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TAYSE FERREIRA FERREIRA DA SILVEIRA

PHENOLIC COMPOUNDS AND ANTIOXIDANT CAPACITY OF AÇAÍ PULP (*Euterpe oleracea* Mart.): EFFECT OF HIGH PRESSURE PROCESSING IN COMPARISON TO THERMAL PASTEURIZATION OF PURPLE AÇAÍ PULP AND A STUDY ON "WHITE AÇAÍ"

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Orientadora: Prof[a] Dr[a]. Helena Teixeira Godoy

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A ata da defesa com as respectivas assinaturas dos membros encontra-se no processo de vida acadêmica da aluna.

“Com ele está o braço de carne, mas conosco o Senhor nosso Deus, para nos ajudar, e para guerrear por nós.”

(2 Crônicas 32:8)

“E prá que tu foi plantado
Prá invadir a nossa mesa
E abastar a nossa casa...
Teu destino foi traçado
Pelas mãos da mãe do mato
Mãos prendadas de uma deusa
Mãos de toque abençoado...
És a planta que alimenta A
paixão do nosso povo A
mais magra das palmeiras
Mas mulher do sangue grosso
E homem do sangue vasto
Tu te entrega até o caroço...
Tens o dom de seres muito
Onde muitos não têm nada
Uns te chamam açaizeiro
Outros te chamam juçara...
Põe tapioca
Põe farinha d'água
Põe açúcar
Não põe nada
Ou me bebe como um suco
Que eu sou muito mais que um fruto
Sou sabor marajoara (...)”

Nilson Chaves

DEDICO

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RESUMO

Este trabalho teve como objetivo investigar o efeito da alta pressão hidrostática (APH) (400, 450, 500, 600 MPa/5 min/20ºC) no conteúdo de tocoferóis, compostos fenólicos antociânicos (CFA), não antociânicos (CFNA), e na capacidade antioxidante contra espécies reativas do oxigênio (ROS) e nitrogênio (medida como apparent total nitroso compounds – ATNC) da polpa de açaí. Os resultados foram comparados com amostras controle (sem nenhum tratamento) e pasteurizadas termicamente (PT) (85ºC/ 1min). Primeiramente, um planejamento de misturas simplex centroide com 4 solventes (acetona, metanol, água, etanol) e um planejamento composto central (PCC) 2^3 avaliaram o efeito da composição do solvente, do tempo, da razão sólido-líquido e da porcentagem de ácido no teor de antocianinas totais (AT) e compostos fenólicos totais (CFT) extraídos. Como resultado, as condições ótimas de extração foram solvente extrator composto de 31:24:45 v/v/v acetona:metanol:água, razão sólido:líquido (S/L) 1:54, e tempo de extração de 6 minutos. Para a avaliação do efeito da APH nos CFNA, um método por cromatografia líquida de ultra alta eficiência e DAD (UHPLC-DAD) foi otimizado utilizando um PCC 2^3 (que avaliou o efeito do tempo de gradiente linear e da porcentagem inicial e final de acetonitrila no gradiente), resultando em 8 minutos de análise, começando com 7,8% e terminando com 15,7% de acetonitrila. Os resultados indicaram que comparado ao controle, ambas as técnicas de conservação promoveram redução significativa do teor de CFA (medidos por cromatografia líquida de alta eficiência com DAD, HPLC-DAD), porém, a APH foi mais eficiente para reter a cianidina-3-glicosídeo e a cianidina-3-rutinosídeo do que PT (até 40% mais). PT não afetou significativamente o teor individual dos CFNA em comparação a polpa controle, ao passo que para as amostras pressurizadas observou-se um aumento significativo no teor destes compostos nas amostras processadas a 500 MPa (até 60% mais). Além disso, as amostras tratadas a 500 MPa apresentaram maior capacidade de sequestro do radical peroxila do que controle e PT. As amostras APH e controle exibiram maior capacidade de inibição da ROS HOCl do que PT (até 3 vezes mais). Por outro lado, PT apresentou a maior capacidade inibitória para H_2O_2 dentre todas as amostras, bem como exibiu maior capacidade de inibição de espécies reativas do nitrogênio do que o controle, mas no geral não diferiu das amostras tratadas com APH para esta propriedade. Já os tocoferóis (analisados por HPLC-detecção por fluorescência) não foram significativamente afetados por nenhum dos tratamentos usados. Assim, a APH mostrou-se uma técnica eficiente para preservar os compostos nutricionais e bioativos da polpa de açaí, sendo o processo a 500 MPa/5 min/20ºC a melhor condição testada neste trabalho. Finalmente, os CFNA e a capacidade antioxidante em relação a ROS e ATNC também foram determinados para a polpa de açaí branco. A extração dos CFNA foi otimizada através de um PCC 2^2 que avaliou o efeito do tempo de extração e % etanol no teor de CFT, resultando em 35:65 v/v etanol:água, 10 minutos, S/L 1:54. Os extratos foram analisados por UHPLC acoplado a um triplo-quadrupolo configurado para MRM (monitoramento múltiplo de reações) e ionização no modo negativo. O perfil de CFNA do açaí branco foi similar ao do açaí roxo, sendo a orientina, isoorientina e ácido vanílico os compostos majoritários (90%). Além disso, apresentou capacidade antioxidante contra espécies reativas de relevância fisiológica (ROO e HOCl) e boa capacidade inibitória de formação de ATNC, mostrando que pode ser uma fonte potencial de substâncias antioxidantes para a dieta.

Palavras-chave: açaí, alta pressão hidrostática, compostos fenólicos

ABSTRACT

This study aimed to evaluate the effect of high pressure processing (HPP) (400, 450, 500, 600 MPa/5 min/20ºC) on the contents of tocopherols, anthocyanin (APC), non-anthocyanin phenolic compounds (NAPC) and on the scavenging capacity against reactive oxygen species (ROS) and nitrogen species (measured as apparent total nitroso compounds – ATNC) on the açaí pulp. The results were compared to control (untreated) e thermal pasteurized (TP) (85ºC/ 1min). At first, a simplex centroid mixture design (acetone, methanol, water, ethanol) and a 2^3 central composite design (CCD) were used to study the effect of the solvent composition, time, solid-to-liquid ratio and acid percentage on total monomeric anthocyanins and total phenolic compounds (TPC) extraction. Optimum extraction conditions were: extractor solvent 31:24:45 v/v/v acetone:methanol:water, 1:54 solid to liquid ratio (S/L) and six min long. To evaluate the effect of HPP on NAPC, a method by ultra-high performance liquid chromatography and DAD (UHPLC-DAD) was optimized through a CCD 2^3 (studying the effect of time of linear gradient, acetonitrile percentage at the beginning and at the end of the gradient). The method was 8 minutes long, starting at 7.8% and ending up at 15.2% acetonitrile. According to the results, both preservation techniques caused the APC content to decrease significantly (measured by high performance liquid chromatography with DAD, HPLC-DAD), however, HPP was more effective to preserve cyanidin 3-glucoside and cyanidin 3-rutinoside (up to 40% more). TP did not affect significantly NAPC content compared to the control, whereas the samples treated at 500 MPa were found to exhibit a significant increase in the NAPC amounts (up to 60%). Furthermore, the samples treated at 500 MPa had higher scavenging capacity against peroxyl radical than control and TP. HPP and control samples exhibited higher scavenging capacity towards HOCl than TP. On the other hand, TP displayed the highest scavenging capacity against H_2O_2 among all samples, as well as showed higher inhibition capacity against reactive nitrogen species (ATNC) than the control, but globally it did not differ from HPP samples regarding this property. Tocopherols (measured by HPLC-fluorescence detector) have not been found to differ significantly among the treatments applied. Therefore, HPP was efficient in preserving nutritional and bioactive compounds of açaí pulp, and 500 MPa/5 min/20ºC was the best processing condition tested. Finally, NAPC and the antioxidant capacity against ROS and ATNC was determined for white açaí pulp (WAP). NAPC extraction was optimized using a CCD 2^2, which examined the effect of time and %ethanol on TPC extraction. Final conditions were 35:65 v/v ethanol:water, 10 minutos, S/L 1:54. The extract was analysed by UHPLC coupled with a triple quadrupole mass spectrometer set for operate on MRM (multiple reaction monitoring) and negative ionization mode. NAPC profile of WAP was similar to that of purple açaí, with isoorientin, orientin and vanillic acid as the predominant phenolics (90%). In addition, the WAP showed scavenging capacity against radicals with physiological relevance (ROO and HOCl) and great inhibitory capacity for ATNC formation, indicating that this product might be a potential source of antioxidant substances in the diet.

Key words: açaí, high hydrostatic pressure, phenolic compounds

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INTRODUÇÃO GERAL

As frutas e os alimentos à base de frutas são reconhecidos por seu alto valor nutricional e por serem fontes de componentes bioativos e substâncias que, quando ingeridas, podem promover tanto benefícios à saúde, como auxiliar na prevenção contra o desenvolvimento de certos tipos de câncer, doenças cardíacas, neurodegenarativas e diabetes (Scalbert et al., 2005, FAO, 2015). Nesse contexto, o Brasil se destaca como o terceiro maior produtor mundial de frutas, e em decorrência da sua vasta extensão territorial, permite abrigar uma variada gama de espécies frutíferas de clima temperado e tropical (IBRAF, 2016, Buainain, Batalha, 2007). Dentre estas, destaca-se o açaí, fruto da palmeira *Euterpe Oleracea*, originária da Amazônia brasileira, a partir do qual obtém-se a bebida ou polpa de açaí (Schauss, 2010).

Inicialmente um produto de consumo restrito à região amazônica, a partir do final da década de 90 a polpa de açaí conquistou posição de destaque no mercado de polpas de fruta nas regiões Sul, Sudeste e Nordeste do Brasil, bem como passou a ser exportado para países da América do Norte e Europa (Menezes et al., 2011). Na última década, a produção nacional de açaí dobrou, crescendo a uma taxa de 10% ao ano, transformando o fruto no principal produto de extrativismo vegetal brasileiro, com 216 mil toneladas produzidas em 2015. Nesse mesmo período, a renda gerada a partir da sua produção aumentou 80%, a uma taxa de 37% ao ano, resultando em mais de 400 milhões de reais gerados em 2015 (SIDRA, 2016). Além disso, os valores de exportações de polpa de açaí para a América do Norte, Europa e Ásia também têm crescido significativamente, apresentando um aumento de cerca de 37% entre 2012 e 2014 (Conab, 2016). Portanto, atualmente, o açaí é uma das frutas com maior crescimento de demanda no mercado nacional e internacional, e um importante produto de desenvolvimento da economia Amazônica (Conab, 2015).

O interesse crescente sobre este produto está relacionado as suas características nutricionais, contendo fibras, proteínas, minerais e alto valor energético, bem como nas suas propriedades funcionais relacionadas à presença de compostos bioativos (Rogez, 2000, Schauss et al., 2006). A polpa de açaí apresenta em sua composição química altas concentrações de compostos fenólicos, em especial de antocianinas, os pigmentos responsáveis pela coloração violácea do fruto (Dias et al., 2012, Dias et al., 2013). Níveis significativos de tocoferóis também fazem parte da composição do fruto (Schauss et al., 2006, Costa et al., 2010, Darnet et al., 2011). Esta composição química tem sido associada à atividade biológica relatada em

estudos *in vitro* e *in vivo*, nos quais extratos de açaí apresentaram elevada capacidade antioxidante, antiproliferativa, anti-inflamatória e hipocolesterolêmica (Kazumy et al., 2015).

Contudo, em função das características do fruto e das condições intrínsecas altamente favoráveis ao desenvolvimento de micro-organismos, a polpa de açaí possui elevada perecibilidade, tornando-se indispensável a aplicação de algum método de conservação que permita prolongar a vida útil desse produto (Rogez, 2000, Aguiar, Menezes, Rogez, 2013). Atualmente, a conservação da polpa de açaí é realizada através do emprego de calor (pasteurização) e/ ou resfriamento/congelamento (Kazumy et al., 2015).

No entanto, alguns estudos já demonstraram que os compostos bioativos, bem como a qualidade nutricional e sensorial dos alimentos, podem ser negativamente afetados quando submetidos às altas temperaturas normalmente utilizadas na indústria para efetuar o tratamento térmico, o que pode resultar na degradação destas substâncias (Sanchez-Palência et al., 2005, Pacheco-Palência, Ducan, Talcott, 2009, Hoffman-Ribani, Huber, Rodriguez-Amaya, 2009, Murcia, Jimenez, Martinez-Tomé, 2009).

Nesse contexto, o estudo da aplicação de métodos de conservação que não utilizam calor como princípio de eliminação de micro-organismos, como por exemplo a tecnologia de alta pressão hidrostática (APH), torna-se uma alternativa relevante para atender a demanda crescente por alimentos que apresentem características nutricionais, funcionais e sensoriais próximas ao do produto *in natura*. O processamento por APH caracteriza-se como um método de pasteurização capaz de resultar em produtos microbiologicamente seguros e, por não afetar ligações covalentes, permite maior preservação dos nutrientes, compostos funcionais e substâncias que conferem sabor e aroma à matriz alimentícia em relação ao método tradicional (Balasubramanian, Martínez-Monteagudo, Gupta, 2015). Diversos estudos têm relatado que a APH é capaz de produzir produtos com elevada qualidade sensorial, nutricional e funcional (Oey et al., 2008, Patras et al., 2009, Keenan et al., 2011, Cilla et al., 2012, Huang et al., 2013, Rodrígues-Roque et al., 2015). No entanto, Barba, Esteve e Frígola (2012) revisaram o efeito da APH sobre a composição química de frutas e constataram que apesar de ser possível estabelecer algumas tendências, a resposta de um mesmo composto ao processo pode variar significativamente de acordo com a matriz alimentícia, concluindo-se ser necessário um estudo direcionado para cada alimento.

Assim, tendo em vista a elevada importância biológica da polpa de açaí em função dos compostos bioativos presentes em sua constituição, e partindo da premissa de que o uso da alta pressão para a conservação dos alimentos pode contribuir para a obtenção de produtos com

qualidade sensorial e nutricional elevados, bem como para a preservação dos compostos bioativos presentes, este estudo teve como objetivo avaliar o efeito da aplicação do método de conservação por alta pressão hidrostática sobre os compostos fenólicos antociânicos, não-antociânicos, tocoferóis e capacidade antioxidante frente a espécies reativas do oxigênio (ROS) e do nitrogênio (medidos como capacidade de inibição da formação de compostos nitrosos) da polpa de açaí. Os resultados foram comparados a amostras controle (sem tratamento) e pasteurizadas termicamente. Além disso, com o objetivo de preencher uma lacuna de conhecimento existente na literatura, os compostos fenólicos e a capacidade antioxidante em relação a EROs e a capacidade de inibição da formação de compostos nitrosos também foram determinados em polpa de açaí branco, o tipo de polpa de açaí mais consumido e com maior significância comercial depois do roxo.

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OBJETIVOS

Objetivo geral

Utilizar ferramentas estatísticas de otimização multivariada para estabelecer as condições de extração de compostos fenólicos e as condições cromatográficas de separação de compostos fenólicos não-antociânicos em polpa de açaí roxo e branco e aplicar os resultados obtidos para avaliar o efeito da alta pressão hidrostática sobre os compostos fenólicos, tocoferóis e capacidade antioxidante da polpa de açaí roxo, comparando-o com o efeito do tratamento térmico (pasteurização) e amostras controle (sem tratamento). Além disso, investigar o perfil de compostos fenólicos e a capacidade antioxidande do açaí branco.

Objetivos específicos

Avaliar o efeito de 4 solventes (acetona, metanol, etanol, água), através de um planejamento de misturas simplex centroide, e investigar o efeito do tempo, da concentração de ácido e da razão sólido-líquido, através de um planejamento composto central, para estabelecer um método de extração de compostos fenólicos da polpa de açaí roxo;

Otimizar, usando métodos estatísticos multivariados, e validar um método por cromatografia líquida de ultra-alta eficiência e detector de arranjo de diodos (UHPLC-DAD) para separação e quantificação de 12 compostos fenólicos não-antociânicos (ácido gálico, ácido 3,4-hidroxibenzóico, 4-hidroxibenzóico, catequina, ácido vanílico, ácido cafeico, ácido siríngico, epicatequina, ácido *p*-cumárico, orientina, isoorientina, ácido ferrúlico).

Avaliar o efeito da aplicação da alta pressão hidrostática e do tratamento térmico sobre a concentração das antocianinas majoritárias (cianidina 3-glicosídeo e cianidina 3-rutinosídeo), compostos não-antociânicos e tocoferóis da polpa de açaí roxo;

Investigar o efeito da alta pressão hidrostática e do tratamento térmico sobre a capacidade antioxidante da polpa de açaí roxo frente a espécies reativas do oxigênio, bem como sobre a capacidade de inibir a formação de compostos nitrosos;

Avaliar o efeito do tempo e da concentração de etanol na eficiência de extração de compostos fenólicos da polpa de açaí branco utilizando um planejamento composto central;

Caracterizar e quantificar os compostos fenólicos da polpa de açaí branco por UHPLC-ESI-MS/MS;

Investigar a capacidade antioxidante de extratos de polpa de açaí branco frente a espécies reativas do oxigênio e sua capacidade capacidade de inibir a formação de compostos nitrosos.

CAPÍTULO I

Um *screening* do potencial do Brasil para a produção de sucos e polpas de fruta conservados por alta pressão hidrostática

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1 Introdução

A associação de uma dieta rica em frutas com a diminuição do risco de desenvolvimento de doenças cardiovasculares e câncer tem elevado a demanda por esses alimentos no mundo (FAO, 2015). Em razão disso, a produção global de frutas tem apresentado crescimento contínuo, com um aumento de cerca de 50% nos 20 últimos anos, resultando na produção de 773 milhões de toneladas em 2012 (SEAB, 2015). Nesse contexto, as frutas tropicais e subtropicais possuem um elevado potencial de consumo, em especial as frutas tropicais, as quais vem expandindo significativamente em termos de produção e consumo, em função do reconhecimento de suas características sensoriais, valor nutricional e funcional, tendo atingido a marca de 82 milhões de toneladas produzidas em 2009 (FAO, 2011).

Nesse cenário, o Brasil possui uma grande extensão territorial que abrange condições edafoclimáticas diversificadas, e permite o cultivo de uma grande variedade de frutas. O país destaca-se como o terceiro maior produtor mundial de frutas, com uma produção anual de 43,6 milhões de toneladas em 2013. A produção engloba frutos de clima tropical e temperado, contando com espécies exóticas, isto é, originárias de outras regiões do globo que apresentaram boa adaptação as condições locais, bem como uma variada gama de frutos nativos com elevado apelo sensorial e nutricional (Buainain, Batalha, 2007, Rufino et al., 2010, Bataglion et al., 2015, IBRAF, 2016).

As frutas podem ser consumidas frescas ou processadas na forma de sucos, néctares e polpas. Por prolongar sua vida útil e permitir a elaboração de produtos com sabores diferenciados, o mercado mundial de frutas processadas (incluindo sucos, polpas de frutas e frutas em conserva) cresceu cerca de 89% entre 2005 e 2015, movimentando cerca de US$ 36 bilhões por ano, impulsionado pela demanda por alimentos saudáveis e pela dinâmica da vida moderna que reinvidica refeições práticas e nutritivas (Cunha et al., 2008, Comtrade, 2015).

Dentre as etapas do processamento, o tratamento témico constitui um passo importante para que os produtos atinjam a estabilidade bioquímica e microbiológica necessária para o armazenamento. A pasteurização, realizada a temperaturas entre 65 e 100°C, e a esterilização, conduzida a temperaturas entre 110 e 150°C constituem os métodos de conservação mais utilizados na indústria de alimentos e são comprovadamente eficazes para produzir alimentos estáveis (Jay, 2000). No entanto, estes processos apresentam a desvantagem de causar alterações indesejáveis nos produtos, como modificações de cor, sabor e perdas funcionais ou nutritivas (Ribani-Hoffman et al., 2009, Rawson et al., 2011). Dessa forma, a crescente busca por alimentos naturais, frescos, sem aditivos químicos e com o mínimo de

intervenções industriais possíveis, tem redirecionado a forma como a indústria de alimentos pensa a conservação dos alimentos.

Nesse contexto, o uso de tecnologias emergentes de conservação que não utilizam calor, como irradiação, campo elétrico pulsado, ultrassom, e alta pressão hidrostática, integram um elenco de novos métodos de conservação capazes de destruir micro-organismos e inativar enzimas com mínimos efeitos deletérios sobre as características sensoriais, nutricionais e funcionais dos alimentos (Sanchez-Moreno, Canos del Anco, 2010). Atualmente, a alta pressão hidrostática (APH) constitui a técnica mais estudada, bem estabelecida e utilizada industrialmente (Huang et al., 2015). A técnica de APH consiste em submeter alimentos líquidos ou sólidos a pressões entre 100 MPa e 800 MPa, associada ou não à elevação da temperatura. A pressão aplicada não afeta ligações covalentes e portanto moléculas de tamanho pequeno como vitaminas, compostos voláteis e compostos bioativos não são significativamente afetadas (Delgado et al., 2013, Tadapaneni et al., 2014, Balasubramanian, Martínez-Monteagudo, Gupta, 2015).

Dessa forma, tendo em vista o enorme potencial do país para a produção de produtos à base de frutas, o uso da alta pressão hidrostática pode contribuir significativamente para tornar estes alimentos e seus benefícios disponíveis para o mundo de forma segura e com elevada qualidade sensorial, nutricional e funcional (IBRAF, 2009). Assim, esta revisão teve como objetivo abordar os princípios da APH, seus efeitos sobre nutrientes e compostos bioativos de frutas, com enfoque em frutas produzidas no Brasil com elevado apelo nutricional e funcional e com potencial para serem utilizadas na crescente indústria de processamento por alta pressão hidrostática.

2 O mercado de frutas *in natura* e processada no Brasil

A fruticultura tornou-se uma importante fonte de receita para a economia brasileira, e constitui uma fonte de renda fundamental para milhares de pequenos agricultores no país, tendo movimentado mais de R$ 26 bilhões em 2015 (Buainain, Batalha, 2007, IBGE, 2015). A Tabela 1 mostra as principais frutas produzidas no Brasil em termos de volume de produção e comercialização. Banana, laranja, uva, abacaxi, maçã e melancia lideram a lista e juntos representam cerca de 70% do valor da produção total. O País lidera a produção mundial de laranja e maracujá, além de destacar-se em nível global como produtor de banana, mamão, abacaxi e coco (Oliveira, Souza, Coelho, 2009, Martins, Jesus Júnior, 2014, FAO, 2009). Já o

melão constitui a principal fruta *in natura* exportada pelo país (IBRAF, 2015). Naumov (2009) destaca que embora o Brasil ainda se encontre em posição de desvantagem no cenário mundial em relação a produtividade, o país tem grande potencial para investir em tecnologias para aumentar a produção.

Tabela 1. Principais frutas produzidas no Brasil

Principais frutíferas	Área (ha)		Quantidade produzida (t)	Rendimento médio (kg/ha)	Valor da produção (1 000 R$)
	Plantada ou destinada à colheita	Colhida (ha)			
Total	**2 633 472**	**2 581 097**	..	..	**26 454 293**
Banana	484 430	475 976	6 844 491	14 380	5 790 992
Laranja	668 189	665 174	16 746 247	25 176	5 635 413
Uva	78 026	78 011	1 497 302	19 193	2 322 996
Abacaxi (1) (2)	69 565	69 165	1 801 415	26 045	2 218 862
Maçã	35 872	35 842	1 264 651	35 284	1 311 868
Melancia (2)	97 910	95 965	2 119 559	22 087	1 233 944
Mamão	30 445	30 285	1 463 770	48 333	1 164 872
Coco-da-baía (1)	253 383	251 665	1 958 663	7 783	1 114 109
Maracujá	51 187	50 837	694 539	13 662	921 275
Limão	47 391	46 078	1 180 271	25 615	847 030
Manga	64 412	64 305	976 815	15 190	841 125
Tangerina	50 936	48 975	999 686	20 412	753 552
Goiaba	17 688	17 603	424 305	24 104	476 807
Melão (2)	20 837	20 762	521 596	25 123	470 921
Pêssego	17 451	17 436	216 241	12 402	394 768
Caqui	8 613	8 588	192 327	22 395	290 666
Castanha de caju	619 196	586 523	102 485	175	265 177
Abacate	10 381	10 354	180 636	17 446	246 461
Figo	2 855	2 855	29 063	10 180	81 936
Pera	1 453	1 453	21 160	14 563	38 804
Noz (fruto seco)	3 136	3 129	5 201	1 662	31 555
Marmelo	116	116	841	7 250	1 161

Fonte: IBGE, 2015.

Em complemento às tradicionais frutas tropicais e de clima temperado cultivadas racionalmente, o país abriga uma variada gama de frutas nativas, algumas pouco exploradas, em geral provenientes exclusivamente da extração vegetal diretamente da floresta (Tabela 2). Dentre estas, o açaí (*Euterpe oleracea)* destaca-se como o principal produto do extrativismo vegetal não-madeireiro, tendo movimentado em 2015 R$ 480 milhões com uma produção de cerca de 216 mil toneladas, o que representa um aumento de 9% em relação a 2014, reflexo da

crescente demanda pelo fruto (IBGE, 2015). Além do açaí, observa-se que a mangaba, fruto do cerrado brasileiro, e o umbu, oriundo da Caatinga, ocupam posição de destaque dentre as frutíferas provenientes do extrativismo vegetal. Ademais, o país abriga uma variada gama de frutas tropicais (Tabela 3) as quais possuem grande aceitação no mercado interno como fruta *in natura* e processada, porém ainda não apresentam significância comercial elevada, de forma que a sua produção é absorvida para consumo doméstico (Genovese et al., 2008, Rufino et al., 2010, Costa et al., 2015, Neves et al., 2015).

Tabela 2. Produtos brasileiros provenientes exclusivamente da extração vegetal.

Produto	**Quantidade produzida em 2015 (t)**
Açaí (fruto)	216 071
Castanha de caju	2280
Castanha-do-pará	40 643
Erva-mate	338 801
Mangaba (fruto)	663
Palmito	4669
Pequi (fruto)	18 866
Pinhão	8393
Umbu (fruto)	8094
Outros	2412

Fonte: IBGE, 2015.

Mais de 90% da produção de frutas no Brasil é destinada ao consumo interno, sendo que 47% destinam-se ao consumo *in natura*. A indústria de processados, que atende basicamente aos segmentos de sucos, néctares e polpa, é responsável por consumir 53% do total produzido. Deste total, 24% se destina para o mercado interno, e 29% para o mercado internacional, o qual é liderado pela produção de suco de laranja concentrado, que tem o Brasil como maior produtor e exportador, com 1,9 milhões de toneladas exportadas em 2014 (IBRAF, 2015, IBGE, 2015). Em 2012 o Brasil exportou 2 milhões de toneladas de frutas processadas, sendo os principais compradores Bélgica, Holanda e Estados Unidos (Gutierrez, 2012). Embora

o país ainda não possua uma posição hegemônica no mercado internacional de frutas processadas, estes números indicam um grande potencial de crescimento a ser explorado (Cunha et al., 2008).

Tabela 3. Nome de algumas frutas tropicais não tradicionais produzidas no Brasil.

Nome popular	Nome científico
Açaí	*Euterpe oleracea*
Araçá-boi	*Eugenia stipitata*
Araticum	*Annona crassiflora*
Bacuri	*Platonia insignis*
Biribá	*Rollinia mucosa*
Cajá	*Spondias lutea L.*
Caju	*Anacardium occidentale*
Cambuci	*Byrsonima dealbata*
Cagaita	*Eugenia dysenterica*
Cupuaçu	*Theobroma grandiflorum*
Guariroba	*Syagrus oleracea (Mart.) Becc.*
Jaboticaba	*Myrciaria cauliflora*
Jambolão	*Syzygium cumini*
Inajá	*Maximiliana maripa* Aublet Drude
Lobeira	*Solanum lycocarpum*
Mangaba	*Hancornia speciose*
Maná-cubiu	*Solanum sessiliflorum*
Murici	*Byrsonima crassifolia*
Pitanga	*Eugenia uniflora*
Uxi	*Endopleura uchi*
Umari	*Andira spinulosa*
Umbu	*Spondias tuberosa*

De acordo com o Instituto Brasileiro de Frutas (IBRAF) o consumo de frutas processadas cresce de forma mais rápida do que o de frutas frescas, movimentando R$ 52 milhões ao ano (IBRAF, 2009, Gutierrez, 2012). Nesse cenário, a produção de polpas e sucos naturais vem apresentando aumento significativo, impulsionado pela demanda crescente por

produtos naturais com apelo à saúde. Um levantamento realizado pela consultoria Euromonitor indicou que este segmento aumentou suas vendas em 98% nos últimos 5 anos no país, enquanto as de produtos tradicionais cresceram 67%. O consumo de bebidas à base de frutas alcançou 1.152.670.000 l em 2012, o que representa um aumento de 8,1% em comparação com o ano anterior (IBRAF, 2013). Por sua vez, a produção de polpas de fruta constitui uma parcela importante no mercado de frutas processadas no país, uma vez que corresponde a primeira etapa na produção de sucos, néctares e outros produtos processados (Cunha et al., 2008). As polpas de fruta mais produzidas no mercado nacional são abacaxi, manga e maracujá. As polpas de goiaba, caju, cupuaçu, acerola, açaí, graviola, bacuri, entre outras, também constituem produtos tropicais com alta aceitação no mercado, porém algumas delas apresentam consumo restrito às regiões em que são produzidas (Cunha et al., 2012, Gutierrez, 2012, IBGE, 2015).

3 Frutos produzidos no Brasil: potencial nutricional e funcional

O Brasil é reconhecido mundialmente por sua rica flora nativa e por abrigar espécies originárias de outras regiões do planeta mas que apresentaram excelente adaptação ao país. De acordo com o IBGE, a produção nacional de frutíferas reúne 22 produtos principais, apresentados na Tabela 1. Estas frutas são reconhecidas por serem fontes de minerais, vitaminas e compostos antioxidantes como flavonoides, antocianinas e ácidos fenólicos, e por produzirem efeitos benéficos a saúde do consumidor, o que já foi bem documentado em trabalhos anteriores (Dembitsky et al., 2011).

Além destas, o país possui espécies tropicais com menor expressão econômica, como a acerola, e uma ampla gama de frutíferas nativas oriundas dos diferentes biomas brasileiros, como o cerrado, a caatinga e o amazônico. Rufino et al. (2010) utilizaram a definição “frutas tropicais não-tradicionais” para nomear estas espécies. Na Tabela 3 estão descritas algumas delas. Informações sobre a produção dessas frutas são escassas, e algumas ainda não são utilizadas em escala industrial (Costa et al., 2015).

Estima-se que a Amazônia possui aproximadamente 220 espécies de frutos comestíveis, o que representa 44% da flora nativa nacional, a maioria delas pouco exploradas comercialmente ou conhecidas fora da região (Neves et al., 2015). Por outro lado, espécies como o açaí (*Euterpe oleracea* Mart.) vem aumentando sua participação na economia nacional e a sua composição química e os potenciais efeitos à saúde já foram bem documentados (Dias et al., 2012, Dias et al., 2013, Gordon et al., 2012, Kazumy et al., 2015, Carvalho et al., 2016).

Souza et al. (2012) demonstraram que a polpa do murici contém 16 mg.100-¹ (b.s.) de carotenoides (expresso como β-caroteno) e é capaz de fornecer 5,5%, 10% e 100% da ingestão diária recomendada de cálcio (Ca), magnésio (Mg) e potássio (K), respectivamente. Por sua vez, Berto et al. (2015) investigaram a composição nutricional de frutos amazônicos e encontraram que a casca e a polpa de biribá, uxi, umari e cubiu apresentam minerais como manganês, zinco, cobre, magnésio, sódio e fósforo, com predominância do manganês, sódio e fósforo. Além disso, os autores verificaram a presença de ácidos graxos mono e poli-insaturados (MUFA e PUFA) na composição de ambas as frações destes frutos, com destaque para o uxi e umari, que apresentaram 13659 mg.100g-¹ (MUFA casca do umari) e 575 mg. 100g-¹ (PUFA polpa de uxi). Rodrigues, Mariutti e Mercadante (2013) estudaram o perfil de carotenoides, compostos fenólicos e a capacidade antioxidante do maná-cubiu frente a espécies reativas do oxigênio e nitrogênio. Dezessete carotenoides foram encontados nos extratos da fruta, sendo o
β-caroteno o composto majoritário (7,15 µg.g b.s.), e o 5-cafeoilquínico foi o composto fenólico majoritário (1351 µg.g b.s.). Além disso, os extratos apresentaram capacidade antioxidante contra todas as espécies reativas testadas (peróxido de hidrogênio, radical hidroxila, ácido hipocloroso, radical peroxila e peroxinitrito).

