimenta *in natura* devido a ruptura da matriz estrutural da pimenta que proporcionou maior biodisponibilidade desdes carotenóides. O aumento da temperatura a secagem 70 ºC e 80 ºC mostrou-se prejudicial às propriedades bioativas quanto ao teor de carotenoides como, por exemplo, para o α-caroteno que nas temperaturas de 70 ºC e 80 ºC não foi possível a sua quantificação. A estabilidade dos carotenoides e dos frutos está relacionada tanto com a magnitude quanto com a duração do tratamento térmico. O melhor entendimento dos fatores envolvidos na degradação de tais substâncias é, portanto, fundamental para maximizar a qualidade nutritiva e sensorial de produtos alimentícios.

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