Neves et al. (2015) reportaram níveis de vitamina C, compostos fenólicos totais e capacidade antioxidante (pelo método ORAC) em polpa de araçá-boi, cajá, inajá, murici e uxi variando entre 0 (inajá e uxi) e 15.9 mg. 100 mL-¹ (caju), 182 (uxi) e 2991 mg EAG.100 mL-¹ (murici), e 19 (uxi) e 130 (caju) µmol Trolox Eq 100 g-¹, respectivamente. Rufino et al. (2010) determinaram a capacidade antioxidante (DPPH, ABTS, FRAP, branqueamento do β-caroteno) de alguns frutos não tradicionais brasileiros e verificaram que a acerola e o camu-camu apresentaram resultados muito superiores ao bacuri, cajá, caju, açaí, provavelmente devido ao elevado teor de vitamina C destas frutas (Rufino et al., 2010). Bataglion et al. (2015) investigaram o perfil de compostos fenólicos em frutos tropicais brasileiros e observaram a presença de ácido gálico em cajá, caju e camu-camu; ácido cafeico e ácido ferrúlico em acerola, ácido *p*-cumárico e quercetina nas quatro espécies; miricetina e luteolina em caju e camu-camu, e kaempferol em caju e acerola, sugerindo que estes frutos podem contribuir para a ingestão de compostos antioxidantes da dieta.

Por sua vez, o cerrado constitui o segundo maior bioma brasileiro (204 milhões de hectares) e abriga cerca de 4400 espécies de plantas (Bailão et al., 2015). Embora sejam consumidos há décadas pela população local, os frutos do cerrado vêm conquistando a atenção de pesquisadores e processadores de fruta apenas recentemente, e têm se mostrado com elevado

potencial de exploração comercial por apresentar características sensoriais e nutricionais de boa aceitabilidade. Rufino et al., (2010), Siqueira et al. (2013) e Genovese et al. (2008) investigaram a atividade antioxidante de diversos frutos do cerrado, e observaram que cagaita, lobeira, mangaba e araticum apresentaram capacidade antioxidante por diferentes métodos (FRAP, DPPH, ABTS), resultados que sugerem que o consumo destas frutas pode contribuir para a ingestão de substâncias antioxidantes. Bailão et al. (2015) revisaram os compostos bioativos encontrados em frutos do cerrado e destacaram a presença de ácido ascórbico em algumas espécies (araticum, cagaita, mangaba), carotenoides (cagaita), flavonoides como catequina, epicatequina, quercetina, e ácidos fenólicos como ácido cafeico, ferrúlico e gálico, assim como tocoferóis e trocotrienois (mangaba, cagaita).

Além disso, a jaboticaba, uma fruta genuinamente brasileira encontrada na região da mata atlântica, tem sido consistentemente associada a diversos efeitos benéficos, como diminuição do colesterol, controle da diabetes, atividade antioxidante e anticâncer, os quais são relacionados a composição química da fruta, rica em antocianinas (Wu, Long, Kenelly, 2013). A pitanga, outro fruto da mata atlântica, também já foi estudada quanto a sua composição química e capacidade antioxidante por Denardin et al. (2015), que encontraram flavonoides como cianidina-3-glicosídeo, quercetina, kaempferol e compostos derivados destes (ligados a açúcares) em diferentes variedades de pitanga (roxa, vermelha), além de capacidade antioxidante pelos métodos DPPH, FRAP e TRAP.

Schwartz et al. (2010) comentam que há uma demanda cada vez maior no mercado internacional por frutas com novos aromas, sabores e texturas, e o Brasil é um país com imenso potencial para fornecer esses recursos naturais vegetais. No entanto, em vista da dificuldade de disponibilizar estes frutos para localidades distantes da sua região de produção, o processamento é uma alternativa chave para tal questão. Assim, em complemento às frutas tradicionalmente processadas no país, estes estudos indicam que as espécies nativas possuem um significativo potencial de industrialização, a qual aliada a técnicas de conservação como a alta pressão hidrostática, poderá fornecer produtos diferenciados e com alto valor agregado no mercado (Paz et al., 2015).

4 Alta pressão hidrostática: princípios e aspectos regulatórios

A tecnologia de alta pressão hidrostática (APH) é um método de conservação emergente, desenvolvido como uma alternativa ao tratamento térmico. Apesar do efeito da alta

pressão sobre a destruição de micro-organismos ser reconhecido há mais de 100 anos, este método de conservação permaneceu inutilizada por muito tempo, mantendo-se, posteriormente, limitado a laboratórios de pesquisa e desenvolvimento durante um longo período. Somente na década de 1990 começou a ser utilizado em escala industrial no Japão, época a partir da qual as pesquisas sobre o APH e seus efeitos nos alimentos começaram a se intensificar (Balasubramanian, Martínez-Monteagudo, Gupta, 2015).

Os processos comerciais atualmente existentes consistem na aplicação de pressões entre 200 e 600 MPa sob temperaturas baixas a elevadas (0 a 80°C), por aproximadamente 5 minutos (Balasubramanian, Martínez-Monteagudo, Gupta, 2015, Delgado et al., 2013, Huang et al., 2017). Alimentos líquidos, sólidos, pastosos e viscosos, embalados ou não, são admitidos no equipamento. O sistema de APH (Figura 1) é composto dos seguintes itens: 1) uma câmara de pressão, usualmente um cilindro de aço inox; 2) um fluido transmissor, geralmente água potável, no qual o alimento será submergido, responsável por transmitir a pressão gerada no sistema até o produto 3) o reservatório do fluido transmissor 4) um sistema de bombas, que transportará o fluido transmissor até a câmara 5) um dispositivo intensificador de pressão, que elevará a pressão do sistema até o nível desejado. O processo é regido pelo princípio isostático, que indica que a compressão do produto ocorre de forma instantânea e uniforme, independentemente da sua composição, tamanho e formato, resultando em um processo mais homogêneo (Balasubramanian, Martínez-Monteagudo, Gupta, 2015).

O principal fator que diferencia a APH dos tratamentos térmicos tradicionais é a habilidade para manter ligações covalente intactas após o processamento. Assim, compostos voláteis e vitaminas, como a vitamina C, folatos, e compostos antioxidantes como antocianinas e carotenoides são preservados, resultando em produtos com características sensoriais, nutricionais e funcionais mais próximas ao produto *in natura,* uma premissa que constitui o fator impulsionador da expansão de sua utilização (Sanchez-Moreno et al., 2011, Balasubramanian, Martínez-Monteagudo, Gupta, 2015).

Isto é possível devido ao outro princípio que rege os processos com alta pressão: o princípio de Le Chatelier, segundo o qual qualquer fenômeno (transição de fase, mudança de conformação molecular ou reação química) acompanhado por uma redução de volume é favorecido pelo aumento de pressão (e vice-versa). Na prática, isto significa que a alta pressão beneficia reações que resultam em uma diminuição de volume. Por exemplo, no caso de uma reação, a pressão alterará o equilíbrio na direção do sistema de menor volume (Medina-Meza et al., 2014). Assim, a alta pressão afeta interações inter e intra moleculares de componentes

dos alimentos os quais a força da interação muda em função da distância, como interações eletrostáticas, de van der Waals e pontes de hidrogênio. Por outro lado, as ligações covalentes, as quais o comprimento da ligação não é sensível a compressão, são minimamente afetadas pela alta pressão (Balasubramanian, Martínez-Monteagudo, Gupta, 2015). A Tabela 4 sumariza as principais vantagens e desvantagens da APH.

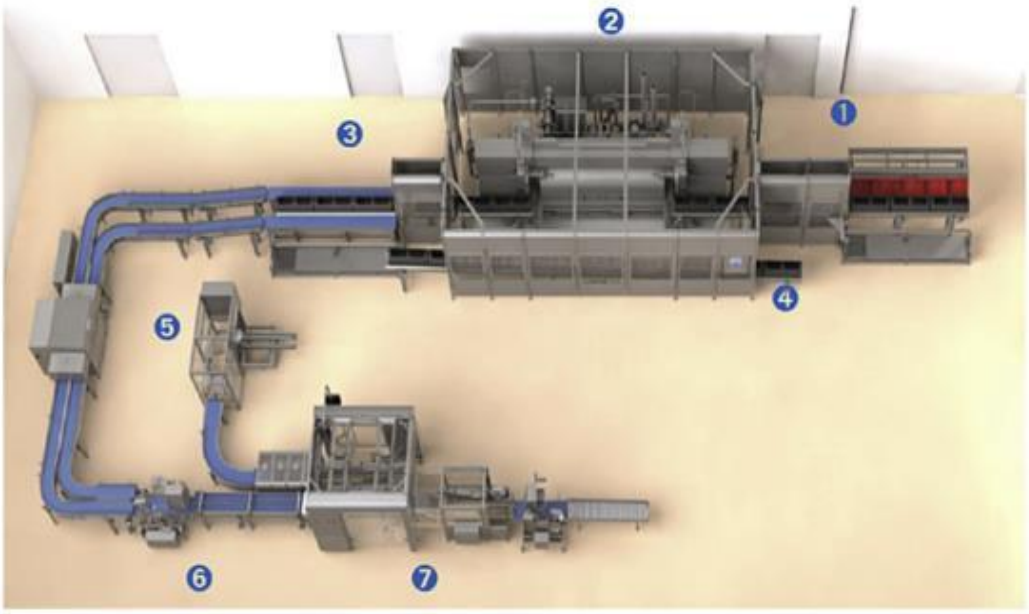

Figura 1. Esquema de uma linha de produção contendo uma unidade de alta pressão hidrostática. 1) Cestas de carregamento de produto, 2) câmara de pressurização, 3) Descarregamento, 4) Esteira de retorno das cestas de carregamento, 5) Secagem de embalagens, 6) Rotulagem, 7) Processamento final de empacotamento. **Fonte**: Balasubramanian, Martínez-Monteagudo, Gupta, 2015.

Por sua vez, o efeito da alta pressão sobre enzimas é altamente dependente das condições de processamento, de modo que a ineficiência da técnica para promover a inativação enzimática tem sido apontada como uma das principais limitações da APH (Vervoort et al., 2011, Toepfl et al., 2006). Em função das condições de processo, diferentes efeitos sobre a atividade enzimática tem sido observados, com aumento ou redução da atividade, podendo haver inativação completa e irreversível, completa e reversível, inativação incompleta e irreversível, ou inativação incompleta e reversível (Hendrickx, 1998). Isso significa que após o

processamento, pode haver uma atividade enzimática residual significativa no produto e que durante o armazenamento, enzimas que haviam sido inativadas durante a pressurização podem recuperar sua atividade e contribuir para diminuir a vida-de-prateleira do produto (Terefe et al., 2013).

Tabela 4. Vantagens e desvantagens da APH

Vantagens	Desvantagens
Rápida, instântanea distribuição da pressão na amostra	Opera somente em batelada ou semi-contínuo
Mínima ou reduzida exposição do produto ao calor	Se a alta pressão for utilizada juntamente com calor, necessita de pré-aquecimento e a temperatura não é uniformemente distribuída no produto durante o processo
Aumento de temperatura momentâneo e posterior resfriamento após despressurização	
Adequado para alimentos com alta umidade	Não adequado para produtos com compressibilidade diferentes, como marshmallows
Adequado para alimentos líquidos e viscosos bombeáveis	Eficiência variável sobre a inativação enzimática; a alta pressão não destrói esporos bacterianos
O processo independe da forma e do tamanho do produto	Elevados custos de processo e operação impedem a expansão da técnica
Oportunidade de desenvolvimento de novos produtos através dos diferentes efeitos da alta pressão, como desnaturação protéica, gelatinização do amido e cristalização da gordura	
Aceleramento da inativação microbiana	
Produtos com elevada aceitação do consumidor	

Fonte: Balasubramanian, Martínez-Monteagudo, Gupta, 2015.

No que se refere ao efeito sobre os micro-organismos, desde os trabalhos pioneiros realizados por Hite (1899) e Hite, Giddings e Chas (1914), em que a sua sensibilidade a pressões acima da atmosférica foi utilizada na preservação de leite, frutas e vegetais, a eficácia da APH

para a eliminação de micro-organismos deterioradores e patogênicos tem sido muito bem documentada (Chen, Yu, Rapasinghe, 2012, Bicosin-Júnior, Rosenthal, Monteiro, 2013, Huang et al., 2013, Uckoo et al., 2013, Pinho et al., 2015, Andrés et al., 2016). Quando aplicadas condições de processo adequadas, a alta pressão é eficiente para eliminar formas vegetativas de bactérias (patogênicas e deterioradoras), bolores e leveduras (Bicosin-Júnior, Rosenthal, Monteiro, 2013, Huang et al., 2013, Pinho et al., 2015, Huang, Ye, Chen, 2013, Lowder, Waite-Cusic, Dewitt, 2014).

O mecanismo de destruição é complexo, uma vez que várias mudanças morfológicas são observadas com o aumento da pressão: compressão de vacúolos gasosos, alongamento da célula, separação da membrana da parede celular, contração da parede celular com a formação de poros, modificações no citoesqueleto, modificações no núcleo e em organelas intracelulares, coagulação de proteínas citoplasmáticas, liberação de constituintes intracelulares (especialmente os de origem nuclear) para fora da célula, entre outros, o que pode ocasionar danos à membrana celular e inativação de enzimas importantes para a sobrevivência do organismo (Campos, Dosualdo, Cristianini, 2003). Além disso, a pressão causa algumas desnaturações protéicas na membrana, modificando a permeabilidade e a seletividade da membrana plasmática, podendo resultar na morte da célula. Entretanto, a alta pressão sozinha não é capaz de eliminar esporos bacterianos, de modo que os níveis de destruição microbiana alcançados correspondem àqueles da pasteurização térmica (Campos, Dosualdo, Cristianini, 2003, Balasubramainian, Martínez-Monteagudo, Gupta, 2015). Assim, é imprescindível que os produtos conservados por alta pressão, principalmente aqueles de baixa acidez, sejam mantidos sob refrigeração durante o transporte, estocagem e manuseio doméstico.

No que concerne a aspectos regulatórios, a alta pressão segue a mesma regulamentação de outros métodos de conservação de alimentos, o que significa que a técnica deve ser eficiente para reduzir a população de micro-organismos indicadores a níveis seguros para o consumidor (redução de 5 ciclos logarítmicos). Atualmente, a União Européia (European Commission), os Estados Unidos (USDA, FDA), o Reino Unido (Food Standards Agency), o Canadá (Health Canada), a Alemanha (Eisenbrand), Austrália e Nova Zelândia (FSANZ) têm uma regulamentação estabelecida para produtos processados por alta pressão.

A FDA estabeleceu a APH como uma tecnologia de conservação alternativa à pasteurização térmica, e instituiu a *Escherichia coli* O157:H7, a cepa de patogênicos mais resistente encontrada em estudos realizados pela agência, como o micro-organismo indicador de segurança alimentar. As condições de processo devem ser suficientes para reduzir a

população em 5 ciclos logarítmicos. Já na Europa, a APH é considerada como uma nova tecnologia sujeita à regulamentação prevista na Novel Food Regulation, e as embalagens dos produtos processados por esta técnica devem conter uma descrição das condições de processo utilizadas (Huang et al., 2017). Portanto, é essencial que estudos continuem avançando em investigar as condições de processamento de APH adequadas para promover a destruição de micro-organismos importantes para a saúde pública.

5 APH vs. tratamento térmico: aspectos econômicos

Para definir o processo a ser utilizado na produção de um alimento, o produtor leva em consideração vários aspectos, como por exemplo custo de equipamento, automação, instalação, plano de preparo de funcionamento, embalagem, mão-de-obra e design do produto. Na APH os custos com compra do equipamento e instalação correspondem a fração mais significatva do total, e podem ser entre 4 e 7 vezes mais elevados do que aqueles verificados para os tratamentos térmicos tradicionais (Sampedro et al., 2014). Além disso, a APH é uma tecnologia recente, conduzida em batelada, fato que impede a implantação de linhas de produção de elevada produtividade. Em face dessas características muitas empresas ainda adotam uma postura conservadora em relação a APH, o que impede sua expansão pelo mundo.

Os custos de produção ainda são elevados para a APH, com preços podendo variar em função do produto e das condições de processo entre U$ 5 e 20 centavos.L^{-1} ou kg^{-1}, frente à U$ 2 a 4 centavos.L^{-1} ou kg^{-1} do processo térmico tradicional, o que resulta em produtos com preço unitário mais elevado para o consumidor (Rostgi et al., 2010, Bermúdez-Aguirre, Barbosa-Canóvas, 2011). Porém, o crescimento da demanda por produtos desse segmento tem levado os fabricantes de equipamentos de alta pressão a investir em tecnologias para produzir sistemas mais produtivos e de preço reduzido, de forma que a tendência futura é a diminuição dos custos operacionais (Bermúdez-Aguirre, Barbosa-Canóvas, 2011).

Uma pesquisa realizada pela companhia norte-americana Universal Pasteurization entre empresários, fabricantes e varejistas revelou que as maiores barreiras para a utilização da técnica estavam relacionadas aos custos de aquisição do equipamento e produção. Entretanto, de acordo com a fabricante Avure Technology, a utilização adequada da APH, que resulta em alimentos seguros e com elevada qualidade nutricional e sensorial, pode auxiliar no aumento das vendas e minimizar a incidência de produtos fora das especificações de qualidade (que chega a 100 unidades por lote no tratamento térmico), contribuindo assim para a recuperação

do investimento realizado, o que segundo a empresa pode ocorrer em um ano. Além disso, Rastogi et al (2010) ressaltaram que o elevado custo inicial de investimento é compensado pelo menor gasto energético requerido pela APH em comparação com a pasteurização térmica, enquanto Toepfl et al. (2006) estimaram que a economia energética em um processo envolvendo pressão e calor vs. somente calor é de até 20%. Rodriguez-Gonzáles et al. (2015) destacaram que ainda há muitos parâmetros de processo a serem otimizados para a alta pressão hidrostática, os quais podem contribuir significativamente para a redução dos custos operacionais no futuro.

A despeito do valor mais elevados, observa-se a existência de um mercado consumidor disponível a pagar mais por produtos com qualidade sensorial, funcional e nutricional diferenciada, de forma que o uso da alta pressão tem se tornado uma tendência crescente no segmento de sucos e polpas. Outros fatores como o não uso de conservantes químicos e a ideia de que a alta pressão é uma tecnologia que causa menos impacto ao meio ambiente também contribuem para tornar os produtos deste segmento mais atrativos ao consumidor (Toepfl et al., 2006, Pereira, Vicente, 2010). Diversos estudos têm relatado a sua elevada aceitabilidade no mercado, destacando que o consumidor, ao ser alertado sobre as qualidades e benefícios do produto, declarou que o custo mais elevado não prejudicaria a sua decisão de compra (Olsen, Grunet, Sonne, 2010, Lee et al., 2010, Romano, Rosenthal, Deliza, 2015).

Assim, é possível afirmar que existe um mercado em expansão pronto para absorver os produtos processados por APH, sugerindo que ainda que o custo atual da técnica seja mais elevado do que os métodos tradicionais, a qualidade do que é produzido aliado à significância que produtos naturais vêm ganhando no mercado, constituem fatores com grande potencial para superar tal limitação e ser um excelente ramo de investimento.

6 APH vs. tratamento térmico: efeito sobre produtos à base de frutas

O efeito da APH e sua comparação com o tratamento térmico para a preservação de frutas de clima temperado tem sido objeto de diversos estudos experimentais e de revisão de literatura, os quais têm focado principalmente em produtos à base de maçã e frutas vermelhas (maçã, morangos, framboesa, amoras, romã), como sucos, bebidas lácteas, formulações de frutas, polpas e purês. Porém, estudos de comparação entre a APH e o tratamento térmico para frutas tropicais ainda são escassos. A banana e a manga são os frutos tropicais com maior

relevância internacional (FAO, 2009), sendo que esta última é a mais estudada até o momento no que se refere ao efeito da alta pressão em suco e polpa. Por sua vez, produtos à base de laranja, principalmente o suco, lideram o número de estudos (Sanchéz-Moreno et al., 2010).

Em geral, existem três vertentes principais de resultados que vêm sendo demonstrados quando da comparação da APH com tratamento térmico. Um primeiro grupo tem demonstrado que a APH é capaz de melhor reter a qualidade sensorial (cor, sabor, aroma), de nutrientes como vitaminas (C, E), e compostos antioxidantes (flavonoides, ácidos fenólicos, antocianinas, carotenoides) do produto fresco em comparação ao produto tratado termicamente (Oey et al., 2008, Plaza et al., 2011, Ramaswamy, Balasubramanian, Kaletunç, 2011, Patraz et al., 2009, Terefe et al., 2012, Yi et al., 2017). Em contrapartida, alguns estudos observaram resultados opostos, com a APH mostrando menor retenção de compostos antioxidantes do que amostras tratadas termicamente, ou não diferindo significativamente das mesmas, o que sugeriria que não há vantagens na aplicação desta técnica (Moreira et al., 2015, Vervoot et al., 2011, Cilla et al., 2012).

Uma terceira vertente de resultados mostra que a APH resulta em produtos com teor suplementado de vitaminas e compostos antioxidantes em comparação ao controle ou tratamento térmico, o que foi justificado pelo efeito da alta pressão sobre a parede celular vegetal, a qual pode promover a liberação de compostos de suas organelas, modificação da sua localização na célula, quebra de ligações entre moléculas conjugadas, ou rompimento da parede celular, deixando-os mais acessíveis para extração (Suárez-Jacobo et al., 2012, Hernández-Carrión et al., 2014).

Por exemplo, Patras et al.(2009) avaliaram o efeito do processamento por alta pressão (400, 500 e 600 MPa por 15 minutos a 20°C) e do tratamento térmico tradicional (pasteurização a 70°C por 2 minutos) sobre o teor de compostos fenólicos totais (CFT) e sobre as antocianinas presentes em purê de amora (cianidina-3-glicosídeo) e morango (pelargonidina-3-glicosídeo). Os autores concluíram que a APH contribuiu para a retenção destes compostos em ambos os produtos, independente da pressão empregada, e que as amostras processadas a 600 MPa apresentaram teor detectável de CFT e pelargonidina-3-glicosídeo mais elevado (em média 10% e 29%, respectivamente) do que aquelas submetidas ao tratamento térmico. Além disso, os resultados indicaram que a APH resultou em produtos com menor variação de cor em relação ao produto sem tratamento, indicando que esta técnica foi superior a pasteurização térmica na preservação deste parâmetro sensorial.

Keenan et al (2012a), estudando o efeito do processamento térmico (70 °C por 10 minutos) e do APH (450 MPa por 5, a 20°C) sobre o teor de CFT, vitamina C e antocianinas totais e cor de *fruit smoothies* preparados com morango, maçã, suco concentrado de maçã, banana e laranja, observaram que em comparação ao produto não processado houve a redução significativa do teor de vitamina C e CFT após o tratamento térmico e elevação do conteúdo quantificável dos mesmos nas amostras submetidas ao APH. Em contrapartida, o tratamento térmico não alterou significativamente a concentração das antocianinas, porém, verificou-se que a aplicação da APH (450 MPa por 5 minutos) aumentou 15% o teor desses compostos em comparação ao controle. Além disso, a APH preservou melhor as características de cor e aroma do produto (Keenan et al., 2012b)

Em um outro estudo realizado para *fruit smoothies* (com morango, maçã, suco concentrado de maçã, banana e laranja), avaliou-se o efeito do APH (450 MPa por 1, 3 e 5 min a 20ºC) sobre o conteúdo individual de compostos fenólicos (procianidina, hesperidina, ácido clorogênico e ácido p-cumárico) frente ao efeito do tratamento térmico tradicional (90°C por 10 minutos). Os resultados indicaram que o comportamento e o teor individual podem variar significativamente em função do composto e do tempo de processamento empregado. Em comparação ao produto *in natura* e ao tratamento térmico, as amostras tratadas por 5 minutos apresentaram as menores concentrações de ácido *p*-cumárico (0,025 mg.100 g-1), procianidina (14,97 mg.100 g-1) e hesperidina (11,21 mg.100 g-1), enquanto as concentrações destes compostos nas demais condições (1 e 3 minutos) não diferiram significativamente dos tratamentos testados. Já o ácido clorogênico apresentou concentrações mais elevadas nas amostras pasteurizadas do que em todos os demais tratamentos (Keenan et al., 2011).

Em contrapartida, Sanchéz-Moreno et al. (2005) observaram que o processamento de suco de laranja por APH (400 MPa/40ºC/1 min) foi mais efetiva do que a pasteurização tradicional (90ºC/1 min e 70ºC/30 s) para preservar a vitamina C e os compostos bioativos de suco de laranja, enquanto Plaza et al. (2011) observaram um aumento no teor de carotenoides, pró-vitamina A e flavonoides extraídos de suco de laranja (até 30%) submetido sob as mesmas condições de pressão, ao passo que amostras tratadas termicamente (70ºC/30 s) não diferiram significativamente do suco controle em relação a estes parâmetros. Entretanto, ressalta-se que em todos os estudos anteriores, as condições de tratamento térmico e pressão foram estabelecidas visando atingir a inativação microbiana (redução de cinco a seis ciclos logarítimicos de micro-organismos patogênicos, como por exemplo *Listeria monocytogenes* e *Escherichia coli* CECT 515). Porém, nestes dois últimos estudos, este parâmetro não foi

utilizado para a determinação das condições de processamento de APH e portanto pode não representar uma situação real a ser aplicada na indústria a fim de atingir a destruição microbiana, o que inviabiliza a comparação com o tratamento térmico.

Nesse sentido, Vervoot et al. (2011) realizaram um estudo de comparação entre a APH (600 MPa/1 min/ 5ºC) e a pasteurização térmica (72ºC/20 seg) de suco de laranja de forma que ambos os métodos resultassem em níveis equivalentes de destruição microbiana (enterobactéricas, *E. coli*, bolores e leveduras). Os autores demonstraram que o suco submetido ao tratamento térmico não apresentou diferença significativa no que se refere ao conteúdo de vitamina C e carotenoides comparado ao suco processado por alta pressão. Além disso, os autores destacaram a incapacidade da APH para inativar a enzima pectinametilesterase, ao contrário da pasteurização térmica que atingiu 100% de inativação.

Andrés et al. (2016) desmonstrou que não houve diferenças significativas em *smoothies* à base de soja contendo suco de mamão, de laranja e melão, processados a 550 MPa e 650 MPa/3 min/20ºC, no que se refere ao teor de ácido cafeico, narirutina e hesperidina em bebidas, enquanto os ácidos clorogênico e *p*-cumárico, daidizina e genisteína tiveram teores inalterados ou aumentados. Já o tratamento térmico (80ºC/3 min) causou um decréscimo destes compostos fenólicos (até 18%), da epicatequina e da catequina. O teor de vitamina C permaneceu inalterado nas amostras pressurizadas, enquanto um decréscimo significativo foi observado devido ao tratamento térmico. Além disso, estes autores observaram que as amostras submetidas APH mantiveram a coloração (medida pelo sistema CIELAB L*) mais próximas do produto fresco do que o pasteurizado termicamente. Os autores informaram que os processos foram realizados para atingir equivalência de inativação microbiana, porém não forneceram detalhes sobre a condução do tratamento térmico, o qual pode influenciar os resultados obtidos.

Cilla et al. (2012) investigaram o efeito da APH (400 MPa/5 min/36ºC) em formulações de fruta (abacaxi, laranja, kiwi e maga) com leite de soja e leite de vaca (integral, semi-desnatado) e constataram uma elevação significativa nos teores de α-tocoferol em bebidas à base de soja, assim como um aumento no conteúdo de carotenoides (totais, zeaxantina, luteína, zeinoxantina) nas formulações à base de leite integral e semi-desnatado, enquanto o teor de β-caroteno e β-criptoxantina permaneceu inalterado em relação ao controle. O teor de vitamina C foi reduzido em 30% pelo tratamento térmico (90ºC/30 s) em bebidas à base de leite integral ao passo que a APH manteve níveis iguais ao do produto sem tratamento, enquanto as formulações com leite semi-desnatado e soja apresentaram teores similares ou reduzidos aos das amostras sem tratamento independente do processo de pasteurização empregado.

Rodríguez-Roque et al. (2015) produziram uma formulação semelhante de frutas (abacaxi, kiwi, laranja manga) com leite de vaca e de soja e verificaram que o teor de vitamina C não diferiu significativamente entre as amostras controle e pressurizadas (400 MPa/5 min/40ºC), enquanto o tratamento térmico (90ºC/ 60 s) produziu uma redução significativa no teor da vitamina nas bebidas. Em relação aos compostos fenólicos investigados, os autores observaram respostas que variaram em função do composto e da matriz, mas em geral, foi observado um aumento entre 10 e 44% no seu teor quantificável após a aplicação da APH com relação ao controle, com exceção dos ácidos ferrúlico, *p*-cumárico e *p*-hidroxibenzoico, que sofreram redução. Já as amostras conservadas por pasteurização térmica apresentaram uma redução de até 36% ou não diferiram das amostras *in natura*.

Kaushik et al (2014) reportou que o processamento de polpa de manga (600 MPa/5 min/30ºC) resultou em um produto com 85% retenção de vitamina C e 93% de retenção de CFT. Liu et al. (2014) comparou APH (600 MPa/1 min) e tratamento térmico (110ºC/8,6 s) em néctar de manga, e não verificou diferença significativa nos teores de carotenoides, fenolicos totais, vitamina C entre as técnicas utilizadas, porém, o parâmetro de cor *L* (luminosidade no CIELAB L*) sofreu decréscimo significativo nas amostras tratadas termicamente, de forma que aquelas submetidas a alta pressão mostraram-se com coloração mais próxima ao produto *in natura.* Por sua vez, Jacobo-Velásquez, Hernandéz-Brenes (2012), produziram pasta de abacate seguida de conservação a 600 MPa/3 min, verificando um aumento significativo nos teores extraíveis de carotenoides em comparação ao controle (entre 40% e 523%).

No que se refere às frutas nativas do Brasil, não há estudos investigando a viabilidade de uso ou o efeito da APH sobre os seus nutrientes e compostos bioativos, e mesmo para o efeito do processamento térmico os trabalhos ainda são escassos. Alguns trabalhos investigaram o efeito da pasteurização industrial e verficaram que esta promoveu perdas significativas de quercetina, miricetina e kaempferol em polpa de pitanga, acerola e caju (Hoffman-Ribani, Huber, Rodriguez-Amaya, 2009), reduziu em até 30% o teor de antocianinas do açaí (Pacheco-Palência- Ducan, Talcott, 2009) e resultou em perdas significativas ácido ascórbico no cupuaçu (Vieira, Teixeira, Silva, 2000).

De fato a maioria das frutas nativas são sazonais e apresentam baixa expressão comercial devido ao pequeno volume de produção e a limitações de disponibilidade fora das suas regiões naturais (Roesler et al., 2007, Genovese et al., 2008). Em vista disso, a incorporação destas espécies para a produção de polpas, sucos e formulações em grande escala torna-se um desafio, entretanto, o potencial de industrialização destas espécies deve ser

considerado. Nesse sentido, o açaí constitui um exemplo de fruto nativo e de consumo inicialmente restrito a região Norte do Brasil, mas que atualmente é mundialmente reconhecido como uma "super-fruta" devido a sua composição química e efeitos à saúde, com demanda crescente de produção e exportação (Kazumy et al., 2015).

Moreira et al. (2017), produziram uma formulação de bebida probiotica utilizando açaí e manga da região de Ubá, em Minas Gerais, e avaliaram o produto conservado por pasteurização tradicional (82ºC/1 min) e APH (600 MPa/5 min/ 25ºC). Como resultado, o produto apresentou antocianinas, compostos fenólicos e capacidade antioxidante pelo método ABTS, os quais foram maiores nas amostras tratadas termicamente. Tais resultados foram associados a ocorrência de atividade enzimática residual em amostras tratadas por APH, indicando que a atividade enzimática consiste em um parâmetro importante a ser estudado quando do desenvolvimento de novos produtos a serem conservados por alta pressão hidrostática. Ambas as bebidas probioticas atingiram as concentrações necessárias de micro-organismos de um produto deste tipo mesmo após 30 dias de armazenamento a 4ºC, demonstrando a viabilidade do produto. Além disso, a análise sensorial indicou que a bebida conservada por APH foi a preferida pelos provadores, sugerindo que as características do produto original (sem tratamento) foram melhor retidas nessas amostras. Assim, este trabalho mostra que o uso de frutas nativas como ingrediente na produção de um produto com apelo funcional pode constituir uma alternativa de utilização destes frutos nativos do Brasil, de forma a agregar valor aos mesmos (Paz et al., 2015).

Em suma, é possível concluir que a alta pressão constitui uma alternativa ao tratamento térmico para a destruição de micro-organismos. No que concerne ao efeito sobre a inativação enzimática, nutrientes e compostos bioativos, os resultados indicam grande variação em função das condições de processamento, das características da matriz e do composto sendo examinado, de modo que a inativação enzimática constitui atualmente o maior entrave nesta técnica. Isto também é válido para a comparação entre tratamento térmico e APH, sendo que os resultados apontam que dependendo das condições de processo, a APH pode oferecer vantagens na conservação de características sensoriais, nutricionais e de compostos funcionais, mas é possível observar em alguns casos que a utilização de condições otimizadas de temperatura e tempo na pasteurização térmica tendem a diminuir as diferenças entre as técnicas. Nesse sentido, mais estudos que considerem a equivalência de processos na eliminação microbiana são necessários para a realização de uma comparação mais justa entre as técnicas.

Além disso, devido ao aumento da extratibilidade dos componentes dos alimentos devido ao rompimento da parede celular vegetal, tem sido investigada a hipótese de que a maior torna-os mais bioacessíveis ao organismo, o que pode elevar o seu potencial nutricional e funcional (Briones-Labarca et al., 2011, McInerney et al., 2007). Recentemente, Cilla et al. (2017) revisaram o efeito do processamento térmico e da alta pressão hidrostática sobre a bioacessibilidade *in vitro* de compostos fenólicos, carotenoides, minerais, ácido ascórbico e tocoferóis e concluíram que o efeito depende da matriz alimentícia e das condições de processo, podendo o tratamento térmico exercer efeitos positivos ou negativos, e a APH afetar este parâmetro positivamente, como já demonstrado em alguns trabalhos (Cilla et al., 2012, Rodriguez-Roque et al., 2015). Entretanto, os autores ressaltaram a necessidade de mais estudos na área e uma maior padronização dos métodos de bioacessibilidade utilizados, para que resultados comparáveis possam ser gerados. Além disso, é importante considerar que até o momento os estudos têm sido conduzidos exclusivamente em sistemas *in vitro*, sendo inexistentes estudos em humanos. Portanto, apesar de a APH ser uma técnica com premissas interessantes do ponto de vista nutricional e funcional, estudos que suportem a efetividade de tais premissas ainda são bastante escassos.

7 A APH no Brasil e no mundo

A alta pressão hidrostática é considerada uma das mais importantes inovações tecnológicas no processamento de alimentos dos últimos 50 anos (Dunne, 2005). O seu uso está em expansão e atualmente movimenta um mercado de US$ 2,5 bilhões no mundo com aproximadamente 500 mil toneladas de produtos variados. A previsão é de que até 2025 esse mercado aumente para $ 54 bilhões (Lee, Lusk, Mirosa, Oey, 2015, Haung et al., 2015). As principais companhias globais produtoras de equipamentos de alta pressão são Avure (Middletown, OH, USA), Hiperbaric (Burgos, Spain), e Multivac (Germany, Technology). Os principais segmentos envolvidos são carnes (fresca e processada, como presunto e bacon), frutos do mar, bebidas, vegetais prontos para consumo, frutas e produtos derivados de frutas, como sucos, polpas e purês (Huang et al., 2015, Balasubramanian, Martinéz-Monteagudo, Gupta, 2015).

Atualmente, mais de 300 unidades de alta pressão estão em atividade no mundo, porém, devido ao seu elevado custo de implantação (US$ 0,5 a 2,5 milhões dependendo da capacidade e parâmetros de operação do equipamento), estas estão estabelecidas

predominantemente em países desenvolvidos, com destaque para os Estados Unidos, que detém 54% das plantas processadoras, seguido por países europeus (18%) e asiáticos (8%). A América Latina representa apenas 3% desse total.

Dentre os segmentos mais expressivos da conservação por APH está o de sucos, polpas e purês de frutas. O mercado de produtos derivados de frutas e vegetais corresponde atualmente a uma fatia de 40% do mercado de produtos conservados por APH (Visiongain, 2015). As premissas desta tecnologia em preservar as características sensoriais e nutricionais, resultando em produtos com características mais próximas ao produto fresco resultou num crescimento de 80% no número de companhias produtoras de sucos entre 2010 e 2015 (Huang et al., 2015).

Nesse contexto, é possível verificar que o uso da APH no Brasil para a conservação de produtos à base de frutas encontra-se em estágio inicial, ainda em escala piloto e com pouca extrapolação para a escala industrial. Isto está em contraste com o fato de o Brasil ser um grande produtor mundial de frutas e se destacar com várias fruticulturas, como laranja, manga, coco, abacaxi, uva e maçã, além de se diferenciar pela produção de frutos tropicais com apelo sensorial reconhecidos no mundo inteiro como goiaba, acerola e maracujá, e possuir uma rica flora nativa, com características de sabor e composição química que sugerem significativo potencial de industrialização (Romano, Rosenthal, Deliza, 2015, Costa et al., 2015).

Em escala piloto, existem algumas plantas processadoras no país localizadas em instituições de ensino e pesquisa. A Embrapa (Empresa Brasileira de Pesquisa Agropecuária), em parceria com a Universidade Federal do Rio de Janeiro (UFRJ), tem desenvolvido alguns estudos com foco na preservação de frutas tropicais relevantes para a fruticultura nacional, como laranja, manga, abacaxi, maracujá e açaí. Os trabalhos mostram a eficácia da alta pressão (300, 400 e 500 MPa, por 5, 10, 15 minuto a 25ºC, 30ºC, 35ºC) para gerar produtos de elevada aceitabilidade sensorial e destruir a carga microbiana, porém verificaram que a inativação enzimática dependeu das condições aplicadas, sendo observada diminuição ou estímulo da atividade enzimática em função das condições aplicadas (Rosenthal et al., 2004, Rosenthal et al., 2005, Rosenthal et al., 2006, Menezes et al., 2008).

Biscosin Junior et al. (2013), em trabalho do mesmo grupo de pesquisa, investigaram o efeito da APH na atividade da enzima pectinametilesterase e na contagem microbiana de suco de laranja Pera Rio, verificando que o processo a 500 e 600 MPa, 6 minutos e 60ºC promoveu maior inativação enzimática e destruição microbiana. Os autores avaliaram ainda o efeito da APH sobre vitamina C e atividade antioxidante, e verificaram uma retenção

de 70% e 80% desses parâmetros, respectivamente, quando o suco foi submetido a pressões entre 100 e 250 MPa/ 30-40ºC/30 a 125 s, indicando que a melhor condição de inativação enzimática e microbiana pode diferir daquela que favorece a maior retenção de nutrientes (Biscosin-Júnior et al., 2015).

Por sua vez, a Universidade Estadual de Campinas também possui uma planta piloto de processamento por alta pressão e homogeneização a alta pressão, e realiza estudos relacionados ao efeito destas técnicas em produtos lácteos (Oliveira et al., 2014, Pedras et al., 2013); frutas e vegetais (Augusto et al., 2013, Augusto et al., 2014, Leite et al., 2017); destruição de micro-organismos (Pinho et al., 2015, Cavalcante et al., 2014) e inativação enzimática (Tribst et al., 2016, Tribst et al., 2013).

Em escala industrial, atualmente, existe apenas uma indústria processadora de sucos no Brasil que utiliza a alta pressão. Os produtos estão disponíveis em mercados comuns e incluem principalmente suco de laranja e formulações compostas por maçã, uva, framboesa, manga, limão, maracujá e goiaba, todos integrais, isto é, sem adição de água. Por ser um setor iniciante no País (cerca de 3 anos atuando no mercado), dados de produção e mercado da alta pressão hidrostática não foram encontrados. De acordo com a Euromonitor (2016) 28% dos brasileiros consideram o valor nutricional o fator mais importante na decisão de compras, enquanto 22% dão preferência a alimentos naturais. Assim, é possível observar que o Brasil dispõe de matéria-prima e possui um potencial mercado consumidor para absorber a produção de produtos conservados por APH.

8 Considerações finais e conclusão

O Brasil é um grande produtor mundial de frutas de clima tropical e temperado. Dentre estas é possível destacar a laranja, a qual o país lidera a produção mundial de fruto *in natura* e suco. Tendo em vista que estudos demonstraram que a conservação por APH resultou em produtos com propriedades sensoriais, nutricionais (Vitamina C) e funcionais (compostos bioativos e atividade antioxidante) preservadas, o Brasil apresenta um elevado potencial para a produção de suco de laranja utilizando esta técnica de conservação. Uma vez que a laranja é considerada uma fruta de baixo valor comercial, a utilização da APH e a produção de sucos com elevada qualidade sensorial e funcional seria fundamental para agregar valor ao produto no mercado nacional e internacional, de forma a aumentar a representatividade do Brasil nesse setor.

Similarmente, outras espécies como uva, maracujá, goiaba, manga, coco, mamão, abacaxi, possuem uma representatividade significativa na fruticultura nacional e no segmento de sucos e polpas. No entanto, o estudo do efeito da alta pressão na conservação destas frutas ainda é escasso ou inexistente. Devido ao seu grande apelo sensorial e nutricional, os produtos à base destes frutos têm grande potencial para APH na geração de produtos de alta qualidade.

Existem ainda as espécies chamadas ‘‘não-tradicionais’’, as quais são frutas nativas do Brasil. A qualidade sensorial, nutricional e funcional de algumas destas frutas tem sido cada vez mais valorizada em função de avanço no estudo destas espécies, de modo que a industrialização das mesmas ou o seu uso como ingrediente na indústria de sucos e polpas deve ser considerado. Nesse contexto, o açaí constitui atualmente o produto de maior valor proveniente do extrativismo vegetal e em função da alta demanda pelo fruto, possui um alto valor de mercado. Tendo em vista que os efeitos à saúde mediante o consumo de açaí são associados com a sua composição química rica em compostos antioxidantes e lipídios bioativos, o uso da APH para conservação da polpa e de outros produtos derivados revela-se como uma alternativa interessante para preservar os seus compostos bioativos e nutrientes, entregando alimentos de maior qualidade para o consumidor.

Portanto, observa-se que o Brasil possui um enorme potencial para o uso da alta pressão hidrostática para a conservação de frutos processados, pois além de ser um grande produtor de frutos mundialmente apreciados, possui frutos nativos com elevado apelo nutricional, funcional e sensorial, os quais chamam a atenção do consumidor em função dessas características, e que podem ser incorporados em preparações industriais. Dada a baixa participação internacional do Brasil no setor de frutas processadas e todo o potencial de frutíferas do País, a APH pode agregar valor aos produtos produzidos, elevando o seu valor comercial e contribuindo para aumentar a relevância do país no segmento de sucos e polpas de frutas.

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CAPÍTULO II

MULTI-STEP OPTIMIZATION OF BIOACTIVE COMPOUNDS EXTRACTION FROM FREEZE-DRIED AÇAÍ PULP (*Euterpe oleracea* Mart)

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Abstract

In the present work, a simplex-centroid mixture design was used for the first time to study the effect of solvent composition (acetone, methanol, water and ethanol) on the extraction of total monomeric anthocyanins (TMA) and total phenolic compounds (TPC) content from freeze-dried açaí pulp (*Euterpe oleracea* Mart.). Next, the effect of time, solid-to-liquid ratio and hydrochloric acid concentration on these responses was investigated through a central composite design. Finally, the number of re-extractions (maximum 3) to achieve maximum extraction yields was examined. The simplex-centroid design indicated significant synergistic effect of binary and ternary solvents, being methanol and acetone the most important contributing solvents for increasing extraction efficiency in the blending. Desirability function was applied to obtain a solvent composition with lower concentration of methanol and acetone as maintaining high yields of TPC and TMA. Then, acetone:methanol:water (31:24:45 v/v/v) 0.1% HCl was chosen as the best solvent. Regarding the CCD, TMA has not been affected by none of the variables considered, whereas TPC content raised with time up to 9 minutes, and started to decrease thereafter. Moreover, solid-to-liquid ratio was another important variable for this response, which had a significant negative interaction effect with time, indicating that by using low solid-to-liquid ratios, extraction times can be shortened. Then, optimal conditions were: half a gram of freeze-dried sample mixed with 27 mL (1:54) of acetone:methanol:water (31:24:45 v/v/v) 0.1% HCl and sonicated for 6 minutes. Finally, results indicated that two extraction (n=2) succeeded in extracting 99% and 83% of TPC and TMA, respectively, confirming that multivariate optimization tools are effective to give extraction protocols for saving time, reducing costs and use of toxic solvents.

Key-words: simplex-centroid design, Derringer & Suich, RSM, anthocyanins, phenolic compounds

1 Introduction

Euterpe Oleracea Mart., a palm tree growing abundantly on the Amazon floodplain, gives açaí fruits, which are widely consumed in the North region of Brazil and have become largely recognized across the country and overseas as a highly nutritive food (Schauss 2010, Dupureur et al., 2012). In viewing of possessing high perishability, açaí fruits are hardly found fresh for consumption, being chilled or frozen pulp, obtained by mechanical pulping and addition of different proportions of water, its main form of commercialization (Rogez et al., 2012). Moreover, the pulp is used as an ingredient in dietary supplement manufacturing, açaí beverages, and other açaí-based products (Menezes et al., 2011, Carvalho et al., 2016).

Açaí pulp extracts have been reported to possess the highest hydroxyl radical and superoxide anion *in vitro* scavenging capacity of any fruit (Schauss et al., 2006). Moreover, it has been related to own potent anti-inflammatory activity, antiproliferative, artheroprotective and anticarcinogen effects (Pacheco-Palencia et al., 2010, Hogan et al., 2010, Kang et al., 2012, Schauss, 2013, Fragoso et al., 2013, Romualdo et al., 2015, Martino et al., 2016). These properties have been linked to the high contents of phenolic compounds found in the açaí pulp, rich in anthocyanins (averaging 5 $mg.g^{-1}$ dry matter), phenolic acids and other flavonoids (27 mg GAE.g^{-1} dry matter) (Rufino et al., 2010, Gordon et al., 2012, Garzón et al., 2017).

Given the importance of its phytochemicals composition, phenolic compounds content in açaí pulp have been extensively investigated. In order to characterize the fruit composition, different extraction conditions, including solvent composition, time, solid-to-liquid ratio, among others, have been used. Ethanol, methanol, acetone and water, either combined or pure, used isolatedly or in sequential extraction, acidified with HCl, formic or acetic acid, have been reported to extract efficiently phenolic compounds from açaí pulp (Schauss et al., 2006, Borges et al., 2011, De Rosso et al., 2008, Pompeu et al., 2011, Mulagabal, Calderón, 2012, Borges et al., 2013, Gordon et al., 2012, Romualdo et al., 2015, Borges et al., 2016, Garzón et al., 2017).

Since a successful phytochemical characterization of any food matrix is highly dependent on extraction conditions, studies on how extraction parameters affect efficiency is of great importance for a proper quantification of the bioactive content and correlation with health benefits. However, studying the influence of such variables separately is time-consuming, high-cost and usually involves high solvents consume; therefore, statistical tools of multivariate optimization aim at securing a proper optimization, evaluating interaction terms and minimizing such drawbacks (Ferreira et al., 2007).

A few studies on multivariate optimization for extraction of bioactive compounds from açaí pulp have been carried out, including an investigation into *Euterpe edulis,* another species from *Euterpe* genus (Borges et al., 2011). Solvent composition, time and solid-to-liquid ratio have been shown to be important variables, being the study of the solvent composition a key parameter for a successful extraction (Pompeu, Silva, Rogez, 2009, Borges et al., 2011, Borges et al. 2016). However, none of these approaches looked into the solvent effect on the extraction of bioactive compounds using a mixture design, which are not only ideal for studying the effects of pure solvents but also to determine synergic and antagonistic effects among solvents that are relevant to the extraction procedure (Passari et al., 2014). In this sense, previous works have stressed the importance of using simplex-centroid designs to determine the extractor solvent composition, since interaction effects can be more important than pure solvent features, such as basicity, acidity and polarity, which have been thought to only partly contribute to the extraction efficiency (Garcia et al., 2010, Xavier et al., 2011)

Ultimately, it is expected that by combining multivariate techniques in a two-step extraction approach may be useful to give an extraction method possessing high efficiency, reduced time, and use of toxic solvents and cost of analysis. Therefore, this study used a simplex-centroid mixture design comprising 4 commonly used solvents (acetone, methanol, water and ethanol), in tandem with a central composite design to maximize yields of total monomeric anthocyanins (TMA) and total phenolic compounds (TPC) in extracts of freeze-dried açaí pulp.

2 Material and methods

2.1 Chemicals and açaí samples

Methanol P.A, ethanol, acetone, hydrochloric acid, sodium carbonate, potassium chloride and sodium acetate were purchased from Synth (Diadema, São Paulo, Brazil), galic acid from Sigma-Aldrich (St. Louis, MO, USA) and the phenol reagent Folin–Ciocalteu from Merck (Darmstadt, Germany).

Açaí pulp (12% solid content) was acquired in polyethylene bags from local shops in Belém (Pará, Brazil), frozen, and transported under freezing to Campinas. Then, they were overnight defrosted at 5 ºC, allocated in steel plates and freeze dried for 96 hours (-48ºC). The freeze-dried açaí pulp was collected, stored protected from light and oxygen under -20ºC until the analysis (up to 1 month).

2.2 Folin Ciocalteu assay

The Folin-Ciocalteu method as described by Singleton and Orthofer (1999) was used after adaptation to a microplate reader (FLUOstar Omega, BMG LABTECH). The samples or the galic acid standard were set to react with the Folin reagent for 5 minutes, and then sodium carbonate (7.5%) was added to the mixture. After 2 hours, the blue colour intensity of the samples was measured at 760 nm. Results were expressed as mg of gallic acid equivalents (GAE) per gram of freeze-dried sample. Extracts were analysed in triplicate.

2.3 Total monomeric anthocyanins (TMA) assay

Monomeric anthocyanin content was determined using the pH differential method described by Giusti and Wrolstad (2001). Samples were properly diluted in sodium acetate buffer (pH 1.0) and potassium chloride buffer (pH 4.5). The anthocyanin content was calculated according to Equations 1 and 2, and expressed as mg of cyanidin 3-glucoside per gram of freeze-dried sample.

$$A = (A_{510nm} - A_{700nm})_{pH\ 1.0} - (A_{510nm} - A_{700nm})_{pH\ 4.5} \quad \text{(Equation 1)}$$

$$A = \frac{(A \times MW \times DF \times 1000)}{\varepsilon \times 1} \qquad \text{(Equation 2)}$$

In which A = absorbance, MW = 449.2 (molecular weight of cyanidin 3-glucoside), DF = dilution factor, and ε = 26.900, the molar absorptivity coefficient. Results were expressed as mg of cyanidin 3-glucoside equivalent (CGE) per gram of freeze-dried pulp.

2.4 Multivariate optimization

2.4.1 Solvent effect

Different proportions of methanol, ethanol, acetone and water were studied according to the simplex-centroid mixture design displayed in Table 1 and depicted in Figure 1. Hydrocloric acid (HCl) have been majorly used as acidifying reagent of extractor solvents for açaí samples and have been shown to promote rupture of the cell walls (De Rosso et al., 2008, Pompeu, Silva, Rogez, 2009, Rogez et al., 2011, Borges et al., 2011), therefore, all the extractor solvents used in the mixture design were acidified with 0.1% HCl (Mulabagal, Calderón, 2012). At this stage, extraction conditions were based on Mulabagal, Calderón (2012) and Wu et al. (2004). One gram freeze dried açaí pulp was mixed with 30 mL of extractor solvent, vortexed for 30 s and sonicated (Tecnal, Piracicaba, SP, Brazil) for 5 minutes. Afterward, the extracts were centrifuged (15 minutes at 3000 g and 4ºC), transferred to 50 mL flasks and the volume was completed with the proper extraction solution. The sonication temperature was maintained at 25±2 ºC over the experiments execution. Re-extractions have not been carried out to avoid loses during the filtration process that could jumble the results (Bochi et al., 2014).

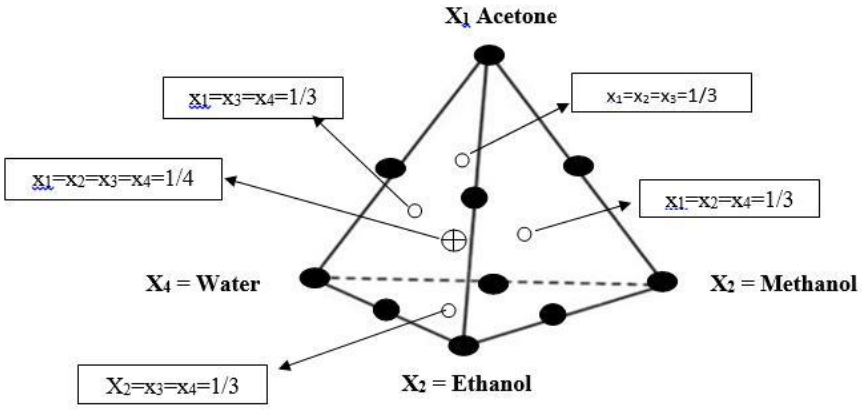

Figure 1. Simplex–centroid mixture design for the acetone, methanol, water and ethanol solvents.

Responses were tested to fit linear, quadratic and special cubic models (Equation 3). The linear model is represented by the first term of the equation, where y is the predicted response measured (total phenolic compounds or total monomeric anthocyanin), x*i* the solvent proportion and b*i* the linear model coefficient. The quadratic model, given by the b*ij* coefficients, indicates the significance of synergic and antagonistic binary effects. The b*ijk* coefficients represent possible ternary effects and are significant when ternary mixtures give significantly different response values compared to those of their corresponding pure solvent and binary mixtures.

$$y = \sum i \quad + \sum i \ \sum < \quad + \sum \sum < \ \sum <$$ (Equation 3)

From the models generated, the global desirability function was used (Derringer and Suich, 1980) to reduce solvent proportion in the extractor solvent but keeping maximum yields of bioactive compounds (TMA and TPC). Hence, for each of the responses studied, a desirability value for the goal settings was calculated according to the Equations 4, 5 and 6; next, they were combined to establish a global desirability. This means that the algorithm by Derringer and Suich used the mathematical models to combine the extractor solvent composition containing the lowest amounts of organic solvent along with the highest contents of TPC and TMA.

$= 0 \quad < \quad$ (Equation 4)

$= \left(\frac{\quad - \quad}{\quad} \right), \leq \leq$ (Equation 5)

$= 1 \quad \geq \quad$ (Equation 6)

where di corresponds to desirability i between 0 and 1, Ri are the predicted values by the models for TPC or TMA, Rmax are the maximum values for content of TPC or TMA, Rimin is the minimal value for the TPC or TMA content. Multiple regression analyses, analysis of variance (ANOVA) and significance test were performed using the Design Expert 6.0 (Stat-Ease, Minneapolis, USA).

2.4.2 Time, acid concentration and solid-to-liquid ratio effect

After the optimization of the extractor solvent composition, a study of the effect of the time, concentration of HCl and solid-to-liquid ratio on the extraction of TPC and TMA from freeze dried açaí pulp was carried out using a 2^3 central composite design. Codified and decodified values for each of the variables investigated are shown in Table 2. Ranges studied were based on previous tests and related literature (Rogez, 2000, De Rosso et al., 2008, Pompeu, Silva, Rogez, 2009, Agwa et al., 2011, Borges et al., 2011, Wu et al., 2004, Rufino et al., 2010, Pacheco-Palencia, 2010, Gordon et al., 2012)

Half a gram freeze dried açaí pulp was mixed at different solid-to-liquid ratio (1:10 – 1:60) with the optimized extractor solvent containing varying HCl concentrations (0.1 – 2%), then vortex for 30 s to promote good homogenization, and sonicated according to the time indicated by the experimental design (1 – 21 min). Afterward, the extracts were centrifuged (15 minutes at 3000 G and 4 ºC), transferred to 50 mL flasks and the volume was completed with the proper extraction solution. The extraction temperature was maintained at 25±2 ºC throughout experiments execution. Re-extraction steps were avoided at this phase in order to

decrease the experimental errors (Bochi et al., 2014).The extracts were stored under -18ºC until analysis being performed.

Linear and quadratic models were investigated, according to the Equation 7, where x_1, x_2 and x_3 are the independent variables affecting the responses Y; b_o, bi (i = 1, 2, 3), bii (i = 1, 2, 3), and bij (i = 1, 2, 3; j = 2, 3) are the regression coefficients for the intercept, linear, quadratic and interaction product terms, respectively.

$$Y = b_0 + \Sigma_i b_i x_i + \Sigma b_{ii} x^2 + \Sigma\Sigma b_{ij} x_i x_j \qquad \text{(Equation 7)}$$

Multiple regression analyses, analysis of variance (ANOVA) and significance test were performed using Statistica 7.0 (StatSoft, Tulsa, OK, US).

3 Results and discussion

3.1 Solvent effect

The results for the simplex centroid mixture design are described in Table 1. As the composition of the extractor solvent changed, TPC and TMA varied from 5.23 to 24.81 $mg.g^{-1}$ and from 0.15 to 4.14 $mg.g^{-1}$, respectively, suggesting that the solvent composition might have affected the extraction of the compounds studied. Figures 2a and 2b show bar graphs of the content of TPC and TMA for each simplex-centroid design experiment.

Among the pure solvents, methanol had the highest extraction efficiency for both TPC and TMA, averaging twice the concentration of TMA than that of the others pure solvents, and 30% more TPC than pure water or ethanol, and 70% more than acetone. This result confirms previous studies in which methanol was shown to possess high performance for extraction of anthocyanins and other phenolic compounds (Revilla et al., 1998, Wu et al., 2004, Borges et al., 2011, Truong et al., 2012). Binary mixtures, for instance water:ethanol and water:acetone caused TPC to increase 15%, whereas TMA gave similar values for these blends compared to those of pure methanol. In turn, ternary mixtures such as water:acetone:methanol (experiment 11) and water:acetone:ethanol (experiment 13) had outcomes in average 30% higher for TPC and TMA than pure methanol. The former stood out among pure, binary and

ternary mixtures by yielding 20% more TMA than experiment 13, 32% more than 6 and 10, and 50% more than experiment 2.

These results suggest that binary and ternary synergistic effects may have occurred between these solvents and stress the importance of the interaction between the solvents molecules for the performance of the extraction process (Passari, Scarminio, Bruns, 2014). To confirm the existence of significant synergic effects between solvent molecules, the data were fitted to linear, quadratic and special cubic mixture models and the generated coefficients are shown in Table 2. The analysis of variance (ANOVA) showed that the special cubic model was significant for both TPC and TMA, and that the models did not present evidence of lack of fit (at the 95% confidence).

For both TPC and TMA, linear methanol coefficients had the highest values, whereas acetone had the lowest ones, supporting that methanol was the best pure extractor solvent. Significant binary (acetone:water, acetone:ethanol and water:ethanol) interaction coefficients for TPC had values as high as three times than those of the pure solvents, moreover, TPC and TMA contents were in average twice higher when these solvents were combined compared to the single ones, confirming the existence of a synergistic effect between the solvents. On the other hand, the combination of acetone:ethanol and methanol:ethanol had antagonistic effect on the extraction of TMA (negative coefficients values).

Significant third order coefficients were even higher (in average four times for TPC and ten times for TMA), in such a way that there was a substantial increase in the TPC and TMA content when a combination of three solvents was used compared to pure or binary systems, indicating that ternary interactions posetively affected the extraction. TMA was found to be positively affected, at different extents, by all ternary interactions, whereas TPC was highly affected by one ternary combination only (ABC). Acetone:methanol:water (ABC) had the highest coefficient value and gave the highest outcomes for both of the responses. Although the mixture acetone:ethanol:water (ACD) have yielded similar TPC contents, coefficient values evidenced the importance of methanol for TMA extraction, which is supported by the experiments 13 and 11 that significantly differed ($p<0.05$) one from another.

Table 1. Simplex-centroid mixture design solvent proportions, total phenolic compounds and total monomeric anthocyanins contents.

Runs	Independent variables								Responses	
	Acetone		**Methanol**		**Water**		**Ethanol**			
	Codified	*Decodified*	*Codified*	*Decodified*	*Codified*	*Decodified*	*Codified*	*Decodified*	TPC (mg.g-1 DW[a])	TMA (mg.g-1 DW)
1	1	100%	0	0.0	0	0.0	0	0.0	5.27	1.08
2	0	0.0	1	100%	0	0.0	0	0.0	18.59	2.31
3	0	0.0	0	0.0	1	100%	0	0.0	13.04	1.19
4	0	0.0	0	0.0	0	0.0	1	100%	11.65	1.61
5	1/2	50%	1/2	50%	0	0.0	0	0.0	11.79	0.91
6	1/2	50%	0	0.0	1/2	50%	0	0%	20.83	2.37
7	1/2	50%	0	0.0	0	0.0	1/2	50%	17.47	0.15
8	0	0%	1/2	50%	1/2	50%	0	0.0	16.78	1.44
9	0	0%	1/2	50%	0	0.0	1/2	50%	13.57	0.49
10	0	0%	0	0%	1/2	50%	1/2	50%	22.16	2.27
11	1/3	33%	1/3	33%	1/3	33%	0	0%	24.90	3.29
12	1/3	33%	1/3	33%	0	0.0	1/3	33%	16.40	1.51
13	1/3	33%	0	0%	1/3	33%	1/3	33%	24.32	2.74
14	0	0%	1/3	33%	1/3	33%	1/3	33%	22.11	2.66
15	1/4	25%	1/4	25%	1/4	25%	1/4	25%	23.41	4.00
16	1/4	25%	1/4	25%	1/4	25%	1/4	25%	23.41	4.09
17	1/4	25%	1/4	25%	1/4	25%	1/4	25%	24,63	3.81

a: DW – dry weight

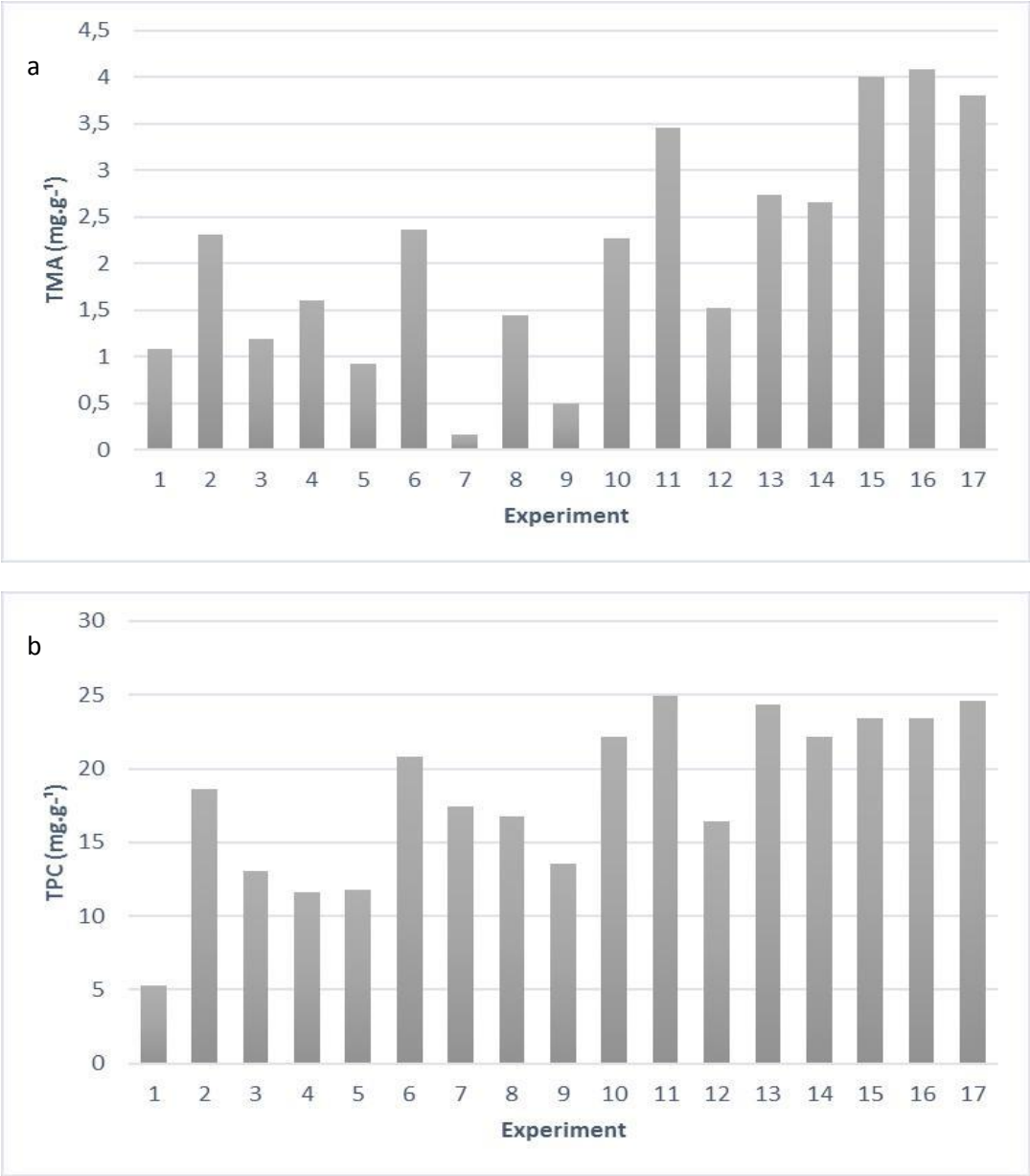

Figure 2. Bar graphs for (a) total monomeric anthocyanins and (b) total phenolic compounds for each solvent extractor of the mixture design.

Table 2. Significant mixture model coefficients for validated models of total phenolic compounds and total monomeric anthocyanins.

Coefficient	TPC	TMA
*A (*Acetone)	5.26	1.09
B (Methanol)	18.58	2.32
C (Water)	13.02	1.20
D (Ethanol)	11.63	1.61
AB	-	-
AC	47.24	4.70
AD	36.61	-4.98
BC	-	-
BD	-	-6.06
CD	39.86	-
ABC	174.51	56.78
ABD	-	43.41
ACD	-	34.53
BCD	-	42.88
$F_{regression}/F_{residue}$ [a]	39.94	24.97
$F_{lack\ of\ fit}/F_{pure\ error}$ [b]	2.25	8.63

a: Fcritical $_{13,3}$ for model validation: 5.89

b: Fcritical $_{1,2}$ for lack of fit test: 18.51

At 95% of confidence level

Therefore, results pointed the mixture acetone:methanol:water as the solvent of choice for simultaneous extraction of TPC and TMA from freeze-dried açaí pulp. The importance of acetone solutions for anthocyanins and phenolic compounds extraction from açaí pulp have been consistently demonstrated, as it is capable of easily extracting cyanidin 3-glucoside and other phenolic substances (Gordon et al, 2012, Borges et al., 2016).

Since the mixture design indicated acetone:methanol:water at the approximate proportion 33:33:33 (v/v/v) as the best solvent combination to yield approximately 3.5 mg.g DW TMA and 24.9 mgGAE.g-¹ DW, the algorithm by Derringer and Suich was used to find a

solvent composition with a minimal concentration of methanol and acetone keeping the high outcomes of TPC and TMA. Thus, the models generated were used to make predictions within the experimental range according to the desirability criteria described in Table 4. The optimal conditions found were 31% acetone, 24% methanol and 45% water. The responses predicted for the TPC and TMA concentrations using this solvent combination presented good concordance with the values obtained experimentally, as can be observed in Table 3. Therefore, by using the algorithm, the final extractor solvent composition had a higher water proportion (45%) and in average 22% less organic solvent concentration than the aforementioned blend, but maintaining the extraction efficiency.

Organic solvents are found to enhance TMA and TPC yields by degrading plant cell walls and therefore facilitating compounds release from the plant. Acetone is able to give high extraction rates for high molecular weight compounds, such as proanthocyanidins, whereas lower weight substances (anthocyanins, phenolic acids) are efficiently extracted with methanol (Mané et al., 2007). In addition, there is a decrease in the water dieletric-constant, improving interaction between the extractor solvent and target molecules (Pompeu, Silva, Rogez, 2009, Truong et al., 2012). According to the solvent selectivity triangle by Snyder (1993), organic solvents are classified in function of their ability to interact with the solute as a proton donor (basicity, β), as a proton acceptor (acidity, α) or a dipole (dipolarity, Π). In this regard, the higher the difference between such properties, the greater the difference in selectivity will be find on mixing them. Acetone has high Π and β values, whereas water has high α and dipole values, methanol has strong acidity values and ethanol has strong acidity and β (Snyder, 1993). Moreover, solvent polarties diverge among the solvents (5.4 acetone, 6.6 methanol, 9 water and 5.2 ethanol), so that mixing them leads to different polarities extractor solvents. Hence, varying such characteristics by combining these solvents differently affects significantly mixture extraction efficiency and result in synergic or antagonist interactions.

Table 3. Desirability settings for simultaneous optimization of total phenolic compounds and total monomeric anthocyanins using Deringer and Suich function.

Variable/ Response	Goal	Inferior limit	Superior limit	Importance	Predicted	Observed	Prediction interval [a]	
Acetone	minimize	0	1	1	-	-		
Methanol	minimize	0	1	1	-	-		
Water	Maximize	0	1	1	-	-		
Ethanol	Is equal to 0.0	0	0	1	-	-	Low	high
TPC	Maximize	5.27	24.72	5	24.04	26.40	20.97	28.48
TMA	Maximize	0.15	4.1	5	3.6	3.14	2.39	4.59

[a] 95% confidence interval

Mixtures of acidified acetone:water and methanol:water have been commonly used for extraction of anthocyanins and phenolic compounds from *Arecaceae* family fruits (Rufino et al., 2010, Abadio Finco et al., 2012, Gordon et al., 2012, Rezaire et al., 2014, Carvalho et al., 2016b). Nonetheless, studies evaluating the effect of these solvents using a statistical approach able to properly identify their synergistic or antagonistic behaviour have never been carried out. Recently, Borges et al. (2016) used a Plackett-Burnman design to study the interaction between acidified acetone, ethanol and methanol through a sequential extraction (n=3) procedure, for the extraction of TMA and TPC from defatted freeze-dried açaí pulp. However, such approach does not allow assessment of solvent interactions. Pure solvent features, such as basicity, acidity and polarity, as considered separately, have been thought to contribute to extraction efficiency, but taking advantage of solvents chemical proprieties differences by mixing them has been shown to effectively modify solvent selectivity and enhance extraction rates (Garcia et al., 2010, Lonni et al., 2012). Therefore, statistically significant interaction terms involve solvents with different selectivity properties. Therefore, as demonstrated in the present work, mixture designs are the best statistical tool to define the solvent composition for extraction of phytochemicals (Soares et al., 2011, Passari et al., 2014).

3.2 Time, HCl concentration and solid-to-ratio effect

Table 4 contains codified and uncodified levels of the variables investigated and the TPC and TMA concentrations in each central composite design experiment. Data were fitted to linear and quadratic models and the significant coefficients generated, along with the analysis of variance (ANOVA) results, are described in Table 5. The regression models did not suffer from lack of fit at 95% confidence level. In general, there was a narrow fluctuation between TPC contents in the mixture design and CCD.

However, minimal values were higher than those ones from the mixture design, confirming that the optimized solvent composition was efficient for TPC extraction from freeze-dried açaí pulp. Furthermore, the oscillation verified for this response (13.98 to 28.98 mg GAE.g^{-1}) indicated that changing other extraction parameters might affect extraction efficiency even with the use of an optimized solvent composition. Likewise, there was an increase in the minimal values of the TMA concentration (fluctuated between 1.36 and 3.06 mg.g^{-1}) compared to the mixture design, however, maximum values barely surpassed the highest concentration found in the mixture design for ternary mixtures, suggesting that the range of the variables studied in the CCD did not affect TMA extraction.

At the 95% confidence level, none of the tested variables significantly affected the TMA content, and neither linear nor quadratic models were able to explain oscillations in the TMA results. According to Pompeu, Silva and Rogez (2009) anthocyanins in açaí are located in the most external region of the fruit and therefore require low energy to be extracted, favouring its prompt solubilisation in the extractor solvent. Therefore, it is possible that the one-step extraction conditions tested may have promoted solvent saturation. Similar behaviour was found by Bochi et al. (2014), studying the effect of time and acid concentration on TMA extraction from Ceylon gooseberry and Borges et al. (2016) for solid-to-liquid ratio, acetone, ethanol and extraction time effect on TPC and TMA of freeze-dried açaí pulp.

As for the TPC content, results indicated that this response was positively affected by the linear effect of time and solvent volume, adversely influenced by the second order term of these parameters and by their interaction.

Table 4. Coded and uncoded extraction conditions of the full factorial design 2^3 and experimental results for total monomeric anthocyanins and total phenolic compounds.

Run	Independent variables						Responses	
	Time (min)		Solvent volume mL		%Hydrocloric Acid			
	coded	*uncoded*	*coded*	*uncoded*	*coded*	*uncoded*	*TMA* (mg.g-¹DW[a])	*TPC* (mgGAE.g-¹DW)
1	-1	5.04	-1	10	-1	0.46	1.80	19.34
2	1	16.95	-1	10	-1	0.46	2.40	26.72
3	-1	5.04	1	24.9	-1	0.46	2.64	28.98
4	1	16.95	1	24.9	-1	0.46	2.67	28.02
5	-1	5.04	-1	10	1	1.59	2.06	16.69
6	1	16.95	-1	10	1	1.59	2.25	21.11
7	-1	5.04	1	24.9	1	1.59	2.97	27.72
8	1	16.95	1	24.9	1	1.59	2.73	26.96
9	0	11	0	17.5	0	1.05	3.06	26.29
10	0	11	0	17.5	0	1.05	2.74	27.58
11	0	11	0	17.5	0	1.05	2.45	26.50
13	1.68	21	0	17.5	0	1.05	2.21	23.47
14	0	11	1.68	30	0	1.05	2.61	28.91
15	0	11	0	17.5	1.68	2	2.60	24.20
16	-1.68	1	0	17.5	0	1.05	2.06	20.83
17	0	11	-1.68	5	0	1.05	1.36	13.98
18	0	11	0	17.5	1.68	0.1	1.81	23.22

a: DW - dry weight

Table 5. Significant coefficients for validated models of TPC and TMA.

Coefficient	TPC	TMA
Mean	25.89*	2.83*
Time (L)	1.06*	0.06
Time (Q)	-0.96*	-0.13
Solvent Vol (L)	3.87 *	0.33
Solvent Vol (Q)	-1.21*	-0.18
%HCl (L)	-0.65	0.13
%HCl (Q)	-0.58	-0.11
*Time*Solvent Vol*	-1.69*	-0.124
Time%HCl*	-0.34	-0.08
Solvent Vol%HCl*	0.74	-0.03
$F_{regression}/F_{residue}$ [a]	38.98	-
$F_{lack\ of\ fit}/F_{pure\ error}$ [b]	10.55	-

[a] $F_{critical\ 9.7}$: for model validation: 3.39

[b] $F_{critical\ 5.2}$: for lack of fit test: 19.30

*siginificant at the 95% of confidence level

The linear coefficient of the solvent volume had the highest positive value (3.87), indicating that this variable had the highest effect on the TPC extraction. In turn, the interaction term (time *vs.* volume) had the highest negative value suggesting that low solid-to-liquid ratios require shorter extraction times and the opposite is true.

The contour plot (Figure 3) for the TPC shows that the extraction was favoured by an increase in the time up to 9 minutes (corresponding to approximately -0.2 in the experimental region), followed by a decrease at higher levels of the experimental design. Time has been shown to be a critical factor for TPC extraction since long extractions favour light and oxygen exposure and may cause degradation (Chan et al., 2009, Pompeu, Silva and Rogez, 2009, Costa et al., 2015, Borges et al., 2011).

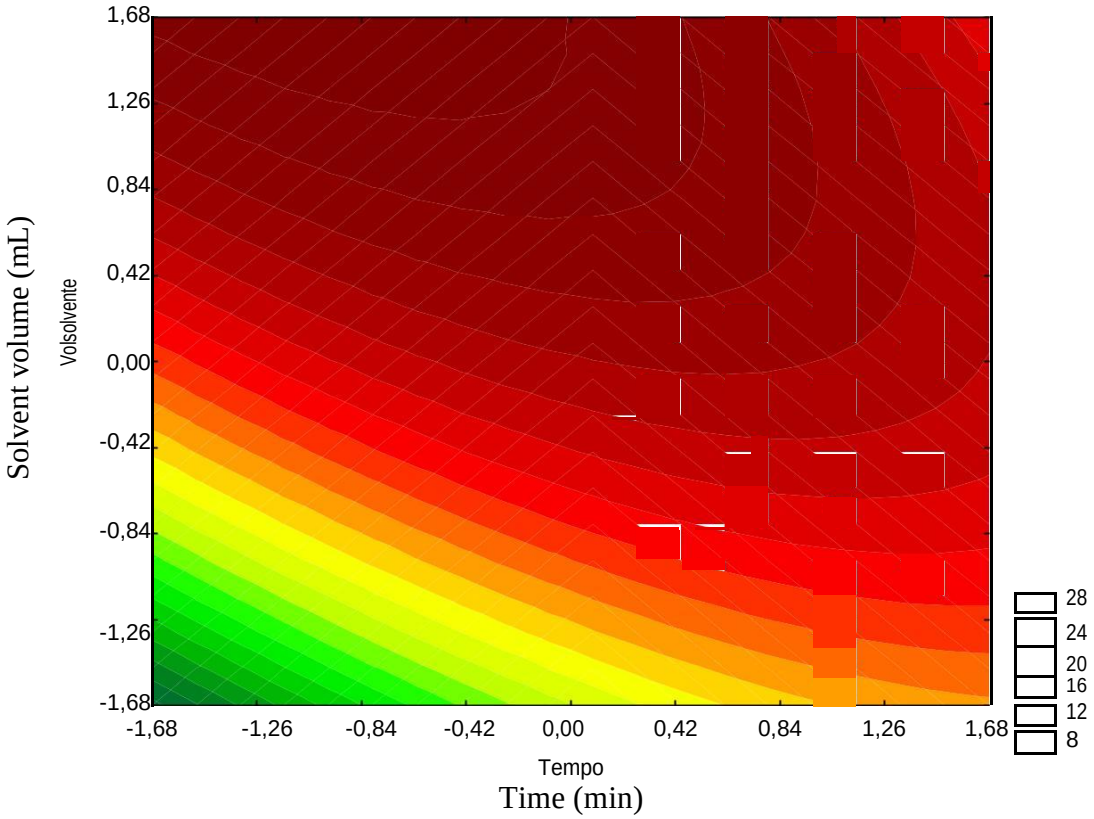

Figure 3. Response surface model plot for effects of time and solid-to-liquid ratio on total phenolic compounds content. R^2 = 0.95955, indicating that the model explained 95% data variation.

In turn, acidification with HCl is required to rupture the cell walls of fruits and facilitate access to phenolic compounds (Pompeu, Silva, Rogez, 2009, Borges et al., 2011, Agwa et al., 2011). Moreover, acidic media provides a favourable condition for the formation of flavylium ions and the stabilization of anthocyanins (He, Giusti, 2010, Truong et al., 2012). Yet, an acid excess may hydrolize phenolic glycosides and promote degradation of the native form by breaking associations with metals and co-pigments, leading to important changes in total anthocyanin content in pigment components of the extracts (Revilla 1998, Castañeda et al., 2009), so low acid amounts are desired. Since this variable did not affect none of responses, it was fixed at 0.1%, the lowest level of the experimental design.

Hence, the extraction conditions of TPC and TMA from freeze-dried acai pulp were defined as acetone:methanol:water (31:24:45 v/v/v) 0.1% HCl, 27 mL of extractor solvent (1:54) and sonication for 6 minutes. Validation experiments were carried out in triplicate using these conditions in order to confirm the suitability of the model for making predictions. The

TPC concentration observed in the confirmation experiments (31.25±1.08 mg GAE.g-¹) had good concordance with the value predicted by the model and fitted within the prediction interval at 95% confidence level (26.16 to 33.06 mg GAE.g-¹), whereas the TMA content was 2.7±0.20 mg.g-¹.

Finally, in order to evaluate extraction efficiency the freeze-dried açaí pulp was subjected to re-extractions (n = 3) and the TMA and TPC were measured in each of the extracts obtained (Figure 4). According to the results, 90% and 77% of the TPC and TMA were extracted in the first extraction (n=1), respectively. In turn, the first re-extraction (n=2) provided 9% and 6% of TPC and TMA, giving an extraction efficiency of 99% and 83%. Since excessive manipulation of the sample may cause degradation of phenolic compounds, the final extraction protocol was fixed to only one re-extration step.

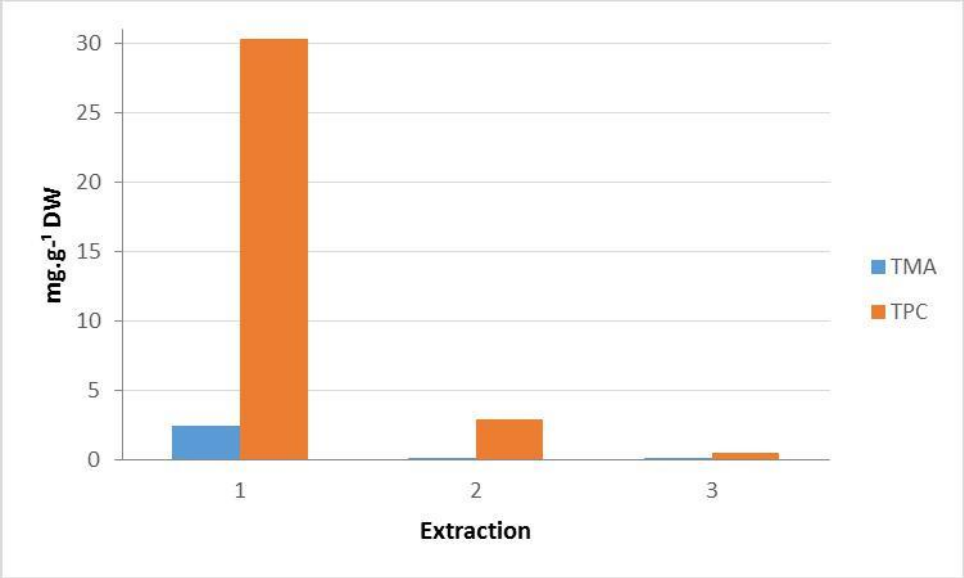

Figure 4. TPC and TMA contents in each of the extractions performed (n=3).

4 Conclusion

This study carried out for the first time a complete study on the solvent effect on the extraction of TPC and TMA from freeze-dried açaí pulp, using the four most common solvents used. Through a simplex-centroid mixture design, methanol showed to be the most efficient single-solvent for both TPC and TMA extraction, however, ternary interaction effects

were highly significant, so that methanol, acetone and water was the selected mixture of solvents. Using the algorithm by Derringer and Suich, it was possible to minimize methanol and acetone proportions in the extractor mixture and maximize the extraction efficiency of both TPC and TMA. The final extractor solvent composition was 45% water, 24% methanol and 31% acetone (v/v/v). Afterwards, a central composite design used to determine the effect of extraction time, solid-to-liquid ratio and HCl percentage on the extraction efficiency of TPC and TMA showed that none of the variables had significant effect on the latter. On the other hand, TPC were affected by the extraction time and the solvent volume, as well as by the interaction between these variables. The final extraction condition using the optimized solvent was: 0.1% HCl, 1:54 solid-to-liquid ratio and sonication for 6 minutes. This study showed the advantages of using combined chemometric tools to optimize extraction processes for saving time, reducing costs and use of toxic solvents.

5 References

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CAPÍTULO III

NON-ANTHOCYNIN PHENOLIC COMPOUNDS IN AÇAÍ PULP BY UHPLC-DAD (E*uterpe oleracea* Mart.): A MULTIVARIATE OPTIMIZATION

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Abstract

Açaí pulp (*Euterpe oleracea)* is a remarkable source of anthocyanins, however, non-anthocyanin phenolic compounds have also shown to play an important role for açaí extracts bioactivity. In viewing of the lack of simple and fast chromatographic methods for the characterization and quantification of this phytochemical fraction of açaí pulp, this study aimed to optimize and validate a chromatographic method using ultra high-performance liquid chromatograph coupled with diode array detector (UHPLC-DAD) for the determination of 8 phenolic acids and 4 flavonoids in açaí pulp. The optimization of a linear gradient elution has been carried out by using a multivariate approach (central composite design), with three independent variables: initial acetonitrile (ACN) concentration in the gradient elution. final ACN concentration at the end of the gradient and linear time of the gradient elution. Resolution between peaks was assessed as response. The optimum chromatographic conditions were initial and final ACN concentration: 7.8 to 15.7% in 8 minutes, with 4 min for column re-equilibration. The method was validated, showing to be suitable for its purpose. Moreover, the method was successfully applied for the analysis of non-anthocyanin compounds in 3 commercial açaí pulp samples, which had significant differences in the content of the quantified compounds. Therefore, it was possible to achieve an efficient and fast chromatographic method, which will result, in medium term, in reduced costs of analysis and lower generation of toxic wastes.

Key words: central composite design, Derringer and Suich, optimization, UHPLC

1 Introduction

Açaí (*Euterpe Oleracea* Mart.*)* is a large palm tree growing abundantly on floodplains in the Amazon estuary (Schauss. 2010). Açaí fruits undergo mechanical pulping along with water to yield açaí pulp, which is widely consumed in the North region of Brazil. In the last years, it has become largely recognized across the country and overseas as one of the new ‘‘superfruits’’ due to i*n vivo* and *in vitro* studies reporting diverse health effects of açaí pulp extracts. It has been shown to possess high antioxidant capacity *in vitro* and *in vivo*, anti-inflammatory, anti-proliferative, anticancer, hypocholesterolemic, artheroprotective, hypoglicemic, anti-obesity capacity, among others properties (Oliveira et al., 2010, Noratto et al., 2011, Hogan et al., 2010, Udani, Singh, Sing and Barrett, 2011, Kang et al., 2012, Sousa et al., 2012, Fragoso et al., 2013. Kazumy et al., 2015).

These health effects have been linked to the high content of phenolic compounds in the açaí pulp, which is a remarkable source of anthocyanins (cyanidin 3-glucoside and cyanidin 3-rutinoside), substances responsible for its violet colour (Pacheco-Palencia et al., 2009, Dias et al., 2012). The açaí pulp has also been pointed as a source of non-anthocyanin phenolic compounds (Gordon et al., 2012. Kang et al., 2011), however, this fraction has received less attention, although its importance for açaí extracts bioactivity has been already demonstrated (Kang et al., 2010).

The characterization and quantification of the non-anthocyanin fraction of açaí pulp has predominantly been carried out by high-performance liquid chromatography (HPLC), a technique presenting several advantages, such as high robustness, reproducibility and sensitivity (Pyrzynska, Sentkowska, 2015). Nonetheless, as main drawbacks, this technique is time-consuming for complex samples, requires the use of large volumes of toxic solvents and greatly generates toxic wastes, demanding high costs for treatment and elimination of residues produced (Kalili, Viliers, 2011). In order to overcome such disadvantages, ultra-high performance liquid chromatography (UHPLC) has been developed aiming at essentially benefit from the separation principles ruling HPLC, but by diminishing columns particle size, improving its performance. As a result, shorter columns can be employed to perform separation, yielding a lower time of analysis and smaller volumes of organic solvents (Kalili, Viliers, 2011, Dias et al., 2012).

However, regardless the used instrumentation, the optimization of chromatographic methods is time-consuming. In this context, the use of multivariate statistical tools allows, with a reduced number of experiments, the determination of chromatographic conditions with

optimised performances parameters, such as the resolution between peaks, symmetry, width, height, and the reduction of time of analysis and costs, in addition to decreasing the use of toxic solvents (Ballus, Meinhart, Bruns & Godoy, 2011). Yet, in the process of optimising a chromatographic method for many compounds, it is necessary to observe a high number of responses, for which it is very unlikely that the optimum region for all of them will be the same. In this case, the methodology of simultaneous optimisation proposed by Derringer and Suich (1980) becomes a valuable statistical tool. With the use of mathematical models it allows the optimisation of a great number of responses by combining individual desirability of each response in a global desirability (Ballus, Meinhart, Bruns & Godoy, 2011).

UHPLC has been barely used for the characterization and quantification of non-anthocyanin compounds from açaí pulp. Prior studies on this approach have either utilized complex and high cost equipment, or have not considered non-anthocyanin phenolic compounds of great importance in the açaí pulp composition (Dias et al., 2013, Bataglion et al., 2015). Therefore, taking into account the lack of a simple and fast chromatographic method for the determination of non-anthocyanin phenolic compounds in this food matrix, we considered worth the optimization and validation of a chromatographic method by UHPLC-coupled to diode array detector (DAD) to determine phenolic acids and flavonoids in freeze-dried açaí pulp (Figure 1). Multivariate statistical tools were used to achieve optimization and the optimized method was applied to determine phenolic compounds in three commercial samples of açaí pulp.

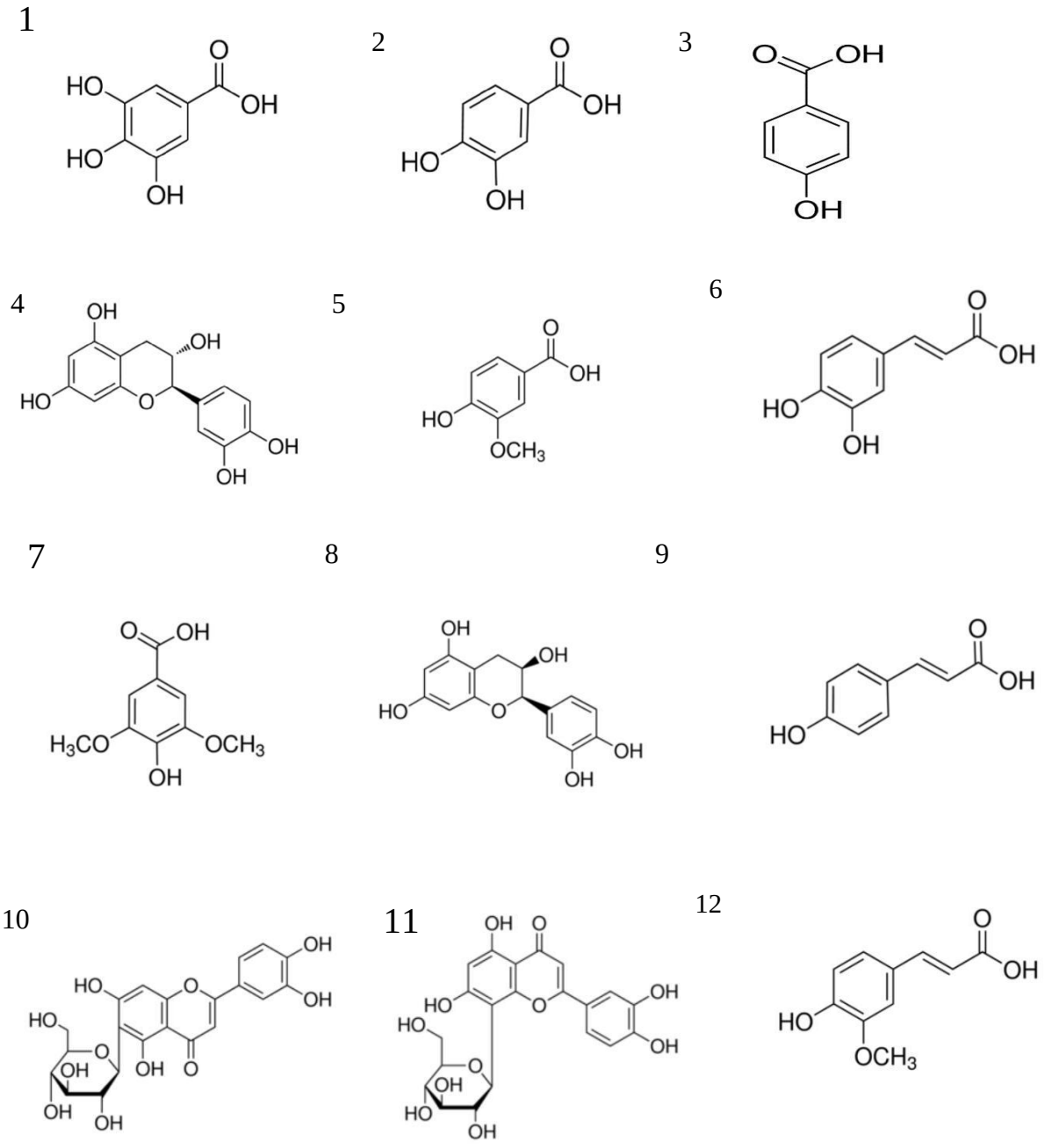


Figure 1. Chemical structure of the phenolic compounds considered in the chromatographic method: 1) gallic acid; 2) 3.4-dyhydroxibenzoic acid; 3) 4-hydroxibenzoic acid; 4) (+)-catequin; 5) vanillic acid; 6) caffeic acid; 7) syringic acid; 8) (-)-epicatechin; 9) *p*-coumaric acid; 10) isoorientin; 11) orientin; 12) ferulic acid.

2 Material and methods

2.1 Chemicals and samples

Acetonitrile and formic acid HPLC grade (98%) were acquired from J.T Baker (Germany). Methanol, acetone, hydrochloric acid were purchased from Synth (SP, Brazil). The phenolic standards (gallic acid 99.3%, 3.4-hydroxybenzoic acid 99%, 4-hydroxybenzoic acid 99%, (+)-catechin 98%, vanillic acid 98%, caffeic acid 98.5%, syringic acid 95%, (-)-epicatechin 98%, *p*-coumaric acid 98%, orientin 98%, isoorientin 98% and ferulic acid 99%) were from Sigma and the water was purified using a Milli-Q (Millipore, São Paulo, Brazil). The standard stock solutions were prepared in methanol at a concentration of 1 mg.mL-¹ and kept at -18 ºC for a maximum of 1 month.

Açaí pulp (1kg) was purchased from local markets in Belém (Pará, Brazil), freeze-dried for 96 h and used for analysis.

2.2 Instrumentation

The separation of the 12 studied phenolic compounds was carried using an UPLC Waters Acquity coupled to a diode array detector, binary pump system, automatic injector and heater for temperature control. The compounds were separated on a Kinetex C18 column (100 mm 2.10 mm x 1.7 µM) (Phenomenex, Torrance, CA, US). Detection was performed at 254 and 280 nm (hydroxybezoic acids derivatives), at 325 nm (for hydroxycinnamic acid derivatives) and 350 nm (for flavonoids). The initial and final concentrations of acetonitrile in the mobile phase and the time of linear gradient (time spent between the initial and final concentration of acetonitrile, in a linear progression rate) were optimised. Data analysis was performed using Empower software (Waters).

2.3 Multivariate optimisation and data treatment

The effect of the following parameters was investigated through a 2^3 central composited design with central and axial points: initial concentration of acetonitrile (0% to 12%); final concentration of acetonitrile (20% to 30%); and linear gradient time between both

concentrations, from 5 to 10 min. These values were limited based on preliminary tests performed to determine the more suitable solvent range to promote the elution of all the studied phenolic compounds.

The assays were performed using an extract containing the non-phenolic fraction of açaí added to the standards of phenolic compounds at a concentration of 5 mg L^{-1}. The purpose of this procedure was to verify that the optimum experimental condition obtained for the separation of the compounds would not be affected by the matrix effect, which could hinder the application of the method in real samples (Silveira et al., 2016). Four repetitions were performed at the central point, totalizing 18 experiments. Before the analysis of each of the multivariate design conditions, the chromatographic column was conditioned for 10 min with the initial composition of the mobile phase of its respective experimental level. The tests were performed by injecting 10 µL of sample and the mobile phase was composed of pure acetonitrile and an aqueous solution of formic acid 0.1% (v/v). Based on pressure limits and previous tests, the flow rate was maintained at 0.3 mL min^{-1} and the temperature of the column at 30º C. The chromatographic criteria chosen for optimization was the resolution (R) (Equation 1) between the following pair of peaks, which showed difficult separation: 4-hydroxybenzoic acid/catequin, vanillic acid/caffeic acid, syringic acid/epicatechin, *p*-coumaric acid/isoorientin, and the isomers orientin/isoorientin. The mathematical models were validated by analysis of variance (ANOVA) using Design Expert 6.0 software (Stat-Ease, Minneapolis, USA) at 95% reliability. The validated models were used to determine the optimum conditions through the technique of simultaneous response optimisation by Derringer and Suich (1980). Hence, desired values were set for each individual response and combined to establish a global desirability.

$$= \frac{2(\ 2 - \ 1)}{_{2+1}} \qquad \text{(Equation 1)}$$

where t1 and t2 represent retention time of adjacent peaks, and w1 e w2 the width of the peaks.

The individual desirability was defined to maximise the resolution between the pair of peaks, according to the Equations 2, 3 and 4. The lowest resolution found in the experimental tests for each pair of peaks was considered as R_{min} and the highest resolution obtained experimentally was taken as R_{max}. Furthermore, time of analysis was set to be minimal.

$= 0 \quad < \quad$ (Equation 2)

$= \left(\quad \frac{\quad - \quad}{\quad} \right), \leq \leq$ (Equation 3)

$= 1 \quad \geq \quad$ (Equation 4)

Where di corresponds to desirability i between 0 and 1, Ri are the predicted values by the models for resolution, R_{max} are the maximum values for resolution and R_{imin} the minimal ones.

2.4 Evaluation of the figures of merit of the chromatographic method

Figures of merit of the method was evaluated according to the recommendations of the Harmonised Guide and the National Health Surveillance Agency (Agência Nacional de Vigilância Sanitária – ANVISA) (BRASIL, 2003, IUPAC. 2002). The limits of detection were estimated by the successive dilution of the standard solutions as 3 times the signal/noise ratio, and the limits of quantification were fixed as 2 times the concentration of the limit of detection. Analytical curves, including eight concentration levels for each studied compound, were randomly created at different equidistant concentration ranges, in triplicate. The linearity of the analytical curves was evaluated and the models were validated by analysis of variance (ANOVA) with the respective verification of the adjustments. The method's precision was assessed by injecting a standard solution containing the 12 compounds at concentrations comprising the linear interval of the method, at three different concentrations for each compound, including the limit of quantification, the centre of the analytical curve and its maximum concentration. To evaluate the intra-day precision, 10 determinations were performed in the same day (n = 10). The inter-day precision was evaluated by five determinations at the same concentration levels for the repeatability, on three different days (n = 3).

2.5 Application in samples

The optimized method was used to determine non-anthocyanin phenolic compounds in samples of 3 commercial brands of açaí pulp, purchased at local markets in Campinas, São Paulo. Two packs (100 g each) from the same batch of frozen pulp were acquired for each sample, which were freeze-dried. Extractions were performed as established in the Chapter II. Half a gram of freeze-dried açaí-pulp was mixed with 27 mL of water:acetone:methanol (45:31:24 v/v) acidified with 0.1% HCl, vortexed for 30 seconds and sonicated for 6 min. Following, the samples were centrifuged for 15 minutes at 4ºC and 3000 g and the supernatant was removed. The residue was extracted once more under the same conditions and the extracts were gathered. To separate the non-anthocyanic phenolic compounds from the anthocyanins, the aqueous phase remaining was partitionated with ethyl-acetate (4 times), according to Pacheco-Palencia, Duncan, Talcott, 2009 and Gordon et al., 2012). The solvent fraction was eliminated by rota-evaporation (10 minutes at 35ºC) and the ethyl-acetate was rota-evaporated until dryness and re-suspended in 2 mL of water:acetonitrile (92.2:7.8 v/v), filtered using membrane PVDF 0.22 µM porosity and injected into the UPLC.

Retention time comparison, sample spiking with genuine standards and evaluation of the absorbance spectra of the peaks in standards and samples were used to confirm the identity of the compounds studied.

2.6 Statistical treatment of the data

The phenolic content in the commercial açaí pulp samples was compared by analysis of variance (ANOVA) and Tukey's test using Statistica 6.0 (Statsoft Inc., 2001). The samples were considered to be significantly different when $p < 0.05$.

3 Results and discussion

3.1 Multivariate optimization

Table 1 displays the results of peak resolution obtained in each experimental condition for the pairs of compounds showing difficult separation: (4-

hydroxybenzoic/catequin), (coumaric/orientin), (vanillic/caffeic), (syrigic/epicatechin) and (isoorientin/orientin). Linear and quadratic models were created for each of the responses. The simplest model that presented proper adjustment was used to perform the prediction of the optimum condition by Derringer and Suich. Table 2 shows the significant coefficients in each model and their respective ANOVA results, as well as the adjustment test.

All the responses presented highly significant regression ($F_{calc}>F_{critical}$) with the exception of isoorientin/orientin resolution, for which the variables within the range studied have not exerted a significant influence. However, there was an evidence for lack of fit of all the models ($p > 0.05$). If the model fails to fit, it may not be suitable to perform prediction within the range studied. Nonetheless, the ANOVA indicated that the quadratic mean caused by the pure error value (MSpe) was very low for all the responses (0.010 for 4-hydroxy/catequin, 0.000056 for vanillic/caffeic, 0.0014 for syringic/epicatechin, 0.0012 for coumaric/orientin). As a result, the F value of the models calculated by the ratio MSlof/MSpe (quadratic mean caused by the pure error divided by the quadratic mean caused by the lack of fit) could have been overestimated, resulting in lack of fit.

This can be explained by the fact that the pure error was calculated from the results of genuine injection replicates performed at the central point of the design (4 authentic repetitions). Since the chromatographic system was quite stable (coefficient of variation for retention time and peak width less than 0.5%), the analytical error resulted greatly low, not necessarily corresponding to the actual analytical error of the design, which could have been obtained only if it had been performed entirely using genuine replicates. Therefore, the low pure error values observed may have been calculated from underestimated values of the analytical error, indicating that the lack of fit should be observed with caution. Indeed, the diagnostics residuals vs. predicted value graph for the responses did not present evidence that the residual behaviour was not normal or suffered from heteroscedasticity (Figure 2). Moreover, predict vs. actual values graph showed good agreement between the values (Figure 2).

Table 1. Levels of the studied variables and experimental responses.

Experiment	Initial concentration of acetonitrile in the gradient (%)	Time (min)	Final concentration of acetonitrile in the gradient (%)	**Responses for resolution**				
				4-hydroxy/ catechin	Vanillic/ caffeic	Syringic/e picat	Coumaric/ isoorientin	Isoorientin/ orientin
1	(-1)2.4	(-1)6	(-1)17.0	1.73	0.90	1.25	0.00	0.95
2	(1)9.6	(-1)6	(-1)17.0	0.38	0.66	0.72	0.73	1.15
3	(-1)2.4	(1)8.98	(-1)17.0	3.74	1.24	2.23	2.56	0.87
4	(1)9.6	(1)8.98	(-1)17.0	0.00	0.73	1.04	1.84	1.08
5	(-1)2.4	(-1)6	(1)22.98	1.24	0.73	0.66	1.22	1.32
6	(1)9.6	(-1)6	(1)22.98	0.77	0.50	0.00	1.49	1.37
7	(-1)2.4	(1)8.98	(1)22.98	2.25	0.93	1.34	0.78	1.12
8	(1)9.6	(1)8.98	(1)22.98	0.45	0.66	0.58	0.00	0.82
9	(0)6	(0)7.5	(0)20	1.26	0.88	1.27	1.05	1.11
10	(0)6	(0)7.5	(0)20	1.15	0.84	1.29	1.02	1.15
11	(0)6	(0)7.5	(0)20	1.31	0.90	1.35	1.07	1.11
12	(0)6	(0)7.5	(0)20	1.09	0.87	1.27	1.06	1.12
13	(1.68)12	(0)7.5	(0)20	1.24	0.00	0.47	1.30	1.54
14	(0)6	(1.68)10	(0)20	1.60	0.89	1.78	2.53	0.83
15	(0)6	(0)7.5	(1.68)25	0.82	0.76	0.84	0.55	0.55
16	(-1.68)0	(0)7.5	(0)20	2.64	1.03	1.33	0.00	0.89
17	(0)6	(-1.68) 5	(0)20	0.58	0.74	0.73	0.87	0.87
18	(0)6	(0)7.5	(-1.68) 15	2.03	0.98	2.22	0.80	0.89

Table 2. Statistical model coefficients and F-distribution parameters for model validation.

Responses	**Indicated Model**	**Intercept**	**Coefficients**										
			A	B	C	A^2	B^2	C^2	AB	AC	BC	Regression significance ($p<0.05$)	Model fit ($p>0.05$)
4-hydroxy/ catechin	2fl	1.35	-0.71	0.30	-	-	-	-	-0.46	0.35	-	0.0006	0.0116
Vanillic/caffeic	Quadratic	0.87	-0.22	0.075	0.079	-0.11	-	-	-	-	-	0.003	0.0066
Syring/epicat	Quadratic	1.31	-0.34	0.32	-0.37	-0.19	-	-	-	-	-	0.0004	0.0053
Coum/isoo	Quadratic	1.04	-	0.33	-	-	0.25	-	-0.31	-	0.064	0.0054	0.0001
Isoo/ori	Quadratic	1.11	-	-0.071								0.28	0.0006

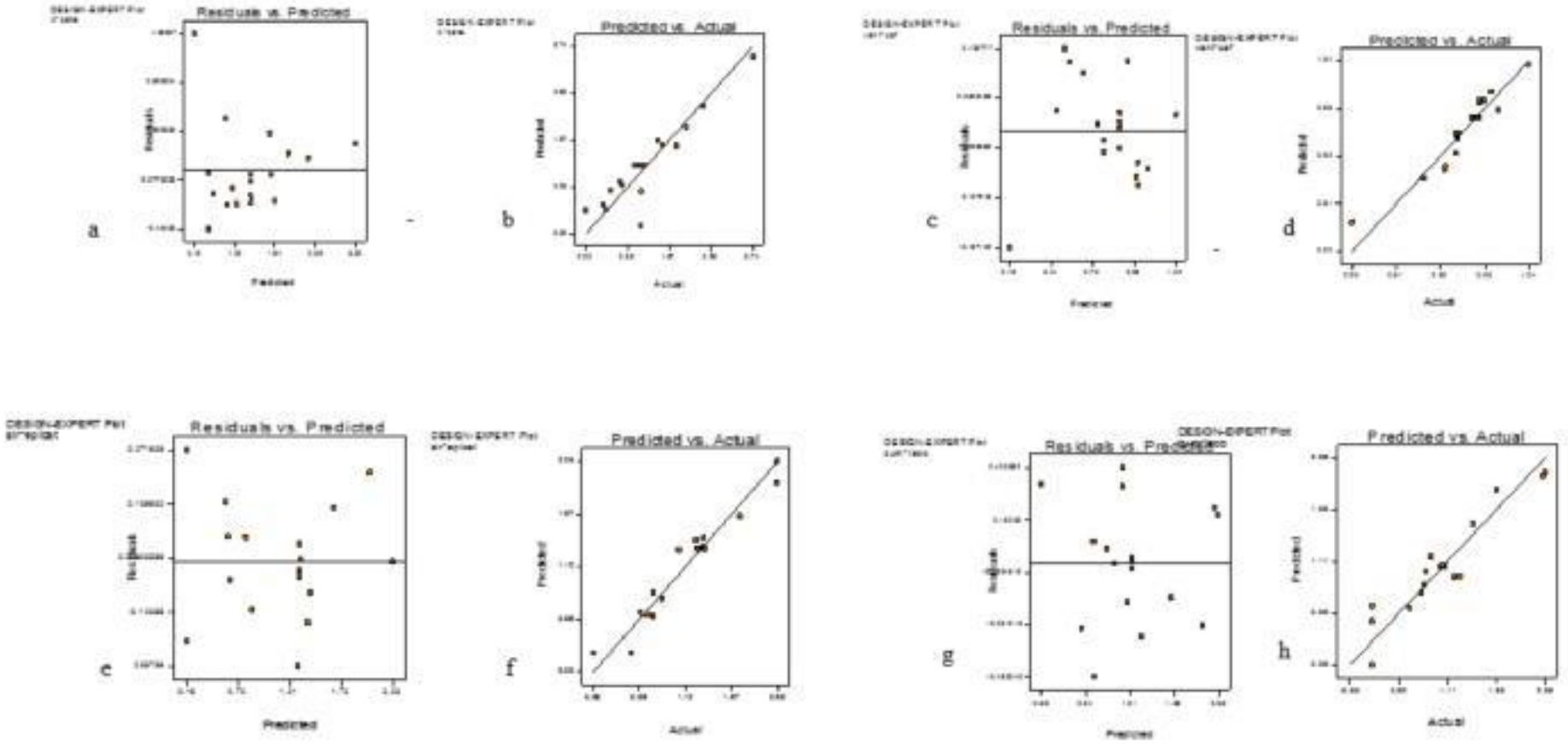


Figure 2. Statistic diagnosis for responses. Graphic of residues and predicted vs. experimental values for: a), b) 4-hydroxi/catequin, c),d) vanillic/caffeic, e), f) syringic/epicatechin, g), h) *p*-coumaric/isoorientin.

Finally, since it did not adversely affect the search for the optimum conditions and there was a good concordance between predicted and actual values, the generated models were further used to perform the prediction using Derringer and Suich. Ballus et al (2011) and Coutinho et al (2015) have successfully used experimental models suffering from lack of fit but with similar statistical diagnosis as those of the present study, and did not have the search for the optimum conditions adversely affected.

We observed that a linear increase of the initial acetonitrile (ACN) percentage (coefficient of regression A) was the most important parameter for the resolution between 4-hydroxybenzoic acid and catechin, showing a negative effect on their separation, as a linear increase in this parameter caused a reduction in the resolution. Both linear and quadratic increase in the initial ACN concentration was deleterious for the separation of vanillic/caffeic and syringic/epicatechin. Thus, the use of lower ACN concentrations (-1, -1.68) is indicated for a higher separation efficiency of these three pair of peaks. Silveira et al (2016) reported similar behaviour for the separation of phenolic acids from aqueous extracts of yerba mate. The authors hypothesized that by beginning with a low chromatographic strength (low ACN percentage) causes the compounds to be longer to leave the column, allowing a higher interaction between the analyte and the stationary phase.

The linear coefficient of the final ACN percentage (C) showed to be significant for the separation between vanillic acid and caffeic acid, and syringic acid and epicatechin, which were positively and negatively affected by raising this parameter, respectively. Therefore, to achieve higher resolutions for vanillic/caffeic, final ACN concentration should be elevated, whereas the opposite is true for syringic/epicatechin. Moreover, there was a positive interaction effect between A and C for the resolution 4-hydroxy/catechin, indicating that the simultaneous increase or decrease of these parameters have enhanced the resolution.

Time (B) of linear gradient stood out as an important variable, being significant for all the studied responses. The positive effect showed that the higher the time, the higher the peak resolution, probably because an increase in time enabled a higher interaction between the compounds and the column (Silveira et al., 2016). The resolution 4-hydroxy/ catechin was negatively affected by the interaction effect between A and B, whereas coum/isoo was affected by both AB and BC interaction, in a negative and positive manner, respectively.

The negative interaction indicated that in order to improve separation, a decrease in the initial percentage of ACN should be accompanied by an elevation in the time of linear

gradient, whereas the negative one suggests that the separation is improved by simultaneously elevating the time of linear gradient and the final ACN concentration.

The algorithm by Derringer and Suich was used to simultaneously optimize the separation of the pair of peaks. Various simulations were carried out using the software, from which several conditions were chosen to test experimentally. The best condition feasible to separate the 12 compounds studied was: initial acetonitrile concentration of 7.8%, which increased up to 15.7% in 8 min. Within this time, all the compounds were successfully eluted from the column. Following, the acetonitrile goes up to 50% in 1 minute and then returns to the initial condition. Re-equilibration time was 4 min. The desirability criteria, the values of resolution predicted by the models and the experimental results are shown in Table 3. There was a good concordance between actual and predicted values.

Hence, with 18 experiments, it was possible to establish the chromatographic conditions for separation of 12 phenolic compounds in açaí pulp, confirming that multivariate statistical tools are highly useful for optimization of chromatographic systems with a reduced time of the analysis, costs, use of organic solvent and generation of toxic residues. Figure 3 depicts a chromatogram of a mix solution containing all the studied standards.

Table 3. Desirability conditions employed for the simultaneous optimization of phenolic compounds resolutions and simmetry. Predicted responses by the models and values experimentally observed.

Variable/Response	Goal	Inferior limit	Superior limit	Importance	Predicted	Observed
% Initial ACN	Is equal to 0.50	-1.68	1.68	3		-
Time	Minimize	-1.68	1.68	5	-	-
%Final CAN	Is in range	-1.68	1.68	3	-	-
4-hydroxy/catechin	Maximize	0	3.73	3	1.15	0.98
Vani/caf	Maximize	0	1.24	3	0.87	0.86
Sir/epicat	Maximize	0	2.23	3	1.73	1.65
Cum/isoo	Maximize	0	2.55	3	1.52	1.6

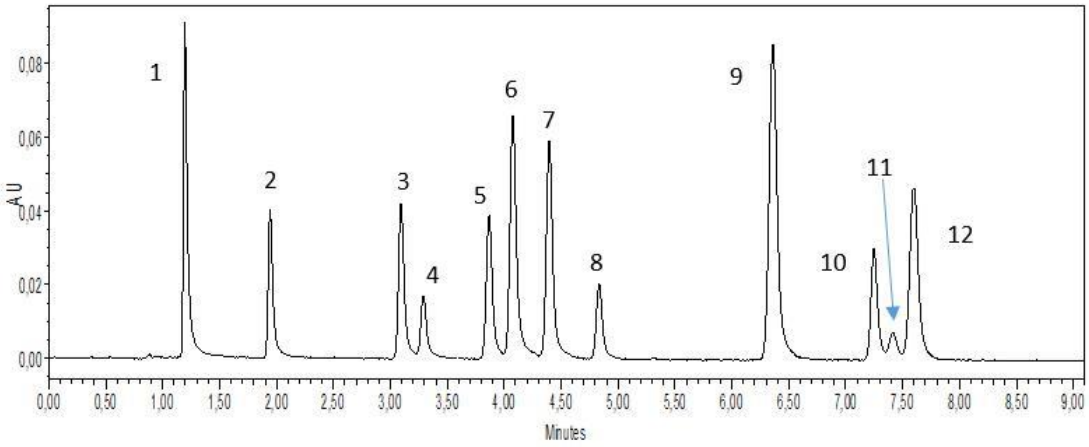


Figure 3. Mix of phenolic compounds standards at the optimum conditions at 280 nm (8 min linear gradien). 1) gallic acid, 2) 3.4-dihydroxibenzoic acid, 3) 4-hydroxibenzoic acid, 4) catechin, 5) vanillic acid, 6) caffeic acid, 7) syringic acid, 8) epicatechin, 9) *p*-coumaric acid, 10) isoorientin, 11) orientin, 12) ferulic acid.

3.2. Figures of merit of the chromatographic method

Figures of merit for the linearity, precision and limits of detection and quantification are depicted in Table 4. The validation was carried out to fit the compounds in their maximum absorption wavelength. LOD varied between 0.001 mg.L-¹ (caffeic acid and 4-hydroxibenzoic acid) and 0.017 mg.L-¹ (epicatechin), whereas LOQ oscillated between 0.002 mg.L-¹ and 0.034 mg.L-¹ for the same compounds. These values are higher than those found for Bataglion et al. (2015) and Dias et al (2013) for non-anthocyanin compounds of açaí, who reported values between 10 times and 400 lower for LOD and up to 255 lower for LOQ. However, these authors used mass spectrometry as a detection system, which is quite more specific and sensible than the detector used herein. On the other hand, Natividade et al., (2013) and Irackli et al., (2012), who used HPLC-DAD for phenolic compounds analysis in wine and cereals, respectively, found higher or similar values as those described in this study.

Linearity ranges comprised the LOQ of the compounds and all linear regressions had R^2 values above 0.99. However, despite the current widespread use of this coefficient as an indication of quality of fit, it is misleading and inappropriate as a test for linearity and should not be used alone (IUPAC, 2002, Natividade et al., 2013). Therefore, the analysis of variance (ANOVA) was applied to the models and results showed that there was a significant linear

regression in the studied concentration ranges, with no evidence for lack of fit ($p > 0.05$), proving that the models are appropriate for quantifications.

Intra-day precision (n=10) and inter-day precision (n=3), studied in three levels including the limit of quantification, exhibited suitable coefficients of variance values (less than 20%). Therefore, results indicated that the chromatographic method was in accordance with validation requirements (BRASIL, 2003; IUPAC, 2002), proving to be adequate for the characterization and quantification of non-anthocyanin phenolic compounds in açaí pulp.

Table 4. Figures of merit for method validation.

Compounds	LOD[a]	LOQ[b]	Linearity			Precision[c]		
			Linear range	R^2	Model Fit	Concentration	Intra-day=10	Inter-day n=3
	($mg.L^{-1}$)	($mg.L^{-1}$)	($mg.L^{-1}$)		(p>0.05)	($mg.L^{-1}$)	(%)	(%)
Galic acid	0.003	0.006	0.006-5.3	0.9979	0.71	0.006	20.00	16.63
						3.21	2.96	0.44
						5.35	2.71	0.63
3.4-dyH acid	0.002	0.004	0.4-2.5	0.9982	0.67	0.004	7.96	6.27
						1.28	2.02	0.65
						2.14	2.07	0.90
4-hydroxiB	0.001	0.002	0.002-3.2	0.9988	0.12	0.002	18.94	23.11
						1.43	2.35	0.86
						2.39	2.37	1.15
Catechin	0.007	0.015	0.015-6.4	0.9984	0.70	0.015	19.31	1.98
						3.21	3.41	1.83
						5.35	0.56	0.90
Vanillic acid	0.007	0.014	0.014-7.2	0.9990	0.53	0.014	4.83	2.83
						3.24	2.31	0.99
						5.40	1.85	0.90
Caffeic acid	0.001	0.002	0.002-7.2	0.9985	0.08	0.002	17.58	1.18
						3.24	2.20	0.69
						5.40	2.68	1.23
Syringic acid	0.003	0.006	0.06-2.1	0.9978	0.17	0.006	9.93	4.81
						1.27	3.30	0.46
						2.12	2.57	1.15
Epicatechin	0.017	0.034	0.034-6.9	0.9973	0.41	0.034	15.65	6.19
						3.49	3.01	1.29
						5.82	3.89	1.08
p-coumaric acid	0.003	0.007	0.007-7.0	0.9989	0.076	0.007	5.16	1.90
						3.15	2.45	0.67
						5.25	2.28	0.96
Isoorientin	0.007	0.014	1.1-7.5	0.9982	0.25	0.014	15.19	2.88
						3.40	3.42	0.48
						5.67	3.19	1.09
Orientin	0.07	0.014	1-6.7	0.9984	0.43	0.014	11.18	3.12
						3.00	3.19	1.08
						5.00	2.78	0.68
Ferulic acid	0.003	0.006	0.006-2.1	0.9977	0.91	0.006	6.49	1.54
						1.26	2.35	2.31
						2.1	1.84	1.69

a: Limit of detection, b: limit of quantification, c: expressed as coefficient of variation (CV)

3.3 Application in samples

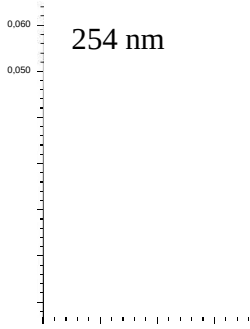
Table 5 shows the concentration of the non-anthocyanin compounds found in the freeze-dried commercial samples of açaí pulp. Gallic acid, (+)-catequin and (-)-epicatechin have not been detected in none of the samples. The phenolic profile is in agreement with previous works on açaí pulp (Pacheco-Palência et al., 2009, Garzón et al., 2016). Vanillic acid was the major compound found in the samples (between 33.65 µg.g-¹ and 49.65 µg.g-¹) and *p*-coumaric acid was the minor compound (ranging from 4.99 µg.g-¹ to 6.68 µg.g-¹).

There was a significant difference among the commercial samples with regard the content of 3.4-dihydroxy benzoic acid, 4-dihydroxy benzoic acid, vanillic acid and isoorientin content. This may be due to differences such as ripening stage, climate, growing conditions and industrial processing features of the pulp (Gordon et al., 2012, Lichtenthaler et al., 2005). Moreover, solid contents in açaí pulp may vary significantly, a fact that might greatly affect its chemical composition (Carvalho et al., 2016). The values found in the present study are in agreement with Gordon et al. (2012) and Carvalho et al (2016), who found vanillic acid content fluctuating between 30 and 69 µg.g-¹ and 4-hydroxybenzoic acid between 9 and 19 µg.g-¹. On the other hand, both authors had lower contents of 3.4-dyhydroxibenzoic acid (6.5 to 9.7 µg.g-¹), caffeic acid (0.2 to 1.8 µg.g-¹), syringic (11 µg.g-¹), *p*-coumaric acid (3.0 µg.g-¹) and ferulic acid (7.00 µg.g-¹). Figure 4 shows typical chromatograms of açaí pulp samples at all the monitored wavelength.

Table 5. Phenolic compounds in freeze-dried samples of açaí pulp (µg.g-¹ dry weight).

Compound	Samples		
	A	**B**	**C**
3.4 dihydroxy benzoic acid	46.09±5.46 [a]	25.40± 5.26[b]	4.87± 0.86[c]
4-dihydroxy benzoic acid	18.97± 1.9 [a]	19.41±3.6 [a]	12.10±0.82[b]
Vanillic acid	49.65±5.40[b]	44.29±8.99 [ab]	33.65±2.04 [a]
Caffeic acid	6.86±1.10 [a]	8.96±1.81[a]	9.04±1.48 [a]
Syringic acid	26.21±2.83 [a]	19.78± 3.83[a]	25.49±2.33 [a]
p-coumaric acid	4.99±0.40 [a]	5.47±1.80 [a]	6.68±0.54 [a]
Isoorientin	13.52±1.84b	24.29±2.98b	36.73±6.71a
orientin	13.74±1.85a	17.37±2.76a	20.99±4.05a
Ferulic acid	15.00±1.52a	14.78±3.06a	16.12±1.85a

Equal letters in the same line indicate no significant difference between samples.


0,060
0,050
254 nm

4 Conclusion

This work succeeded in optimizing a chromatographic method for the separation of non-anthocyanin phenolic compounds in freeze-dried açaí pulp by UHPLC-DAD. The multivariate statistical techniques employed proved to be efficient tools because they promoted the maximisation of the resolution of the peaks of interest. The obtained mathematical methods were useful to describe the modifications in the resolution of the studied compounds that were caused by changes in the investigated variables. The optimum separation condition was an initial acetonitrile concentration of 7.8%, a final concentration of 15.7% and a linear gradient time of 8 minutes. The validation study presented satisfactory results, with the conclusion that the method is adequate for the determination of the 12 phenolic compounds studied in açaí pulp. The method was successfully applied for the analysis of non-anthocyanin compounds in three commercial açaí pulp samples and 9 out of 12 phenolic compounds have been detected and quantified. Therefore, by using ultra-high performance chromatograph coupled to a diode array detector, it was possible to achieve an efficient and short method, which will result, in medium term, in reduced costs of analysis and lower generation of toxic wastes.

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CAPÍTULO IV

ANTHOCYANINS, NON-ANTHOCYANINS, TOCOPHEROLS AND ANTIOXIDANT CAPACITY OF AÇAÍ PULP (*Euterpe oleracea)* AS AFFECTED BY HIGH PRESSURE PROCESSING AND THERMAL PASTEURIZATION

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Manuscrito em preparo para ser submetido ao periódico Food Chemistry

Abstract

This study aimed to investigate the effect of high pressure processing (HPP) (400, 450, 500, 600 MPa/5 min/20ºC) on the contents of anthocyanins, non-anthocyanins compounds (NAPC), tocopherols, antioxidant capacity (AC) against reactive oxygen species (ROS) and inhibition capacity against nitroso compounds (NOCs) formation of açaí pulp. The results were compared to samples subjected to thermal pasteurization (TP) (85ºC/1 min) and freezing only (control). The control samples showed higher contents of anthocyanins (total and individual) than the HPP and TP ones. TP yielded up to 40% losses of C3G and C3R over 20% of HPP. With regard NAPC, there was no significant difference between TP and control, however, there was a significant increment in their measurable content as pressure increased (up to 69% in comparison with the control). Globally, no influence of the preservation method was found on the tocopherol contents and vitamin E activity of the samples. Moreover, total phenolic compounds in HPP did not differ from control but it was significantly higher than TP. HPP samples had higher scavenging capacity against peroxyl radical than control and TP (up to 30%). Furthermore, both HPP and TP samples showed lower scavenging capacity against HOCl compared to the control. As for H_2O_2, globally, no significant difference has been found between the treatments. With regard the inhibition of nitroso compounds formation, control showed lower inhibition capacity than TP and no difference from HPP samples. Overall, processing at 500 MPa was the best condition found. Therefore, HPP is capable of providing açaí pulps with high functional and nutritional quality in comparison to untreated and thermal pasteurized samples.

Key-words: process effect, bioactive compounds, reactive oxygen species, total apparent nitroso compounds.

1 Introduction

Açaí pulp is defined as the product obtained from the edible parts of the fruits of the *Euterpe oleracea* palm tree by using proper technological procedures (MAPA 2000). It is an important source of nutrition for vast populations inhabiting the Amazon and, in the last years, it has been entitled as a functional food due to various bioactivities shown in *in vitro* and *in vivo* studies, such as anticancer, anti-proliferative and anti-inflamatory properties **(**Schauss et a., 2006a**,** Kang et al., 2011**,** Pacheco-Palência et al., 2009, Schauss, 2010, Fragoso et al., 2013**)**. Moreover, açaí has a higher antioxidant capacity than that of most of fruits known (Schauss et al., 2006a, Pompeu, Silva, Rogez, 2009). These properties have been related to the chemical composition of açaí pulp, which is high in anthocyanins, non-anthocyanin flavonoids, phenolic acids and vitamin E (Schauss et al., 2006b, Costa et al., 2010, Darnet et al., 2011, Dias et al., 2013). On the basis of such nutritional and health appeals, nowadays açaí pulp is consumed all over Brazil and in countries across the globe such as United States, Japan, Holland, Spain, Russia, Israel and Australia, where it is traded as frozen pulp (Menezes et al.. 2011). Annually, more than four thousand tons of açaí pulp are exported to these countries, yielding in 2014 US$ 22 millions, which represents an average increment of 37% compared to the previous two years (Conab, 2016).

Given this demand, açaí production has been growing at a rate of 10% a year, raising from 100 thousand tons to 216 thousand tons in the last decade (IBGE, 2015). Within the same period, production values from açaí commercialization increased 37% a year, achieving US$ 480 millions in 2015 over US$ 100 milions in 2006. Currently, açaí holds the status of more profitable Brazilian extractive vegetable, generating (IBGE, 2012). However, alongside the increasingly claim for açaí pulp, concerns on microbiological security of this product has also been focus of interest, since açaí fruits possess intrinsic characteristics highly favourable to microorganisms growth (Aguiar, Menezes, Rogez, 2013). Moreover, the absence of an exocarp for pulp protection combined with low hygienist practices for fruit harvesting and transporting make the açaí pulp a highly perishable product, so that using conservation methods is essential to reduce microbial load and increase shelf life (Aguiar, Menezes. Rogez, 2013). Currently, thermal pasteurization (TP) in association with chilling or freezing have been majorly used to achieve such goals.

Nevertheless, deleterious effects of heat on thermal-labile nutritional compounds, such as vitamins, have been reported (Sánchez-Moreno et al., 2006, Rawson et al., 2011, Barrett, Lloyd, 2011). Furthermore, claims for healthier foods have brought about for discussion non-

nutritional components capable of benefiting human health, which for being heat-sensitive may loose their bioactive properties due to the severe conditions occurring in the pasteurization process (Hoffman-Ribani et al., 2009, Vegara et al., 2013, Chen et al., 2012, Barrett, Lloyd. 2011). In this context, alternative technologies to thermal pasteurization have been developed such as pulse electric field, irradiation and high pressure processing (HPP), which have been a focus of growing interest **(**Balasubramanian, Farkas, 2008, Sanchéz-Moreno et al., 2011).

Among these non-thermal innovative techniques of elimination of micro-organisms, HPP is the most widespread and can provide food products with microbiological safety equivalent to that of thermal pasteurization, but with minimal effects on nutritional, functional and organoleptic properties. This is explained by the fact that the pressure applied is unable to affect covalent bonds and small molecules, so that products freshness characteristics are better retained (Sanchéz-Moreno et al., 2011, Tadapaneni et al., 2014).

Various studies have examined the effect of HPP on beverages, puree and fruit pulps, finding that compared to TP, HPP samples had a higher retention of vitamins (C, D, E), carotenoids and phenolic compounds (Sanchéz-Moreno et al., 2006, Patras et al., 2009**,** Barba, Esteve, Frigola, 2012, Tadapaneni et al., 2012, Terefe et al., 2013). Given that the açaí pulp has been increasingly valued due to its chemical composition, HPP may be an interesting alternative to thermal pasteurization to deliver a high quality product in terms of nutritional and bioactive contents. In this regard, Barba, Esteve, and Frígola (2012) reviewed the influence of HPP on the nutritional properties of different fluid foods, such as juices, purees/pastes based on fruits and vegetables. They concluded that although some general trends were observed, the effect of HPP depended on both the treatment intensity and the food matrix. Therefore, as novel processing techniques are developed, the effect of food processing must be separately studied in each food matrix. Moreover, it is of great interest examining whether it is able to surpass the traditional method used, in this case, thermal pasteurization.

HPP for preservation of açaí pulp has been considered by Menezes et al. (2008) who studied the effect of 300 MPa. 500 MPa, for 5 or 15 minutes, under 25ºC or 35ºC on peroxidase (POD) and polifenoloxidase (POF) of açaí pulp. The authors found that high pressure (300 MPa for 5 minutes at 35ºC) was effective to reduce POF activity up to 70%, but failed to achieve POD inactivation. Nevertheless, to the best of our knowledge, no studies on the effect of HPP on the chemical composition of açaí pulp have been performed. Therefore, this work aimed to study the effect of thermal pasteurization and HPP on anthocyanins and non-anthocyanins phenolic compounds, tocopherols and antioxidant capacity of açaí pulp, and compare both

treatments to control samples, which have not undergone other preservation method than freezing.

2 Material and Methods

2.1 Chemicals

Chromatographic grade formic acid was acquired from Merck (Darmstadt, Germany), acetonitrile and methanol from J.T Baker (Center Valley, PA, USA). Water was purified using a Milli-Q system (Millipore, Bedford, MA). Acetone P.A, methanol P.A, ethyl acetate P.A, NaClO, sodium phosphate were from Synth (Diadema, São Paulo, Brazil). HCl was from Ecibra (São Paulo, São Paulo, Brazil).

Phenolic compounds standards (4-hydroxybenzoic acid 98%, syringic acid 95%, 3.4-dihydroxybenzoic acid 98%, ferulic acid 99%, caffeic acid 98%, p-coumaric acid 98%, vanillic acid 98%, orientin 98% and isoorientin 98%) and tocopherols standards (α-tocopherol 97%, β-tocopherol 90%, δ-tocopherol 90% and γ-tocopherol 96%), potassium iodide, DHR, lucigenin, hydrogen peroxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Standards of cyaniding 3-glucoside (96%) and cyanidin 3-rutinoside (96%) were acquired from Extra-Syntense (GenayCedex, France). Phenolic compounds standard stock solutions were prepared by dissolving the appropriate amount of each compound in chromatographic grade methanol to a final concentration of 1 $mg.mL^{-1}$. Tocopherol stock solutions were prepared by dissolving the total amount in each jar in hexane. In turn, anthocyanins standards were dissolved in methanol acidfied with 0.2% hydrochloric acid (HCl). The solutions were filtered using a 0.22 m PTFE membrane (Milipore, São Paulo, Brazil) and were stored at -22 ºC, protected from light or up to one month.

2.2 Samples

A manufacturer located in Belém, Pará, Brazil provided ten kilograms of frozen, non-pasteurized açaí pulp 11.86% solids, packed in 1 kg polyethylene packs. The samples were transported under refrigeration (-20 ºC) to the University of Campinas. Pulps were maintained

under -80ºC until thermal or high pressure processing. Samples belonged to a unique batch and did not undergo any preservation treatment other than freezing.

2.3 Sample preparation and processing

2.3.1 Sample preparation

Five kilograms açaí pulp were thawed overnight at 4ºC. Following, pack contents were mixed using a blender (approximately 5 min) to obtain a homogeneous sample. Then, it was partitioned into three fractions: Control, promptly addressed to freezing (-80ºC) and freeze drying (96 h at – 48ºC), corresponding to the control sample; HPP, treated by high pressure processing; and TP, which was subjected to thermal pasteurization.

2.3.2 High pressure processing (HPP)

The açai pulp was transferred to proper bags for HHP (300 g each bag), vaccum-sealed and subjected to processing on an AVURE QFP 2L-700 (Avure Technologies®. USA) with a 2 L volume treatment chamber (inner vessel diameter 100 x 254 mm), maximum vessel pressure of 690 MPa (6900 bar/ 100,000 psi) and temperature control from 10 to 90 ºC. Pure demineralized water was used as the pressure-transmitting fluid. Two thermocouples located at the top and midway down the treatment chamber monitored the temperature of the pressure transmitting fluid. The average pressurization rate was 5 ± 0.40 MPa s^{-1}. Processing settings studied were 400 MPa, 450 MPa, 500 MPa, 600 MPa for 5 minutes at 20ºC. These conditions corresponded to the most utilized pasteurization condition in the literature and industry. Each assay was carried out in duplicate. Afterwards, the treated samples were immediately frozen under -80ºC, freeze-dried for 96 hours and stocked under -80ºC protected from light and oxygen for no longer than one month until analysis.

2.3.3 Thermal pasteurization (TP)

Thermal processing, performed to achieve sample pasteurization, was conducted based on Rogez (2000) and Sousa et al (2006). Twenty grams açaí pulp were placed in 30 mL sterilized screw-capped glass test tubes (15cm length and 2 cm internal diameter) and fixed in a thermostatic water bath at 90ºC. The temperature at the core of a control test tube (at 7.5 cm height) was monitored using a thermometer. When it reached 85ºC (5 minutes) time counting was triggered using a chronometer up to 1 min. Afterwards, pasteurization was interrupted by immersing the tubes in an ice water bath (4ºC) until 25ºC. Then. the samples were frozen and freeze-dried for 96 hours.

2.4 Phenolic compounds extraction

Anthocyanins and non-anthocyanins were extracted as described in Chapters 1 and 2, by combining 0.5 g sample with 27 mL acidified (0.1% HCl) water:acetone:methanol (45:31:24 v/v/v), vortex for 30 sec and sonicating for 6 min. Following, the samples were centrifuged (Harrier 18/80R MSB080.CR1.K. Loughborough. LE. UK) for 15 min at 3000 g and 4 ºC and the supernatant was taken. Extraction was performed once more under the same conditions, the supernatants were combined, the volume adjusted to 50 mL and the extracts were maintained for no longer than 24 h under -18ºC until analysis being performed.

2.5 Total monomeric anthocyanins (TMA)

Total monomeric anthocyanins (TMA) content was determined using the pH differential method described by Giusti and Wrolstad (2001). Measurements were recorded at 510 and 700 nm and the total monomeric anthocyanin (ATT) was calculated according to Equation 1.

$$ATT = \frac{(A \times MW \times DF \times 1000)}{\varepsilon \times 1} \qquad \text{(Equation 1)}$$

In which $A = (A_{520nm} - A_{700nm})_{pH1.0} - (A_{520nm} - A_{700nm})_{pH4}$, MW = 449.2 (molecular weight of cyanidin 3-glucoside), DF = dilution factor and ε = 26.900, the molar absorptivity

coefficient. Results were expressed as mg of cyanidin 3-glucoside equivalent per gram of freeze-dried pulp.

2.6 Chromatographic analysis

Anthocyanins were determined by High Performance Liquid Chromatography (HPLC) (Agilent 1260). Prior to chromatographic analysis, the solvent fraction was vacuum-evaporated (10 minutes at 35ºC) and the remaining aqueous phase was adjusted to 25 mL with 13.6:86.6 methanol: aqueous formic acid 0.1% v/v. Extracts were filtered using 0.22 µm membranes and injected into the HPCL equipped with quaternary pump, column heater, auto sampler and diode array detector. The gradient elution used was according to Carvalho et al. (2016), consisting of methanol (A) and an aqueous solution of formic acid 5% (B) in the following proportions: from 13.6% to 60% A in 15 minutes and 4 minutes for reconditioning of the column. A Zorbax Eclipse Plus C18 column (Agilent 100 mm x 4.6 mm x 3.5µm) was used for separation. The volume of injection was 30 µL, the temperature and the flow were maintained at 30ºC and 1 ml min^{-1}, respectively. Chromatograms were recorded at 520 nm and data acquisition was carried out using ChemStation software (Agilent). Anthocyanins identification was performed by comparing retention times and spectrum of the peaks of known standards with the peaks present in the samples. Quantification was performed by external calibration.

Figures of merit of the method was examined by studying limits of detection, limits of quantification, linear range, precision and recovery of the anthocyanins (*Supplemental material 1*), which indicated that the method was suitable to perform anthocyanins analysis in açaí pulp extracts.

As for non-anthocyanin phenolic compounds (NAPC), after solvent evaporation, aqueous extracts were partitioned with ethyl acetate (4 x 10 ml) for extraction of phenolic acids and flavonoids (Pacheco-Palencia et al., 2009; Gordon et al., 2012). Ethyl acetate was evaporated to dryness (35ºC), re-suspended in 2 mL 7.8:92.5 acetonitrile:water v/v, filtered using 0.22 µm membrane and injected into the chromatographic system. Extracts were analysed by Ultra-High-Performance Liquid Chromatography (UPLC Acquity, Waters, USA), according to the chromatographic method optmized in Chapter 2. The system was equipped with binary pump, automatic injector, column heater and diode array detector. Compounds separation was accomplished on a Kinetex C18 (100 mm 2.10 mm x 1.7 µm) (Phenomenex,

Torrance, CA, US). The mobile phase consisted of acetonitrile (A) and an aqueous solution of formic acid 0.1% (B). The gradient elution started with 7.8% A up to 15% in 8 minutes, then increasing to 50% in 1 minute, and returning to the initial condition in 1 minute. The column was left to reequilibrate for 4 minutes beteween injections. The flow was 0.3 mL.min-¹, the injection volume was 10 µL and the column temperature was maintained at 30ºC. Compounds identification was performed by comparing retention times and spectrum of the peaks of known standards with the peaks present in the samples. Quantification was performed by external calibration.

2.7 Tocopherols extraction and analysis

The lipid fraction was extracted from 1 g of freeze-dried açaí pulp using the methodology by Bligh and Dyer (1959), yielding in average 400 mg of açaí oil. Following, 40 mg of açaí oil was diluted in 1.5 mL hexane and injected into the HPLC system. Tocopherols were determined according to Pinheiro-Sant´Ana (2011), with modifications to fit açaí samples. Separation was perfomed on a Hypersil silica column (Thermo Scientific,150 mm x 4.5 mm x 5 uM), in an Agilent 1100 containing quaternary pump, automatic injector and column heater (30ºC). Data recording was performed using a fluorescence detector with excitation and emission wavelength at 290 and 390 nm, respectively. Isocratic elution was used and the mobile phase consisted of hexane:isopropanol:acetic acid 98.9:0.6:0.5 v/v/v for 12 minutes. The flow rate was 1.0 mL.min-¹ and the volume of injection was 25 µL. Figures of merit of the method were assessed (limits of quantification and detection, linearity and intra-assay and inter-assay precision) and the results are described in the *Supplemental Material 2*. Results were expressed in µg.g-¹ oil, and vitamin E activity in terms of RRR-α-tocopherol equivalents (α-TE.g-¹) (National Research Council, 1989) according to Equation 2:

Vitamin E acitivity = α-tocopherol (µg) x 1.0 + β-tocopherol x 0.5 + γ-tocopherol x 0.1 + δ-tocopherol x 0.03 (Equation 2)

2.8 Antioxidant capacity

2.8.1 Total phenolic compounds by Folin Ciocalteu

Total phenolic compounds (TPC) as measured by the Folin-Ciocalteu assay gives the reducing properties of extracts. TPC analysis was performed according to Singleton et al. (1999) and adapted for a microplate reader FLUOstar Omega (BMG LABTECH) dimensions. Briefly, 25 µL of extract or standard solution was mixed with 125 µL of Folin-Cioacalteu reagent, left for 5 minutes and then 100 µL of sodium carbonate was added to the reaction medium. The intensity of the blue colour formed after 2 hours reaction was monitored at 760 nm. The extraction solvent was used for the blank. All the results were expressed as mg of gallic acid equivalents (GAE) per gram of freeze-dried sample.

2.8.2 Peroxyl radical scavenging assay (ORAC)

The tests were performed according to the method described by Dávalos et al (2004), using a microplate reader FLUOstar Omega (BMG LABTECH). Fluorescein was used as flourescent molecule and potassium phosphate buffer (pH 7.4, 75 mM) as reaction medium.
The microplates containing 20 µL of açaí extract or different concentrations of Trolox, 120 µL of fluorescein (0.4 µg mL-1) and 60 µL of radical AAPH (2.2'-azobis (2-methylpropionamidine) dihydrochloride) (108 mg mL^{-1}) were subjected to reading every 1 minute for a total of 80 minutes (485 nm excitation and 520 nm emission), under controlled temperature at 37 °C. ORAC results were determined using a regression equation relating Trolox concentrations and the net area under the fluorescein kinetic decay curve. The ORAC value of each solution extract was expressed in µmol trolox equivalent per gram of freeze dried sample.

2.8.3 Hypochlorous acid-scavenging assay

The HOCl-scavenging capacity was measured as Ribeiro et al. (2014), adapted to a microplate reader for fluorescence (FLUOstar Omega BMG LABTECH). The assay verifies the effect of the extracts and standards on HOCl-induced oxidation of DHR to rhodamine 123.

HOCl was prepared by adjusting the pH of a 1% (w/v) solution of NaOCl to 6.2 with H_2SO_4 0.05 M. The concentration of HOCl was further determined spectrophotometrically at 235 nm, using the molar absorption coefficient of 100 M^{-1} cm^{-1}. The HOCl scavenging property of the extracts was expressed in terms of IC_{50} which represents the weight of freeze-dried sample needed to achieve 50% scavenging. Gallic acid was used as positive control (0.3 µg.mL^{-1} to 250 µg.mL^{-1}) and sample concentration range was 7.8, 15.6, 31.25, 62.5 and 125 µg/mL.

2.8.4 Hydrogen peroxide Scavenging assay

The H_2O_2 scavenging capacity was measured by monitoring the effect of the extract or standard (gallic acid) on the increase in luminescence resulting from H_2O_2-induced oxidation of lucigenin (Ribeiro et al., 2014). Reaction mixtures contained the following reagents at final concentrations (final volume of 300 µL): 50 mM Tris-HCl buffer (pH 7.4). 0.8 mM lucigenin in Tris-HCl buffer, 1% (w/w) H_2O_2, and extract (7.8, 15.6, 31.25, 62.5, 125 µg/mL) or gallic acid (0.30 µg.mL^{-1} to 250 µg.mL^{-1}). The chemiluminescence signal was measured in the microplate reader after incubation for 5 min at 37 °C. Gallic acid was used as positive control.

2.9 Effect on the formation of nitroso compounds

Under the acidic conditions of the stomach, nitrite forms nitrous acid (HNO_2) which may decompose to form a variety of nitrogen oxides, including dinitrogen trioxide (N_2O_3). These reactive nitrogen species have been observed to react with amines and can cause rapid nitrosation at neutral or basic pH. N_2O_3 is a powerful nitrosating agent which has been shown to trigger the formation of N-nitroso compounds (NOCs) such as nitrosamines, which are known for possessing potent carcinogenic properties (Lee et al., 2006, Lewi et al., 2008, Pereira, Barbosa, Laranjinha, 2015). Therefore, scavenging these species is of great interest in order to prevent NOCs formation.

Endogenous formation of NOCs has been pointed out as a relevant contributing factor for increasing the risk for gastric-intestinal malignancies, as approximately 70% of human exposure to these substances is endogenously formed (Cummings, Bingham, 1998). Connected to this is the idea that a high red meat intake (both fresh and processed) may increase the risk for contracting gastrointestinal malignancies by providing nitrosable (proteins) and

nitrosating compounds (nitrate, heme group) capable of boosting the endogenous formation of NOCs (Loh et al., 2011, IARC, 2015). In biological samples, NOCs are measured as apparent total nitroso compounds (ATNC), which include, in addition to nitrosamines, other nitroso species as nitrosothiols and nitrosyl heme.

The inhibitory capacity of the açaí pulp obtained through different preservation techniques to prevent NOCs formation was studied using an *in vitro* gastric system according to Kunhle et al (2007). NOCs formation was evaluated by measuring apparent total nitroso compounds (ATNC) in the presence and absence of açaí samples. Half a gram of freezed-dried sample was mixed with 20 mL of a simulated gastric fluid containing NaCl (34 mM), pepsin from porcine (3.2 $mg.mL^{-1}$), myoglobin (final concentration 0.4 $mg.mL^{-1}$), haemoglobin (0.13 $mg.mL^{-1}$) and bovinum serum albumin (0.05 $mg.mL^{-1}$) as source of nitrosable substances. The pH of the mixture was adjusted to 2.2 with HCl 1 M, and the nitrosation reaction was started by adding $NaNO_2$ to a final concentration of 0.015 $mg.mL^{-1}$. After 30 min incubation in the dark at 37º and 120 RPM, samples (digestive) were analysed for ATNC. A selective chemical de-nitrosation with iodine/iodide reagent was performed to detect the ATNCs according to Kuhnle et al. (2007). Briefly, 50 µL of digestive was taken and incubated with 500 µL of sulphanilamide (50 mg/mL in 1 M HCl) for 3 min to remove unbound nitrite. Afterward, the sample (200 µL) was injected into a custom-made purge-vessel containing the iodine/iodide reagent kept at 60ºC. The released NO was transferred by helium to the NOA chemiluminescence analyser (Model 88 et, Eco Physics, Duernten, Switzerland), via a condenser consisting of a NaOH (1 mol/L) trap. For differentiation between the N-nitroso, nitrosothiol and nitrosyl iron compounds, mercury (II) stable (nitrosothiols) and potassium ferricyanide stable (nitrosyl iron) compounds were determined under exactly the same way as described above after an additional incubation with $HgCl_2$ (53 $mmol.L^{-1}$, 100 µL for 2 min) and $K_3Fe(CN)_6$ (4 mM. 100 µL for 2 min), respectively. Results were expressed as micromoles of NO released.

2.10 Statistical treatment of the data

Samples were analysed through ANOVA and Tuckey´s test using Statistica 7.0. Differences were considered to be significant when $p < 0.05$.

The multivariate exploratory technique, Principal Component Analysis (PCA), was also used to correlate chemical and bioactivity features with different processing conditions,

using the software Piroutte (Infometrix, Bothell, WA, United States). The data of phenolic compounds and tocopherol contents, scavenging capacity against radical species and inhibition effect on nitrosation reaction were used as variables in the derivatization of the principal component. Results were presented as mean values of 5 replicates (for HPP data) and 3 replicates (for control and TP). Data were autoscaled, so as to attribute the same weight for all the variables. The IC50 values obtained in the HOCl and H_2O_2 assays were used as the inverse of IC50 (1/IC50) in the data matrix to facilitate the graphical analysis of PCA.

3 Results

3.1 Anthocyanins

Table 1 shows the contents of TMA, cyanidin 3-glucoside and cyanidin 3-rutinoside in açaí pulp from different processing conditions. There was a significant difference between samples with regard the TMA content, with values oscillating between 5.38 (control) and 3.85 $mg.g^{-1}$ (TP).

According to the results, the control samples had a significantly higher TMA content compared to all the other samples, except for those processed at 600 MPa. There was no significant difference in the TMA content between the HPP and TP açaí pulp, except for 600 MPa. With regard the individual content of anthocyanins, results for cyanidin 3-glucoside (C3G) and cyanidin 3-rutinoside (C3R) fluctuated between 0.58 $mg.g^{-1}$ and 0.32 $mg.g^{-1}$ and 0.64 $mg.g^{-1}$ and 0.37 $mg.g^{-1}$, respectively. Both anthocyanins were significantly higher in the control than in the HPP and TP samples. Moreover, the high pressure processed samples yielded significantly higher contents of both anthocyanins than the thermal treated ones. Furthermore, no statistic difference in the content of these parameters has been found among the HPP samples.

3.2 Non-anthocyanin phenolic compounds (NAPC)

Results indicated that NAPC content in TP samples did not differ from the control. In contrast, HPP not only preserved NAPC but also seemed to exert a positive effect, as an increasing trend in the NAPC concentration can be observed with pressure raising. Samples processed at 500 MPa displayed the highest measurable concentration of all the studied

compounds, with an increment compared to control that fluctuated between 28% (isoorientin) and 69% (caffeic acid).

3.3 Tocopherols and vitamin E activity

With regard tocopherols, two out of four of the tested tocopherols were detected in the samples (α-tocopherol and γ-tocopherol). The contents ranged between 309 and 342 µg.g-¹ oil α-tocopherol and 6.97 and 7.96 µg.g-¹ oil γ-tocopherol. Globally, they have not been affected by heat or pressure, as neither α-tocopherol nor γ-tocopherol levels varied significantly compared to the control. By comparing TP and HPP, only 500 MPa MPa-samples showed a lower (p<0.05) α-tocopherol value. Moreover, vitamin E activity has not been affected by the treatments.

Table 1. Anthocyanins, non-anthocyanin compounds and tocopherol contents in HPP and TP açaí pulp samples.

Compound	Control	Thermal Treatment	400 MPa	450 MPa	500 MPa	600 MPa
			Anthocyanins (mg.g^{-1})			
TMA	5.38±0.32^{a}	3.85±0.07^{c}	4.44±0.17 ^{bc}	4.50±0.34 ^{bc}	4.22±0.43^{bc}	4.62±0.37 ^{ab}
Cyanidin 3-glucoside	0.53±0.09^{a}	0.32±0.00 ^{d}	0.46±0.05 ^{c}	0.47±0.05 ^{bc}	0.45±0.08 ^{b}	0.43±0.04 ^{bc}
Cyanidin 3-rutinoside	0.62±0.04 ^{a}	0.37±0.01^{c}	0.50±0.03^{b}	0.53±0.02^{b}	0.53±0.05 ^{b}	0.53±0.03^{b}
			Non-anthocyanins (µg.g^{-1})			
3.4-dihydroxibenzoic acid	3.24±0.35 ^{bc}	3.70±0.24 ^{abc}	3.08±0.40^{c}	3.73±0.33^{abc}	4.41±0.28^{a}	4.13±0.29 ^{ab}
4-hydroxibenzoic acid	12.10±0.81^{bc}	12.15±1.61^{bc}	10.91±0.98^{c}	13.69±1.89 ^{b}	16.45±1.56^{a}	14.25±1.49 ^{ab}
Vanillic acid	40.05±5.07^{b}	43.10±6.15^{ab}	38.87±4.47^{b}	48.04±4.45^{ab}	56.67±3.40^{a}	47.60±5.06^{ab}
Caffeic acid	9.04±1.48^{b}	8.5±1.37^{b}	9.03±0.79^{b}	11.75±1.11^{b}	15.22±3.01^{a}	12.06±1.41^{ab}
Syringic acid	19.01±2.29^{c}	23.97±2.90 ^{bc}	22.49±3.24^{c}	25.69±2.27^{bc}	31.90±4.03^{a}	29.53±4.33^{ab}
p-coumaric acid	6.69±0.54 ^{bc}	5.28±0.73 ^{c}	7.1±0.78 ^{bc}	9.39±1.14^{ab}	10.43±2.09^{a}	8.66±0.92^{b}
Isoorientin	40.21±8.5^{bc}	36.19±3.22^{c}	39.94±5.60^{bc}	45.08±1.72^{abc}	51.74±4.16^{a}	48.35±3.35 ^{ab}
Orientin	20.99±4.05^{bc}	21.48±3.51^{bc}	20.74±2.32 ^{c}	26.96±2.07 ^{bc}	33.92±4.55 ^{a}	27.41±2.52 ^{b}
Ferulic acid	10.55±1.19 ^{b}	11.63±1.77 ^{b}	10.59±1.31 ^{b}	12.64±1.19 ^{b}	17.02±4.68^{a}	13.04±1.58^{b}
			Tocopherols (µg.g^{-1})			
α-tocopherol	321.90±12.04 ^{ab}	342.37±25.99^{a}	331.07±14.68 ^{ab}	322.92±7.90 ^{ab}	309±10.31 ^{b}	313.06±11.81 ^{b}
γ-tocopherol	7.96±0.65 ^{a}	7.9±1.51 ^{a}	8.00±0.37 ^{a}	7.88±1.09 ^{a}	6.97±0.42 ^{a}	7.07±0.53 ^{a}
Vitamin E	0.32±0.01 ^{a}	0.343±0.02^{a}	0.340±0.01 ^{a}	0.322±0.01 ^{a}	0.310±0.010 ^{a}	0.314±0.016 ^{a}

Equal letters in the same line indicate no significant difference between the treatments. at 95% confidence level.

3.4 Antioxidant capacity

TPC values in açaí pulp samples (Table 2) oscillated between 31.66 and 37.97 mg GAE.g-¹. The TP samples had the lowest TPC content (p<0.05), whereas HPP did not differ one from another and from the control. With respect to the capacity of the samples to scavenge the peroxyl radical (Table 2), no significant difference was found between TP, control, 400 and 450 MPa samples. However, samples processed at 500 and 600 MPa had significantly higher ORAC values.

With regard the scavenging capacity against HOCl, IC50 values ranged between 14.16 and 46.12 µg freeze-dried sample.mL-¹. The control samples showed the highest inhibition capacity among all samples. Moreover, regardless the treatment, HPP samples had higher HOCl scavenging capacity than TP. As for the H_2O_2-scavenging capacity assay, the inhibitory concentration fluctuated between 363 and 490 µg dried sample.mL-¹. TP, control and 450-MPa samples had similar IC50 values, which were lower than that from 450, 500 and 600 MPa-samples. The treatement at 600 MPa had the highest IC50 for this ROS.

Table 2. Antioxidant properties and inhibition capacity of açaí pulp extract subjected to thermal pasteurization and high pressure processing.

Assay	Control	Thermal Pasteurization	400 MPa	450 MPa	500 MPa	600 MPa
Total phenolic compounds (mg GAE.g-¹)	37.02±1.81 [a]	31.66±2.22[b]	35.93±1.58 [a]	36.52±1.38 [a]	37.34±1.73[a]	37.97±1.65 [a]
ORAC(µM Eq. Trolox.g-¹)	652.71±7.35[bc]	598.78±22.23[c]	632.421±40.63[c]	588.81±91.50 [c]	841.66±66.46[a]	762.18±18.68 [a]
HOCl[a] (µg dried sample.mL-¹)	14.16	46.12	15.92	17.35	19.16	23.70
Positive[a] control (µg.mL-¹)	**19.13**					
H_2O_2[a] (µg dried sample.mL-¹)	395.2	363	390	438	443	490
Positive control[a] (µg.mL-¹)	**276**					
Nitroso compounds formation inhibition (%)						
ATNC (% inhibition)	54±3.29[c]	72±3.79[a]	69±8.42[ab]	61±4.6[abc]	56±2.71[bc]	62±6.55[abc]
Nitrosothiols %inhibition)	62.85±2.22[b]	85±0.03[a]	65±10.27[b]	65±7.15[b]	60±2.64[b]	66±7.28[b]
Nitroso-heme	100	100	100	100	100	100

Equal letters in the same line indicate nonsignificant difference between the treatments, at 95% confidence level.

a: results expressed as IC50 values, which represents inhibitory concentration, *in vitro*, to decrease in 50% the oxidizing effect of the reactive species in the tested media.

3.5 Effect on the formation of nitroso compounds

The total amounts of apparent nitroso compounds formed, expressed as ATNC, as well as its composition in terms of nitrosothiols and nitrosyl heme is displayed in Figure 1. The nitrosation inhibition capacity of the samples is shown in Table 2. The control sample had a lower inhibition capacity than TP, but did not differ from high pressure processed samples, except for that treated at 400 MPa. In turn, the HPP samples did not differ one from another and from TP, except for 500 MPa. With respect to inhibition formation of nitrosothiols, high pressure did not affect this parameter significantly, whereas TP samples have shown higher inhibition properties. As for nitrosyl heme, all the samples achieved 100% inhibition under the tested reagents concentration.

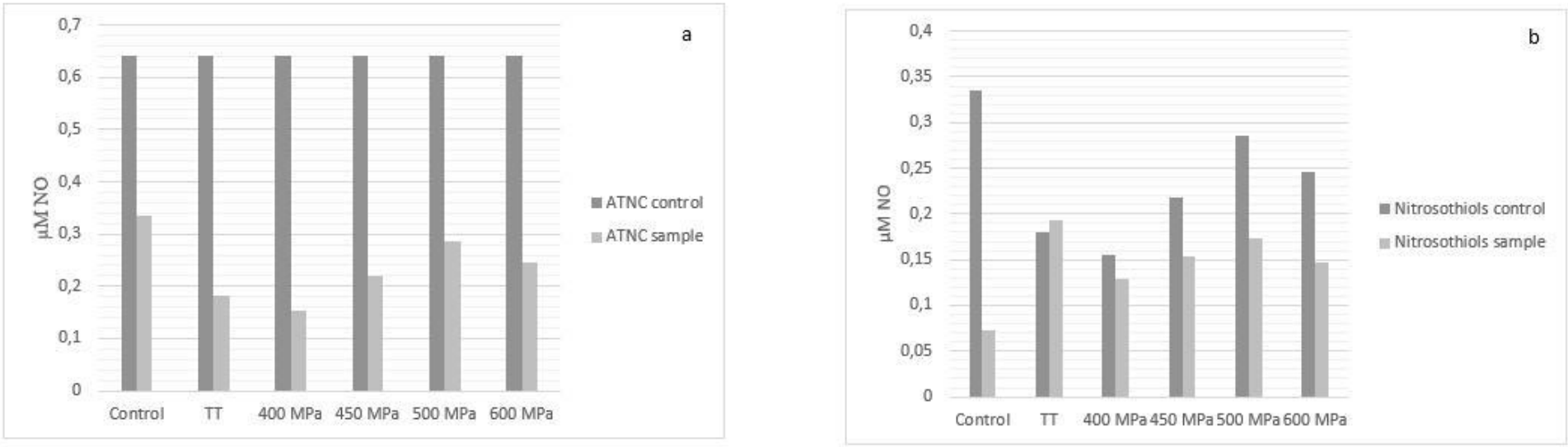

Figure 1. Contents of nitroso compounds formed during incubation of myoglobin + hemoglobin + bovinum serum albumin in the presence (sample) and absence (control) of açaí samples processed under distinct conditions. (a) ATNC formation, (b) nitrosothiols formation.

4 Discussion

4.1 Effect on anthocyanins and NAPC

Various studies on the effect of HPP on the bioactive compounds and antioxidant properties of red fruits and berries have been carried out (Terefe et al., 2013, Ferrara et al., 2010, Patras et a., 2009), but to the best of our knowledge, this is the first report on the effect of high pressure processing on the phytochemical composition of açaí pulp.

The contents of TMA found are in agreement with previous studies on açaí pulp, between 4.58 and 6.93 mg cyanidin 3-glucoside.g-¹ DW (Gárzon, 2016, Rufino et al., 2010). Moreover, the profile of anthocyanin and non-anthocyanin compounds (NAPC) is in line with previous reports on açaí pulp phenolic composition, with anthocyanin-3-rutinoside, vanillic acid and the isomers orientin and isoorientin representing the predominant phenolic compounds (Pacheco-Palência, Ducan, Talcott, 2009, Gordon et al., 2012, Dias et al., 2013). Gallic acid, catechin and epicatechin have not been detected in the samples.

Both TP and HPP caused the anthocyanins (total and individual) content to decrease in comparison with the control. Chen et al (2013) reported significant losses (10%) in TMA following both high pressure and thermal treatment of pomegranate juice (400 MPa/25ºC/5 min, 110ºC/ 8.5 s). In general, anthocyanins are highly susceptible to degradation during processing, being the rate and extent of anthocyanin degradation dependent on extrinsic factors such as processing temperature, light, and the intrinsic properties of the product such as pH, the structure of the anthocyanins and oxidative enzymes (Terefe et al., 2013). Possibly a residual enzymatic activity may have caused the decrease in the HPP samples, as reported by Terefe et al (2013), who found a residual activity (between 80 and 60%, depending on the cultivar) of poliphenoloxidase (PPO) and peroxidase (POD) in HPP samples of strawberry cultivar purées (600 MPa/20ºC/5 min and 88ºC/2 min). The authors reported that the cultivar showing the highest residual activity of PPO displayed higher degradation rates of C3G.

In this regard, Menezes et al (2008) reported that açaí pulp processed at 500 MPa/5 minutes/25ºC showed an increase in peroxidase (POD) and polifenoloxidase (PPO) activity by 40% and 80% compared to unprocessed pulps, respectively, whereas 500 MPa/5 min/35ºC resulted in 10% elevation for POD and 40% for PPO. According to Srivastava et al. (2007) highly hidroxilated anthocyanins, such as those present in the açaí composition, are more susceptible to PPO than other anthocyanins.

Compared to control, TP gave 31% decrease in the content of TMA, whereas for HPP losses averaged between 14% and 21%. Therefore, although significant differences have been detected for 600 MPa-treated samples only, the absolute values suggested that high pressure promoted higher retention of TMA than TP. This was confirmed by examining the results for individual anthocyanins. C3G and C3R had 44 and 42% losses in TP samples, respectively, contrasting to 20% in HPP. Indeed, anthocyanins thermal-sensitivity properties have been widely discussed (Khanal, Howard, Prior, 2010, Patras et al., 2010, Patras et al., 2011, Vegara et al., 2013, Terefe et al., 2013). Accordin to Patras et al (2010) nonenzymatic oxidation and cleavage of covalent bonds are involved in the degradation of anthocyanins during thermal processing. Altough mechanisms have not been yet completely elucidatated, it has been suggested that degradation might be related with hydrolysis of the sugar moiety and aglycone formation, ring opening and formation of a chalcone followed by a breakdown into carboxylic acids and carboxy aldehydes (Patras et al., 2010). Pacheco-Palência, Ducan & Talcott reported extensive anthocyanin degradation in açaí pulp (5 to 34% losses) upon heating treatments of 5, 10, 30 60 minutes/80ºC.

The HPP effectiveness to preserve anthocyanin have been reported previously for various vegetable matrix. For example, Patras et al (2009) reported that high pressure treated blackberry purée samples (400, 500, 600 MPa) showed a higher retention of C3G than TP samples (82ºC/2 min). Studying strawberry purée, Marszalek et al (2015) found that processing at 500 MPa/0ºC/5 min promoted around 14% losses of C3G, Pg3G and Pg3R in relation to the control, whereas TP (90ºC/15 min) resulted in average 43% reduction in the content of these compounds.

With respect the NAPC, globally, they have not been adversely affected by TP. Indeed, Pacheco-Palência, Duncan, Talcott (2009) found no difference in the NAPC concentration (the same ones as studied in this work) in açaí pulp heated for 5, 10, 30 and 60 min at 80 ºC. Odriozola-Serrano et al. (2009) also did not find a significant difference in the content of ferulic acid and caffeic acid between fresh and thermal treated tomato juice (90ºC/30 s). On the other hand, Andrés et al (2016) found the effect of TP on the individual phenolic content of soy-smoothies to vary accordingly the compound, with a significant reduction (10 to 18%) in the chlorogenic acid, *p*-coumaric acid and some flavonoids (catechin, epicatechin, daidzin, genistein) content, but non-significant changes in the caffeic acid content.

As for the HPP, samples processed at 500 MPa displayed significantly higher amounts of NAPC than the control and TP. In agreement with this finding, Huang et al (2013)

reported an increment in the ferulic acid and caffeic acid contents in apricot nectar, with 500 MPa/5 min showing the highest content of the former, whereas the latter showed 25% and 12% more than the control and thermal treated samples (110ºC/8.5 s), respectively. Rodríguez-Roque et al. (2015) also reported an elevation between 10 and 44%, and 9 and 47% in the phenolic acids and flavonoids contents, respectively, in fruit-based beverages after high pressure treatment (400 MPa/ 40ºC/ 5 min).

The results indicated that HPP likely increased extractability of NAPC from the açaí pulp. Some studies have shown an increase in pressure levels accompanied by an elevation in phenolic extraction (Patras et al., 2009, Plaza et al., 2011, Gonzáles-Cerebrino et al., 2013). Indeed, it has been well documented that high pressure exerts a significant influence on plant tissue structure by promoting the breakdown of cell wall that allows increased solvent influx and contact with the analyte (Hernández-Carrión et al., 2014). It also causes changing in the compounds allocation or disposition inside the cell and compounds bond rupture, for example, phenolic compounds binding to proteins or carbohydrates, so that facilitating their extraction (Rodríguez-Roque et al., 2015). Moreover, Morales-de la Peña et al., 2011 hypothesized that processing may cause an increase in the activity of enzymes that participate in the biosynthesis of phenols (i.e. the phenylalanine ammonia-lyse), which might promote an enhacement in the phenolic compounds content.

4.2 Effect on Tocopherols and vitamin E

Açaí pulp is high fat and contains remarkable contents of tocopherols in its composition (Costa et al., 2010, Darnet et al., 2011). The α-tocopherol and γ-tocopherol contents and vitamin E activity found in this study are in agreement with previous report on açaí pulp (Darnet et al., 2011).

Some studies have demonstrated that thermal treatment may cause tocopherols degradation (Romeu-Nadal et al., 2008, Delgado et al., 2013). However, in the present work, thermal treatment did not adversely affect tocopherol contents and vitamin E activity of açaí pulp compared with the control. This result is in agreement with Moltó-Puigmartí et al (2011) and Amador-Espejo et al (2015) who found non-significant difference between α-tocopherol concentration in untreated and thermal treated human (62.5ºC/30 min) and cow (90ºC/15 s, 138ºC/ 4s) milk samples.

High pressure did not alter significantly the levels of α-tocopherol, γ-tocopherol and vitamin E activity compared with the control, which is in line with Moltó-Puigmartí et al (2011), who did not find a significant difference between the content of α-tocopherol in untreated and HPP (400/500/600 MPa/5 min) human milk samples. Compared with the TP sample, only the 500 MPa-processed pulp showed a significant decrease of α-tocopherol. However, in terms of vitamin E activity, there was no significant difference between none of the treatments applied. Little information is known about the effect of high pressure on tocopherols and only a few studies have been carried out on this approach. Results are still controversial, indicating to be highly dependent on food matrix. For exemple, Barba, Esteve and Frigola (2012) reported an increase (7-28%) in the α-tocopherol content and vitamin E activity after high pressure processing of an orange-milk juice (100-400 MPa/9 min), whereas Cilla et al. (2012) reported that HPP samples (400 MPa/36ºC/5 min) showed lower contents of
α- and γ- tocopherol (up to 27%) than control and thermal treated (90ºC/30 s) milk and soy based fruit beverages.

4.3 Antioxidant capacity

Studies have shown that processing might significantly alter antioxidant properties of food (Nicoli et al., 1999, Oms-Oliu et al., 2012). Here, we tested whether the preservation conditions applied influenced the reducing properties (total phenolic compounds by Folin-Ciocalteu) and the scavenging capacity against reactive oxygen species (ROS), $ROO^{\cdot}$, HOCl and H_2O_2.

The total phenolic compounds content (TPC) accorded with those found previously, ranging between 34 and 47 mg GAE.g-¹ (Rufino et al., 2010, Gordon et al., 2010, Garzón et al., 2016). The results showed that the TPC content in TP samples averaged 15% losses in comparison to the control and HPP ($p<0.05$). Similar results have been found for red fruits and other food matrix (Patras et al., 2009, Chen et al. 2013, Terefe et al., 2013, Keenan et al., 2012, Andrés et al., 2016), indicating that HPP succeeded in better retaining reducing substances (e.g. phenolic compounds) from the açaí pulp. Indeed, there was a significant correlation ($p<0.05$) between TPC and cyanindin-3-glucoside (r=0.73) and TPC and cyanidin 3-rutinoside (r=0.72), but no significant correlation between TPC and individual phenolics has been found.

Peroxyl radical scavenging capacity as assayed by ORAC is in the range reported by other authors (Schauss et al., 2006, Kang et al., 2012). Compared to the control, thermal

treatment caused a reduction (~10%), although non-significant, in the radical scavenging capacity of the açaí pulp. Moreover, there was a significant increase in the ORAC values for pulps treated at 500 MPa. These results were consistent to those of Tadapeni et al (2012), who observed that thermal treated (72ºC/20 min) strawberry juice and strawberry-milk based beverages had up to 40% losses in the scavenging capacity compared to control samples, whereas ORAC results for the milk-strawberry based beverage were higher after processing at 600 MPa for 1 and 15 min. The significant higher ORAC values in 500-MPa treated samples might be related to the higher extractability or preservation of antioxidant compounds due to high pressure, as hypothesized by Patras et al (2009) and Tiwari et al (2009). Indeed, there was a low but significant correlation ($p<0.05$) between ORAC and TPC ($r=0.42$) and ORAC and NAPC (between $r = 0.48$ and $r = 0.63$). ORAC assay is based on transfer of one hydrogen atom (HAT) to the free radical, which consists the main mechanism of the phenolic compounds to scavenge ROO· and may explain the observed correlation (Prior, Wu, Schaich, 2005). Indeed, Pacheco-Palencia, Ducan, Talcott (2009) and Kang et al (2011) reported that anthocyanins are the main responsible for the antioxidant properties of the hydrophilic fraction of the açaí pulp, but NAPC also play an important role for the scavenging properties.

To the best of our knowledge, this is the first report on the açaí pulp capacity to scavenge HOCl. Regardless the treatment applied, the açaí pulp samples showed to be effective to scavenge HOCl on a scale of the order of µg of freeze-dried sample, with values similar to that of gallic acid (IC50 = 19.13 µg.mL-¹). Moreover, it had a higher scavenging capacity than other known antioxidants such as chlorogenic acid (IC50 = 56 µg.mL-¹) and trolox (IC50 =134 µg. mL-¹) (Rodrigues et al., 2013, Melo et al., 2015).

The control sample showed the highest scavenging capacity (lowest IC50) among all samples. Compared to the control, TP sample showed a 3.25 decrease in the scavenging capacity of HOCl. In turn, TP exhibited between 2 and 2.9 times lower inhibition properties than HPP samples. There was a significant ($p<0.05$) negative correlation between HOCl and TPC ($r=-0.57$), TMA ($r=-0.61$), C3G ($r=-0.88$) and C3R ($r=-0.75$) indicating that the higher the anthocyanins content, the lower the IC50 values. This might explain the fact that the control had the highest inhibition properties and TP the lowest. However, no correlation or positive correlation values have been observed with regard NAPC, which justifies the fact that 500 MPa-samples did not show a higher scavenging capacity although it had higher NAPC contents than the control.

In addition to HAT, transfer of one electron (SET) from the phenolic compound to the ROS has already been reported and is believed to occur simultaneously. In this regard, the mechanisms of scavenging HOCl involve the transfer of two electrons (Rodriguez, Mariutti, Mercadante, 2013). Anthocyanins are good electron donors owing to their highly hydroxylated structute with conjugated double bonds (Hussein, Almagribi, Al-Rashidi, 2016), which possibly influenced scavenge properties of açaí pulp against this ROS.

With respect to H_2O_2, the açaí pulp also showed inhibition properties towards this ROS, as previously reported by Vissoto et al. (2013). It was lower to that of gallic acid (positive control) and superior to that of quercetin (IC50 = 526 µg.mL-¹) and 5-caffeoylquinic acid (IC50 = 544 µg.mL-¹) (Rodrigues et al., 2013, Ribeiro et al., 2014). Concerning to the studied treatments, TP exhibited the highest scavenging capacity among all the samples. This result indicates that although the control and HPP samples have shown higher amounts of the investigated phytochemicals, this did not influence the scavenging properties towards H_2O_2 of this sample. Indeed, no or a negative correlation has been found between this property and anthocyanins and NAPC, which is in line with the findings of Vissoto et al (2013). Likely, bioactive compounds considered in this study do not contribute greatly to the scavenging capacity of this specific ROS (Vissoto et al., 2013).

Phenolic compounds have also been described to scavenge reactive nitrogen species (Pollard et al., 2006). In the nitrosation reaction, they can readily react with nitrous acid to block its interaction with amines (Bartsch, Ohshima, Pignatelli, 1988). Globally, the açaí pulps showed good inhibitory properties towards the nitrosation reaction measured as apparent total nitroso compound (ATNC). By evaluating the composition of the ATNC formed during the *in vitro* incubation, nitrosothiols were the predominant species produced (~60%), which were also efficiently inhibited in the presence of açaí. Moreover, nitrosyl heme formation has been completely inhibited. Although nitrosothiols and nitrosyl heme are not carcinogenic alone, it has been hypothesized that these species generated at early stages of digestion may act as nitrosating agents on further sites of the gastrointestinal tract, where an amine-rich environment might favour the endogenous production of carcinogenic nitrosamines, and increase the risk for development of colon-rectal cancer (Bartsch, Ohshima, Pignatelli, 1988, Lunn et al., 2007, Kuhnle et al., 2007). Therefore, results display a new potential bioactivity of açaí pulp as a nitrogen reactive species scavenger, and may represent a mechanism of action of this fruit towards endogenous nitrosation. However, the authors emphasize that further studies are

necessary to elucidate mechanisms of action and the effect of phenolic compounds metabolites on the nitrosating reaction.

Both preservation techniques maintained the inhibition properties of the açaí pulp, and TP gave a significant higher inhibition capacity than the control and 500-MPa treated samples, although both have shown higher contents of anthocyanins and the latter a significant increment in the NAPC concentration. This result suggests that in addition to the antioxidant compounds taken into account in this work, possibly other substances played an important role in preventing nitroso compounds formation. Sulfur compounds (e.g. proteins, amino acids) and unsaturated fatty acids are known to act as inhibitors of the nitrosation reaction (Bartsch, Ohshima, Pignatelli, 1988).

In this regard, mechanisms involved in scavenging reactive species are complex and may implicate multistep reactions and pathways. Furthermore, antioxidants may react differently in function of the oxidant source and system (Prior, Wu, Schaich, 2005). Structure features may also affect antioxidant properties and depending on the assay, one specific compound might even if present in lower concentration, contribute more significantly to antioxidant capacity (Mirvish, 1988, Zuleta et al., 2009).

The present study compraised some of the main antioxidant substances of the açaí pulp, such as vitamin E and phenolic compounds. The pulp also contains proteins, amino acids, phytosterols and fibers (Schauss et al., 2006). Recently, Dias et al (2013) and Garzón et al (2017) reported the presence of up to 24 anthocyanin and non-anthocyanins compounds in addition to those studied herein, including other minor anthocyanins, conjugated flavonoids and phenolic acids and their derivatives. Therefore, açaí pulp is a complex food matrix, containing a mixture of diverse substances, which may act against oxidant species through different mechanisms and might present synergistic or antagonistic interactions (López-Alarcón et al., 2013). Moreover, TP and HPP significantly alter food structure to promote liberation of compounds from the cell (Jacobo-Velázquez, Hernandéz-Brenes, 2012), as well as to generate novel substances, such as thermal degradation products of phenolic compounds which still possess a strong antioxidant capacity (Patras et al., 2010).

Hence, other substances exhibiting a strong antioxidant capacity may have played an important role to scavenge H_2O_2 and nitrogen species. Moreover, different reaction mechanisms probably influenced the observed results results in ORAC assay and HOCl-scavenging capacity. These might explain the observed lack of correlation between the studied bioactive compounds concentration and reactive species scavenging properties.

4.4 Principal component analysis (PCA)

Figure 2 displays the scores and loading graphs of the açaí pulp processed under distinctive preservation conditions. PC 1 contributed to explain 64% of the data variability, whereas PC 2 accounted for 26%, totalizing 90% of the computed variance.

According to the results, NAPC and ORAC had high loadings values in PC1 (positive) and PC2 (negative), contributing to differentiate the 500 MPa-samples, confirming that açaí pulp processed under such conditions stood out from the others with regard the NAPC content and peroxyl radical scavenging capacity and reinforcing the correlation between them. Moreover, among the high-pressure treated samples, the 400 MPa-processed showed the lowest values for these variables. In turn, high positive loadings in PC2 for TMA, C3G, C3R and HOCl-scavenging capacity indicated that the control sample had higher values for these variables, corroborating the idea of a the positive correlation between them. Furthermore, the high positive loadings of HOCl in PC2 and high negative loads of H_2O_2 in PC1 confirm the finding that samples high in NAPC presented lower scavenging capacity towards HOCl reactive species, and that H_2O_2-scavenging capacity cannot be correlated with none of the studied bioactive compounds.

Moreover, TP had the lowest concentrations of anthocyanin compounds among all the samples, and showed lower values of NAPC than samples processed at 450, 500 and 600 MPa. In addition, antioxidant capacity by ORAC and HOCl methods was also lower for this sample than for the HPP ones. On the other hand, inhibition of nitroso compounds formation contributed to discriminate TP, confirming that this sample had higher reactive nitrogen species scavenging capacity than those of the others studied treatments, although this difference have been significant for control and 500 MPa-samples only.

Therefore, PCA provided an overview of the obtained results, indicating that HPP is capable of delivering a high quality açaí pulp with a higher retention of anthocyanins and antioxidant capacity than thermal pasteurization and an increment in the content of NAPC and antioxidant capacity compared to the untreated (control) and TP pulps. On this basis, they point out the process at 500 MPa for 5 min at 20ºC the best condition tested in this study. Whether the nutritional and functional increment observed is maintained or might affect metabolism or bioactivity of these compounds *in vivo* is a point still needing further elucidation.

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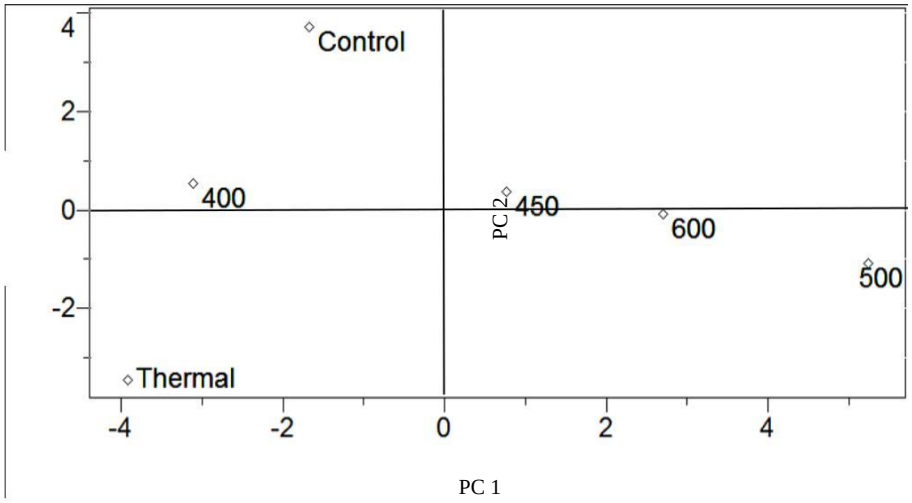

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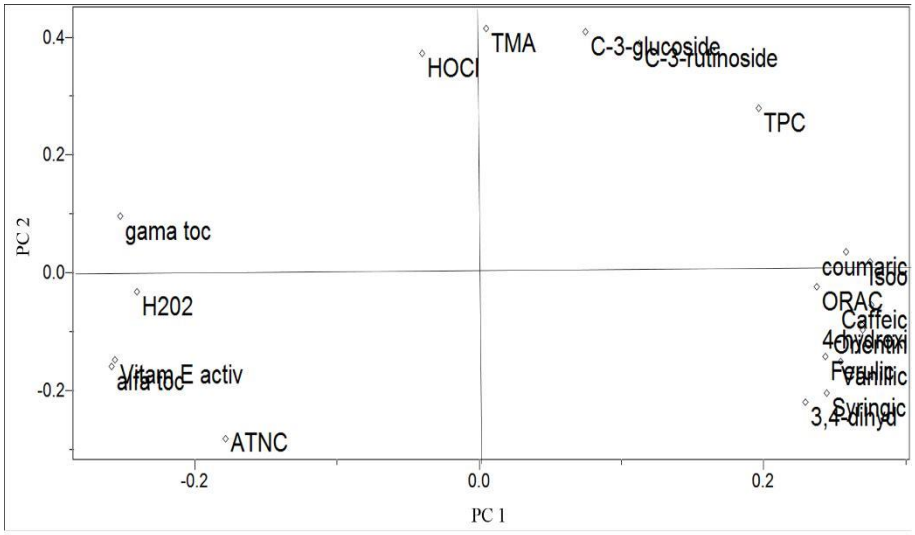

Figure 2. Principal Component Analysis of the effect of different processing conditions on the anthocyanins, non-anthocyanin phenolic compounds, tocopherols content, vitamin E activity and antioxidant capacity of açaí pulp. (a) score graph; (b) loading graph.

5 Conclusion

The present study provided the first data on the effect of high pressure processing on the bioactive compounds composition of açaí pulp. The results showed that this emergent technique was more effective to retain anthocyanins (up to 40%) than TP. Moreover, it was observed that TP did not affect NAPC content, whereas HPP at 500 MPA promoted a significant increase in the non-anthocyanins phenolic compounds content of up to 69% compared to the control. Furthermore, tocopherols and vitamin E activity have not been affected by none of the

treatments applied. Peroxyl radical-scavenging properties was increased with pressure, and HOCl-scavenging capacity was higher in HPP samples than in the TP ones. As for H_2O_2-scavenging, TP showed the highest scavenging capacity whereas 600 MPa had the lowest one. Similarly, inhibition capacity of nitrosation reaction was also higher for TP than for control, but in general did not differ from HPP, demonstrating a lack of correlation between these properties and bioactive compounds concentration that suggests substances other than those considered in this study played to deactivate these reactive species. Therefore, our results point out HPP as an alternative preservation technique capable of providing açaí pulps with high functional and nutritional quality. Whether this may affect açaí bioactivities *in vivo* is worth investigating in future studies.

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SUPPLEMENTAL MATERIAL I

Table 1. Figures of merit of the method for Anthocyanins.

Compound	LOD[a] (mg.L-¹)	LOQ[a] (mg.L-¹)	Linear region (mg.L-¹)	R^2	Lack of fit[b] (*p*)	Concentration level (mg.L-¹)	Intra-day precision [c] (n=10)	Inter-day precision[c] (n=3)	Recovery (%)
Cyanidin 3-glucoside	0.0004	0.0032	0.99-30	0.999	0.06	0.99	0.89	0.16	91.82
						14.1	0.82	0.20	100.5
						30	0.35	0.67	92
Cyanidin 3-rutinoside	0.0003	0.008	0.99-30	0.999	0.04	0.99	0.91	0.18	95.87
						14.1	0.82	0.19	91.82
						30	0.41	0.54	102.72

a - Limit of detection (LOD) and limit of quantification (LOQ) concentrations were determined as those with signal 3 and 10 times the noise.

b – lack of fit for the linear model test ($p>0.05$)

c – Precision values provided in terms of coefficient of variance (%)

SUPPLEMENTAL MATERIAL II

Table 1. Quality parameters of the method for Tocopherols

Compound	LOD[a] (ng)	LOQ[a] (ng)	Linear region (ng)	R^2	Lack of fit[b] (*p*)	Level (ng)	Intra-day Precision[c] (n=10)	Inter-day precision[c] (n=3)
α-tocopherol	0.62	2.06	50-850	0.9985	0.05	50	2.42	9.69
						250	4.35	1.19
						850	8.3	8.09
β-tocopherol	0.95	3.12	5-95	0.9989	0.09	50	3.35	8.55
						250	9.33	4.57
						850	7.01	10.24
γ-tocopherol	0.68	2.29	3.5-66.5	0.9980	0.52	50	8.51	5.54
						250	7.97	2.55
						850	7.21	15.50
-tocopherol	0.61	2.03	3.5-66.5	0.9964	0.67	50	6.91	9.29
						250	8.31	4.39
						850	5.26	4.39

a - Limit of detection (LOD) and limit of quantification (LOQ) were determined as 3 and 10 times the noise.

b – lack of fit for the linear model test ($p>0.05$)

c – Precision values provided in terms of coefficient of variance (%)

CAPÍTULO V

WHITE AÇAÍ PULP (*Euterpe oleracea*): PHENOLIC COMPOSITION BY LC-ESI-MS/MS, ANTIOXIDANT CAPACITY AND INHIBITION EFFECT ON THE FORMATION OF COLORECTAL CANCER RELATED COMPOUNDS

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Abstract

White açaí (*Euterpe oleracea)* is an açaí type presenting a greenish mesocarp in the mature stage, contrasting with the purple one exhibited by the popular açaí berries. Along with the latter, it comprises the most commercially representative types of açaí available in the market. However, white açaí composition and potential bioactivities have been poorly investigated, prompting this study to be carried out. Firstly. the effect of time and ethanol concentration on the phenolic compounds extraction of white açaí was evaluated through a central composite design 2^2, using total phenolic compounds as response. The optimal conditions (10 minutes extraction, 35:65 ethanol:water v/v) were applied for analysis of phenolic profile by ultra-high performance liquid chromatography with electrospray ionisation and triple-quadrupole mass spectrometer in tandem (LC-MS/MS). Moreover, this extract was examined regarding its scavenging capacity towards reactive oxygen species. Finally, white açaí was tested for its capacity to inhibit nitrosation reaction under a simulated gastric environment (pH 2.2), using two systems: (A) pure myglobin, hemoglobin and bovinum serum albumin plus $NaNO_2$; and (B) minced beef plus $NaNO_2$. Phenolic profile of white açaí was similar to that reported for purple açaí, containing vanilic acid and the isomers orientin and isoorientin as major phenolic compounds. In addition, the sample showed a higher scavenging capacity towards ROO· and HOCl than that reported for tropical fruits commonly consumed worldwide. Lastly, it exhibited capacity for inhibiting nitroso compounds formation in both systems investigated, suggesting a potential protective effect of white açaí towards the formation of colorectal cancer-related compounds derived from red meat intake.

Key words: phytochemical profile, nitrosation reaction, LC-MS/MS, MRM, health benefits, central composite design.

1 Introduction

Açaí (*Euterpe Oleracea* Mart.*)* is a large palm tree growing abundantly on floodplains, swamps and upland regions in the Amazon estuary (Oliveira et al., 2002, Schauss, 2010). Dark purple açaí is the most representative fruit of the plant, however, different naturally occurring types or varieties have been reported, which differ one from another regarding palm features, such as bunch weight, size and mainly the colour of the fruits (Oliveira et al., 2002). White açaí fruits (Figure 1a) represent one of these açaí types and contrast from purple açaí for possessing a greenish to yellow colour when ripe and displaying a cream-coloured mesocarp that after undergo mechanical pulping gives a cream-greenish juice, with appearance and distinctive flavour (Figure 1b) (Rogez, 2000, Oliveira et al. 2002, Rodrigues et al., 2006, Wycoff et al., 2015). White açaí is majorly consumed by native populations from the Amazon as a juice, or in desserts such as ice creams and jams. Taken together, purple and white are the main *Euterpe oleracea* types commercially available (Rogez, 2000, Sanches, Oliveira, 2011, Sousa, Oliveira. Neto, 2015)

The chemical composition and health effects of purple açaí have been extensively studied (Dias et al., 2012, Pacheco-Palência et al., 2009, Pacheco-Palência, 2010, Mulabagal, Calderón, 2012, Dias et al., 2013, Schauss et al., 2006, Kang et al., 2012). In contrast, research regarding white açaí has mainly focused on agronomic aspects of the plant, so that information on its composition or potential health effects are minimal (Sanches, Oliveira, 2011, Sousa, Oliveira, Neto, 2015). Rodriguez et al (2006) carried out an investigation into the composition and antioxidant capacity of white açaí seeds, reporting high scavenging capacity towards peroxyl radicals, anion peroxynitrite and hydroxyl radicals. Moreover, protocatechuic acid, epicatechin and procyanidins were determined by LC-MS/MS. More recently, Wycoff et al (2015) also studied the chemical and nutritional composition of white açaí seeds by nuclear magnetic resonance (NMR) and LC-MS/MS. The authors found insoluble fibers, a mixture of saturated and unsaturated fatty acids and no detectable anthocyanins. Aside from these reports, to the best of our knowledge, there is a lack of studies on the chemical composition and potential health benefits of white açaí pulp.

In this context, reporting on benefits of consumption of dietary antioxidants for reducing risks for several diseases such as cancer have encouraged research for new sources of bioactive compounds (Duijhoven et al., 2009, Roleira et al., 2013). Among the numerous types of cancer, colorectal malignancies is a leading cause of deaths worldwide, accounting for 9%

of the total cancer-related death registered in 2012 (Cancer Research UK. 2016). Over the last years, several clinical and epidemiological studies have consistently shown that a high red meat diet, both fresh and processed, leads to an increase in the probability of contracting CRC (Bastide et al., 2010, Oostindjer et al., 2014, IARC, 2015). Among other factors, this association has been linked to the endogenous formation of nitroso compounds (NOCs) in the gastrointestinal tract, such as nitrosamines, a class of substances that has been referred to possessing potent carcinogenic properties by alkylating DNA to give specific DNA-adducts (Bastide et al., 2011, Lewin et al., 2006).

Endogenous formation of NOCs takes place through nitrosation reaction when secondary amines react with nitrite-derived compounds formed under the acidic conditions in the stomach (pH = 2.5- 3.5) (Bartsch et al., 1988). Nitrite may be delivered through vegetables, water, or by the naturally occurring nitrate/nitrite cycle in humans (Espejo Herrera et al., 2016). It is estimated that approximately 45–75% of the NOCs human exposure is attributed to endogenous production (Cummings, Bingham, 1998).

Mechanistically, the role of red and processed meat on CRC has been extensively debated, but there are a few hypothesis mainly being focused: red meat is high in amine compounds prone to undergo endogenous nitrosation; it has heme, the prosthetic group in myglobin and haemoglobin, which is capable of catalysing endogenous nitrosation reaction (Pierre et al., 2013, Bastide et al., 2015). All of these pathways may lead to an increase in NOCs formation (Joosen et al., 2009, Bastide et al., 2010). It has been reported that upon a high red meat meal, NOCs majorly formed in addition to nitrosamines are nitrosothiols and nitrosyl heme (Kuhnle et al., 2007). Collectively, these compounds are measured as apparent total nitroso compounds (ATNC), which have been found to increase in humans and rats stools following a high red meat meal (Hughes et al., 2001, Kuhnle et al., 2007, Holtrop et al., 2012). Moreover, they have been correlated with the levels of DNA adducts associated to CRC (Lewis et al., 2006, Joosen et al., 2009).

On the other hand, epidemiologic studies have suggested that a high intake of fruits and vegetables is inversely correlated with CRC incidence (Duijnhoven et al., 2009, Loh et al., 2011) due to the reducing components in its composition capable of blocking nitrosation reaction, as has been supported by several *in vitro* and *in vivo* studies (Hughes et al., 2002, Ohsawa et al., 2003, Abraham, Khandelwal, 2013, Pierre et al., 2013, Pereira, Barbosa, Laranjinha, 2015). However, there are still few studies on this research field, so that more evidence from different food matrix should be investigated. Given this, the current study aimed

to determine the phenolic composition by LC-MS/MS, the scavenger capacity towards reactive oxygen species and to evaluate the capacity of white açaí pulp to inhibit nitrosation reaction using red meat (beef) in an *in vitro* gastric system.

(a) (b)

Figure 1. (a) white açaí fruits (Mattar, 2016), (b) freeze dried white açaí (right) and purple açaí pulps (left).

2 Material and methods

2.1 Samples and chemicals

Ethanol and NaClO were purchased from Synth (São Paulo, Brazil), phenolic compounds standards (gallic acid 99.3%, 3.4-hydroxybenzoic acid 99%, 4-hydroxybenzoic acid 99%, (+)-catechin 98%, vanillic acid 98%, caffeic acid 98.5%, syringic acid 95%, (-)-epicatechin 98%, *p*-coumaric acid 98%, orientin 98%, isoorientin 98% and ferulic acid 99%, taxifolin 98%), potassium iodide and acetonitrile HPLC grade, DHR were acquired from Sigma-Aldrich (St. Louis, MO, USA). Petroleum ether (40-60ºC), water (HPLC grade), iodine and sulphanilamide were acquired from Fischer Scientific (Hampton, NH, USA). High purity formic acid (98%) was purchased from Merck (Darmstadt, Germany).

One kg of white açaí pulp (11-14% solids) was acquired from two local markets of the city of Belém, Pará, totalizing two kg of sample. The samples were homogenised, frozen, freeze-dried and stored under – 80ºC protected from light and oxygen until analysis.

2.2 Effect of time and ethanol concentration on the extraction of phenolic compounds

Previous tests indicated ethanol:water mixtures as a suitable solvent for extraction of phenolic compounds from freeze-dried white açaí pulp. Then, the effect of different ethanol proportions and extraction time was examined through a 2^2 central composite design, comprising 4 axial (1.44 and -1.44) and 3 central points (0. 0), making up 12 experiments (Table 1) carried out randomly. The response evaluated was total phenolic compounds (TPC) measured by the Folin cicoateaul assay. The significance of linear and quadratic regression models was tested using ANOVA and lack of fit test was performed. both at the 95% confidence level. The validated model was used to predict the optimal conditions for extracting phenolic compounds from freeze-dried white açaí juice.

For the experimental design, 0.5 g sample was mixed with 27 mL of extractor solvent according to the experimental design, and stirred at 225 rpm for the period indicated by the factorial design. Afterwards, the extracts were filtered using paper filter, the volume was made up to 25 mL and stored under -20ºC until analysis.

2.3 Folin cicoateaul assay

Total phenolic compounds (TPC) in white açaí extracts were determined by Folin-Ciocalteu, as described by Singleton et al (1999) and adapted for a microplate reader FLUOstar Omega (BMG LABTECH) dimensions. Briefly, 25 µL of extract or standard solution was mixed with 125 µL of Folin-Cioacalteu reagent, left for 5 minutes and then 100 µL sodium carbonate was added to the reaction medium. The intensity of the blue colour formed after 2 hours reaction was monitored at 760 nm. The extraction solvent was used for the blank. All results were expressed as mg of gallic acid equivalents (GAE) per gram of freeze-dried sample.

2.4 Phenolic compounds profile determination

Phenolic compounds from freeze-dried white açaí were extracted using the optimized extraction conditions established through the experimental design. The sample (0.5 g) was mixed with 27 mL of ethanol:water (35:65 v/v) and stirred for 10 minutes at 225 rpm.

Following, ethanol was evaporated using a rota-evaporator (35ºC for 10 minutes), the volume was adjusted to 20 mL with 7.8% acetonitrile: 92.2% water (0.1% formic acid) v/v, filtered using PVDF 0.22 µM and injected into the LC-MS/MS.

Phenolic compounds were determined using a UPLC Waters Acquity equiped with a binary pump system using the conditions optimized in Chapter 3. The equipment had autosampler and column heater, maintained at 4ºC and 30ºC, respectively. The compounds were separated on a Kinetex C18 column (100 mm 2.10 mm x 1.7 µM) (Phenomenex, Torrance, CA, US). The mobile phase consisted of acetonitrile (A) and an aqueous solution of form acid 0.1% (B). and gradient elution was as follows: 7.8% to 15% A in 8 minutes. then increasing to 50% A in 1 minute. and returning to the initial conditions in 1 minute. The column was left to reequilibrate for 4 minutes before each injection. The flow was 0.3 mL.min-¹ and the injection volume was 5 µL.

The UPLC was coupled with an eletrospray ionization source (ESI) set to negative mode. and a triple quadrupole mass spectrometer (Micromass. Quatro Ultima. Waters) operating in the Multiple Reaction Monitoring (MRM) mode. The settings for the ESI were: capilary voltage 2.4 kV; source and desolvation temperatures were 150ºC and 300 ºC. respectively; desolvation (N_2) and cone gas flow were 1149 L.h-¹ and 100 L.h-¹, respectively. The best MRM transitions were determined by selecting the most abundant product ion for each $[M-H]^-$ precursor ion and the settings of the mass spectrometer were optimized for each transition, which are shown in Table 1.

2.5 Antioxidant capacity

2.5.1 Peroxyl radical scavenging apacity by ORAC assay

The tests were performed according to the method described by Dávalos et al (2004), using a microplate reader FLUOstar Omega (BMG LABTECH). Fluorescein was used as a flourescent molecule and potassium phosphate buffer (pH 7.4, 75 mM) as a reaction medium. The microplates containing 20 µL of açaí extract or different concentrations of Trolox,
120 µL of fluorescein (0.4 µg mL-1), and 60 µL of radical AAPH (2.2'-azobis (2-methylpropionamidine) dihydrochloride) (108 mg mL-1) were subjected to reading every 1 minute for a total of 80 minutes (485 nm excitation and 520 nm emission), under controlled temperature (37 °C). ORAC results were determined using a regression equation relating

Trolox concentrations and the net area under the fluorescein kinetic decay curve. Analysis were performed in triplicate and the ORAC value of each solution extract was expressed as µmol TE g^{-1} DW.

Peak ID	Compound	Precursor ion (*m/z*)	Cone voltage KV	Product ion (*m/z*)	Collision enegy V
1	Gallic acid	168.9	35	124.9	14
2	3.4-dihydroxibenzoic acid	153.1	35	108.9	14
3	4-hydroxibenzoic acid	137.2	35	92.9	15
4	Catechin	288.9	35	245	14
5	Vanillic acid	166.8	35	151.8	14
6	Caffeic acid	178.9	35	134.9	16
7	Syringic acid	197	35	181.8	14
8	Epicatechin	289	35	245	14
9	*p*-coumaric acid	162.9	35	118.9	14
10	Isoorientin	447.1	35	357	20
11	Orientin	447.1	35	327	20
12	Ferulic acid	192.8	35	133.9	16
13	Chlorogenic acid	353	35	190.9	17
14	Taxifolin	303	35	284.9	12

Table 1. Mass spectrometry parameters for MRM transitions.

2.5.2 Hypochlorous acid-scavenging assay

The HOCl-scavenging capacity was measured as Ribeiro et al., (2014). adapted to a microplate reader for fluorescence (FLUOstar Omega BMG LABTECH). The assay verifies the effect of the extracts and standards on HOCl-induced oxidation of DHR to rhodamine 123. HOCl was prepared by adjusting the pH of a 1% (w/v) solution of NaOCl to 6.2 with H_2SO_4 0.05 M. The concentration of HOCl was further determined spectrophotometrically at 235 nm. using the molar absorption coefficient of 100 M^{-1} cm^{-1}. The HOCl scavenging property of the extracts was expressed in terms of IC_{50} which represents the concentration of freeze-dried sample needed to achieve 50% scavenging. Gallic acid was used as positive control (0.3 µg.mL^{-1} to 250 µg.mL^{-1}).

2.5.3 Hydrogen peroxide scavenging assay

The H_2O_2 scavenging capacity was measured by monitoring the effect of the extract or standard (gallic acid) on the increase in luminescence resulting from H_2O_2-induced oxidation of lucigenin (Ribeiro et al., 2014). Reaction mixtures contained the following reagents at final concentrations (final volume of 300 µL): 50 mM Tris-HCl buffer (pH 7.4), 0.8 mM lucigenin in Tris-HCl buffer, 1% (w/w) H_2O_2, and extract (7.8. 15.6. 31.25. 62.5. 125. µg/mL) or gallic acid (0.30 µg.mL^{-1} to 250 µg.mL^{-1}). The chemiluminescence signal was measured in the microplate reader after incubation for 5 min at 37 °C. Gallic acid was used as a positive control.

2.6 In vitro effect on the formation of nitroso compounds (NOCs)

The effect of white açaí on the formation of nitroso compounds was evaluated using an *in vitro* gastric system according to Kuhnle et al (2007), and NOCs formation was measured as apparent total nitroso compounds (ATNC) in the presence (intervened samples) and absence (control) of açaí samples. As source of nitrosable compounds, two systems were purposed: A) Half a gram of freezed-dried sample was mixed with 20 mL of a simulated gastric fluid containing NaCl (34 mM), pepsin from porcine (3.2 mg.mL^{-1}), myoglobin (final concentration 0.4 mg.mL^{-1}), haemoglobin (0.13 mg.mL^{-1}) and bovine serum albumin (BSA, 0.05 mg.mL^{-1}). The pH was adjusted to 2.2 with HCl 1 M, and the nitrosation reaction was started by adding $NaNO_2$ to achieve a final concentration of 0.015 mg.mL^{1}; and B), in which all procedures aforementioned were applied, except for the use of 1 g minced beef (5% fat) rather than myoglobin+haemoglobin+BSA. After 30 min of incubation in the dark, at 37 ºC and 120 RPM, in the presence or absence of white açaí, samples were subjected to a selective chemical de-nitrosation with iodine/iodide reagent for detection of ATNCs, according to Kuhnle et al. (2007). Briefly, 50 µL of digestive was taken and incubated with 500 µL of sulphanilamide (50 mg/mL in 1 M HCl) for 3 min to remove unbound nitrite. Afterward, the sample (200 µL) was injected into a custom-made purge-vessel containing the iodine/iodide reagent kept at 60ºC. The reductive cleavage of nitrosated compounds releases NO into the gas phase, which is dragged by a helium stream. The outlet of helium gas stream containing NO passed through a scrubbing bottle containing 1 M of ice cold sodium hydroxide (NaOH) in order to trap traces of acid and iodine before transfer to the analyzer (CLD 88 Eco Medics, Switzerland). In the

analyser, NO in the gas phase engages in a chemiluminescent reaction with ozone (O_3) to form nitrogen dioxide (NO_2) partially in an electronic excited state (NO_2^*). The decay of (NO_2^*) to the electronic ground state emits light in the near-infrared region that can be quantified in the CLD 88 photomultiplier (Feelisch et al., 2002).

For differentiation between contributing NOC classes to ATNC (N-nitroso, nitrosothiol and nitrosyl heme compounds), mercury (II) stable (for nitrosothiols) and potassium ferricyanide stable (for nitrosyl heme) compounds were determined under exactly the same way as described above after an additional incubation with $HgCl_2$ (53 mmol/L, 100 µL for 2 min) and $K_3Fe(CN)_6$ (4 mM, 100 µL for 2 min), respectively. Calibration curves were obtained injecting freshly prepared sodium nitrite solution. Analysis were carried out in triplicate. Results were expressed as micromoles of NO released.

3 Results and discussion

3.1 Effect of time and ethanol proportion

The results of the central composite design for the extraction of phenolic compounds from white açaí are shown in Table 2. The values fluctuated between 4.39 and 10.17 mg GAE.g-¹ DW.The quadratic model was highly significant as the F-*value* ($MS_{regression}/MS_{residue}$) resulted 23 times higher than $F_{critical}$ at 95% confidence level. Moreover, lack of fit test showed that F-*value* ($MS_{purre\ error}/MS_{lack\ of\ fit}$) was lower than $F_{critical}$ at 95% confidence level, indicating that the model did not suffer from lack of fit. Furthermore, the model R^2 value was 0.97085, demonstrating that it was capable of explaining 97% of the variation in the studied system. Therefore, it was suitable to predict the best condition for phenolic compounds extraction.

Figure 2 displays the contour plot of the quadratic model generated. Within the studied range, time had no significant effect on the TPC extraction, so this variable was fixed at the lowest level of the experimental region (10 min). In contrast, negative linear and quadratic coefficients (Table 3) indicated that ethanol proportion adversely affected the response, with TPC outcomes being improved as ethanol concentration increased up to 50%, from which it tended to drop.

Table 2. Total phenolic compounds response for white açaí samples at each level of the central composite design.

Run	Time (min) Codified	%Ethanol Codified	Time (min) Uncodified	%Ethanol Uncodified	TPC (mg GAE.g-1 DW [a])
1	-1	-1	15.7	21.43	9.28
2	1	-1	44.28	21.43	8.98
3	-1	1	15.7	77.78	5.62
4	1	1	44.28	77.78	6.30
5	0	0	30	50	9.91
6	0	0	30	50	9.95
7	0	0	30	50	10.17
8	1.44	0	50	50	9.87
9	0	1.44	30	90	4.39
10	-1.44	0	10	50	10.13
11	0	-1.44	30	10	7.33

a: Dry weight

Similar quadratic behaviour was reported by Pompeu, Silva, Rogez (2009) for purple açaí fruits, who found ethanol proportions above 80% to decrease TPC yields. Increasing ethanol proportion results in a reduction in the dielectric constant of the solution and a consequent reduction in the energy required to separate the solvent molecules, allowing the solute molecules to enter between them (Pompeu, Silva, Rogez, 2009). Moreover, in extraction procedures, solvent mixtures are used for altering solvent polarity and enhance extraction of target compounds by both improving solubility and increasing interaction between substances and the extractor solvent (Metrouh-Amir et al., 2015).

Table 3. ANOVA results and regression coefficients of the regression model for total phenolic compounds in white açaí extracts.

Source	**SS**	**DF**[a]	**MS**[b]	*F-value*	$F_{critical}$[c]
Regression	40.09	2	20.04		
Residue	1.51	8	0.19	105.47	4.46
Lack of fit	1.47	6	0.245	12.25	19.33
Pure error	0.04	2	0.02		
Total	43.45	10			

Parameter/ Variable	**Significant coefficients**[b]		
	Mean	%Ethanol Linear	%Ethanol Quadratic
%Ethanol	9.9	-1.29	-2.07

a: Degree of freedom; b: Mean square; c: At 95% of confidence level

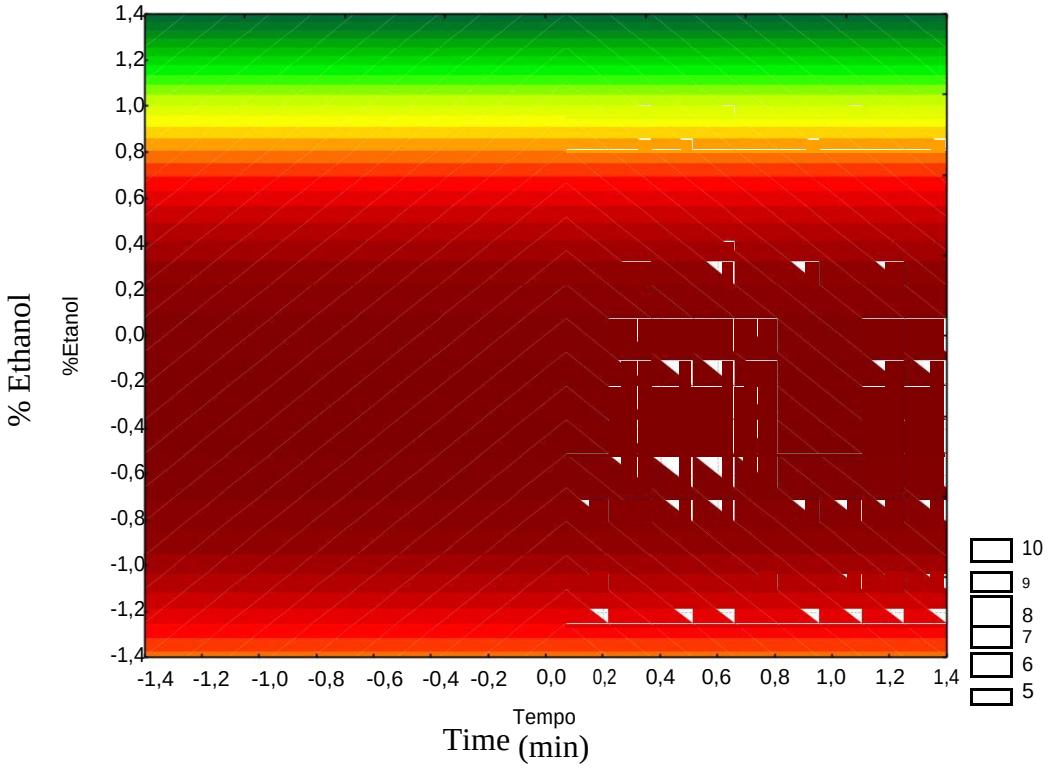

Figure 2. Contour plots generated from the validated model for the extraction of phenolic compounds from white açaí. $R^2 = 0.97085$

Therefore, the optimal condition was 35:65 (ethanol:water v/v) and 10 min of extraction. Validation experiments were carried out using two points of the optimal experimental region, ethanol:water: 35:65 (v/v) and 44:36 (v/v) (n=3). Under such conditions, predicted values for reducing power were of 10.02 and 10.06 mg GAE.g-¹ DW, respectively. After analysis, experimental TPC content were 11.20±0.35 and 10.82±0.44 mg Gallic acid Equivalent.g-¹, respectively, indicating that the model was capable of successfully predicting the TPC content in white açaí extracts.

3.2 Phenolic compounds profile

Phenolic compounds from white açaí were extracted using the optimized conditions set in 3.1. The phenolic profile determined by UHPLC-ESI-MS/MS is shown in Table 4. Although the phenolic composition of purple açaí has been extensively studied, to the best of our knowledge this is the first report on the phenolic profile of white açaí.

Figure 3 contains a typical chromatogram obtained from white açaí samples. Of 14 phenolic compounds studied by MRM, white açaí had eight phenolic acids (peaks 2, 3, 5, 6, 7, 8, 10 and 13). Vanillic acid was the major phenolic acid found (55.61µg.g-¹), followed by ferulic acid (27 µg.g-¹), whereas 3.4-dihydroxibenzoic and caffeic acid contents were found under the limit of quantification of the method (0.01 and 0.0096 mg.L-¹, respectively), and epicatechin and gallic acid have not been detected (limits of detection were: 0.007, 0.021 mg.L-¹, respectively).

In addition to phenolic acids, four flavonoids were identified, including one flavan-3-ol (catechin, peak 4), two flavones and one flavononol. Orientin (luteolin-8-C-glucoside) and isoorientin (luteolin-6-Cglucoside) (peaks 11 and 12, respectively), which are flavones identified as lutein derivatives, were found with typical base peak $[M-H]^-$ at *m/z* 447 and a product ion at *m/z* 327 which is likely due to the fragmentation of the attached C-glycoside (Pacheco-Palencia et al., 2009; Gordon et al., 2012). Taxifolin is a flavononol identified in peak 14, providing a $[M-H]^-$ precursor ion at *m/z* 303 and a corresponding product ion at *m/z* 285 (Gordon et al., 2012). Orientin and isoorientin were the most abundant flavonoids in white açaí (378.49 µg.g-¹ and 178.11 µg.g-¹, respectively), and the predominant phenolic compounds in the sample.

Table 4. Phenolic compounds content in white açaí.

Peak		µg.g-¹DW	LOD[a] (mg.L-¹)	LOQ[b] (mg.L-¹)
1	Gallic acid	ND[c]	0.007	0.025
2	3.4-dihydroxibenzoic acid	NQ[d]	0.003	0.01
3	4-hydroxibenzoic acid	13.38±1.50	0.002	0.008
4	Catechin	5.20±1.08	0.020	0.067
5	Chlorogenic acid	4.23±0.86	0.026	0.04
6	Vanillic acid	55.61±5.26	0.016	0.055
7	Caffeic acid	NQ	0.0021	0.009
8	Syringic acid	0.69±0.09	0.01	0.058
9	Epicatechin	ND	0.021	0.07
10	*p*-coumaric acid	4.67±0.93	0.001	0.004
11	Isoorientin	178.74±11.32	0.031	0.105
12	Orientin	378.49±23.56	0.037	0.123
13	Ferulic acid	27.95±2.48	0.009	0.03
14	Taxifolin	1.57±0.25	0.005	0.040

a: limit of detection, b: limit of quantification, c: not detected; d: not quantified.

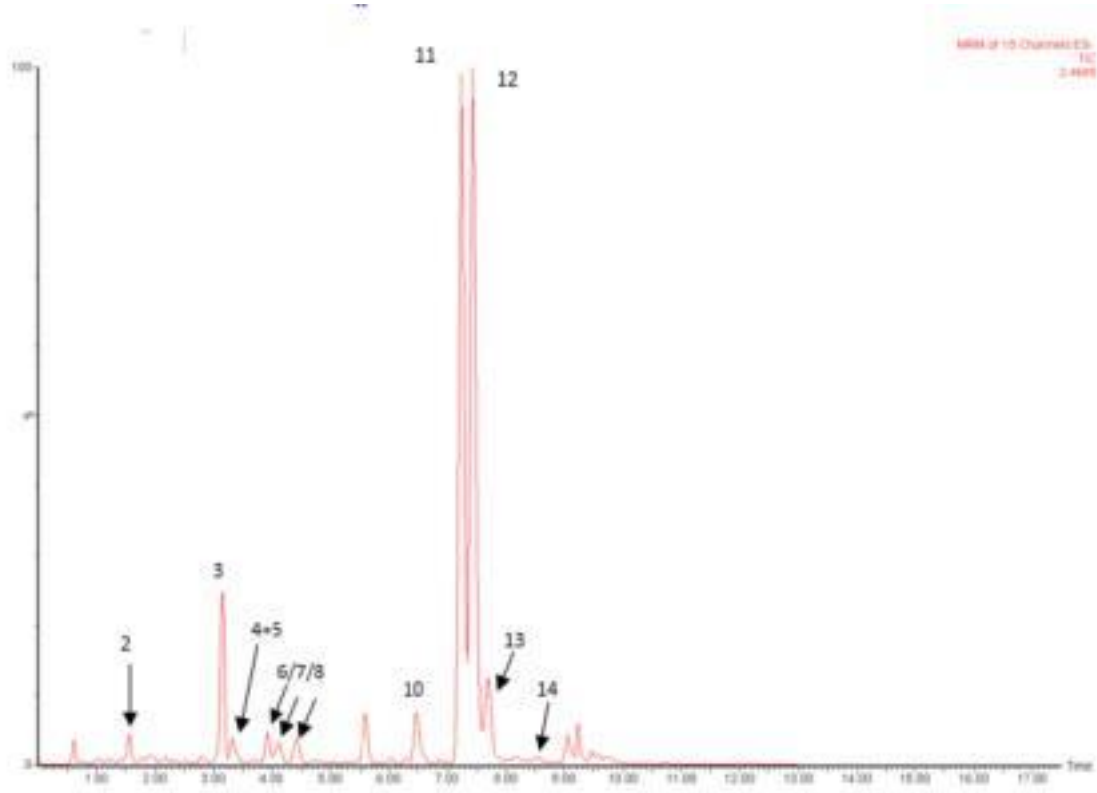

Figure 3. Total ion chromatogram (TIC) obtained for white açaí by LC-ESI-MS/MS. Chromatographic and MS parameters are described in the text. 2) 3.4-dyhydroxibenzoic acid; 3) 4-hydroxybenzoic acid; 4+5) chlorogenic acid + catequin; 6) vanillic acid; 7) caffeic acid; 8) syringic acid; 10) *p*-coumaric acid; 11) isoorientin; 12) orientin; 13) ferulic acid; 14) taxifolin.

The phenolic profile of white açaí is in line with that of previous reports for purple açaí and other species belonging to *Euterpe* genus such as *Euterpe precatoria* and *Euterpe edulis* (Pacheco-Palencia et al., 2009; Gordon et al., 2012, Dias et al., 2013, Schulz et al., 2015, Carvalho et al., 2016, Garzón et al., 2017). Gordon et al (2012), Garzón et al (2017) and Pacheco- Palencia et al (2009) found vanillic acid to be the major phenolic acid in *Euterpe oleracea* (purple) and *Euterpe precatoria* samples, but did not report ferulic acid as an abundant compound in the samples. The contents of syringic and 3.4-dihydroxibenzoic acids found by Carvalho et al (2016) in commercial samples of purple açaí pulp are higher (averaging 11 µg.g^{-1} and 13.24, respectively) than that of white açaí pulp. However, the content of *p*-coumaric acid and 4-hydroxibenzoic acid found were similar (3.08 µg.g^{-1} and 10.78 µg.g^{-1}, respectively) to those found in white açaí (4.67 µg.g^{-1} and 13.38, respectively), whereas Gordon et al (2012)

did not find *p*-coumaric in different açaí ripening stages, as well as Garzón et al. (2017) in Colombian purple açaí pulp samples.

Taxifolin, orientin and isoorientin have been previously detected in *Euterpe* species genus. Pacheco-Palencia et al (2009) first determined a taxifolin conjugated (taxifolin deoxyhexose) in *Euterpe oleracea and Euterpe precatoria*. Later, Gordon et al (2012) found free taxifolin in *Euterpe oleracea* at concentrations close to the present study (2 μg.g-¹), whereas Garzón et al (2017) succeed in identifying and quantifying taxifolin in the free and conjugated form in purple açaí pulp from Colombia. In turn, orientin and isoorientin were first reported in *Euterpe oleracea* by Schauss et al. (2006b) and later by Pacheco-Palencia (2009) and Kang et al. (2011) in both *Euterpe oleracea and Euterpe precatoria*. Studies have found these isomers to be the predominant phenolic compounds in *Euterpe* species, which is in line with the results obtained in the present study (Pacheco-Palencia et al., 2009, Gordon et al., 2012, Carvalho et al., 2016, Garzón et al., 2016). The contents of orientin and isoorientin found in white açaí are higher than those related for *Euterpe oleracea* from Colômbia (150 μg.g-¹ and 99 μg.g-¹, respectevely) (Gárzon et al., 2017) and from the Amazon (112 μg.g-¹ and 30 μg.g-¹) (Gordon et al., 2012). Therefore, the results show white açaí as a source of phenolic acids and flavonoids other than anthocyanins, which have been shown to possess bioactive properties, such as antioxidant and anti-inflammatory capacities (Kang et al., 2011). Given this, white açaí pulp can be used as a novel source of these compounds for diet in order to benefit from their potential health properties.

3.3 Antioxidant capacity

The TPC content in white açaí samples was lower than that for purple açaí of 35 mg GAE.g-¹ DW (Rufino et al., 2010, Gordon et al., 2012, Garzón et al., 2017), but closer to that of bacaba (*Oenocarpus bacaba)* (17.59±1.01 mg GAE.g-¹DW) and buriti (*Mauritia flexuosa)* (16.11 mg GAE.g-¹DW), both palm trees from the *Areceae* family (Finco et al., 2012, Cândido et al., 2015). The scavenging capacity towards peroxyl radical (ORAC) was 282.83 μmol.g-¹ TE. ORAC and TPC values were lower than that reported by Schauss et al. (2006b)
(997 μmol TE g−¹) and Kang et al. (2012) (1014 μmol TE g−¹) for purple açaí. Such difference is likely related to the anthocyanin content, which is much lower in white açaí (Rogez, 2000). Probably, this açaí type lacks key enzymes for the production of cyanidin 3-glucoside and cyaniding 3-rutinoside, the major anthocyanins in purple açaí (Tanaka, Sasaki, Ohmiya, 2008).

It is believed that anthocyanins are the main responsible for the high antioxidant activity of purple açaí, a fact that might explain the difference in ORAC and TPC between the samples (Pacheco-Palencia, Ducan, Talcott, 2009).

In order to compare the antioxidant capacity of white açaí with others fruits commonly consumed, Table 5 shows the TPC content and scavenging capacity of white açaí towards reactive oxygen species. Despite possessing lower TPC and ORAC in comparison to purple açaí and other *Euterpe* species, it is noticeable that white açaí stands out or shows equivalent contents of TPC to most of the fruits displayed. Moreover, ORAC value is between twice and twelve times higher in white açaí than most of the tropical fruits listed.

In turn, HOCl is a non-specific oxidising and chlorinating OCl− radical involved in a variety of chemical and biochemical processes including its reaction with various biomolecules such as proteins, carbohydrates, phospholipids and DNA (Berto et al.. 2015). Thus, effective HOCl scavengers are of interest in the management of oxidative stress. For white açaí, IC_{50} (amount of freeze-dried samples.mL-¹ to achieve 50% inhibition) was achieved at the concentration of 0.027 mg. mL-¹. White açaí has higher scavenging capacity than *Petit verdot* grape rachi (0.035 mg.mL-¹), *Syrah* pomace (0.031 mg.mL-¹) and Syrah rachis (0.042 mg.mL-¹) (Melo et al., 2015). Furthermore, white açaí was more efficient than *Chenin blanc* grape pomace (0.128 mg.mL), trolox (0.134 mg.mL-¹) and 5-caffeoylquinic acid (0.056 mg.mL-¹) (Rodrigues et al., 2013, Melo et al., 2015).

H_2O_2 alone is practically harmless, whereas it may spread easily through the cell membranes, for example, the membrane of the nucleus, and can react with transition metals (Cu^+ and Fe^{2+}) inside cells (Fenton reaction) to generate the most reactive ROS, namely hydroxyl radical (HO·) (Chisté et al., 2012). Samples did not show scavenging capacity towards hydrogen peroxide up to the maximum tested concentration (5 µg dry weight.mL-¹). Vissoto et al. (2013) found no or a negative correlation between this property and phenolic compounds concentration. Likely, the bioactive compounds considered in this study do not contribute greatly to the scavenging capacity of this specific ROS (Vissoto et al., 2013).

Table 5. Total phenolic compounds and scavenging capacity of white açaí towards reactive oxygen species and comparison with other fruits.

Fruit	Scientific name	Assay		
		TPC	ORAC	HOCl
		mg GAE.g-1[a]	µmol TE. g-1 [a]	IC_{50} = mg.mL-1
White açaí	*Euterpe oleracea*	11.20±0.35	282.83±45.58	0.027
Purple Açaí[f]	*Euterpe oleracea*	34.37	1441.67	-
Bacaba[b]	*Oenocarpus bacaba*	30.85	190	-
Buriti[c]	*Mauritia flexuosa*	16.73	132	-
Starfruit[d]	*Averrhoa carambola L*	31.72	279.81	-
Açaí [e]	*Euterpe edulis*	75.55	-	-
Açaí[f]	*Euterpe precatoria*	73.00	1828	-
Cajá[g]	*Spondias lutea L*	5.92	0.15	-
Caju[g]	*Anarcadium sp*	17.19	1.30	-
Araçá-boi[g]	*Eugenia stipitata Mc. Vaugh*	4.13	23.90	-
Guava[d]	*Psidium guajava L.*	11.20	130.60	-
Mango[d]	*Mangifera indica L*	6.31	38.54	-
Papaya[d]	*Carica papaya L.*	0.2	27	-
Inajá[g]	*Maximiliana maripa Aublet Drude*	17.11	27.4	-
Murici[g]	*Byrsonima crassifolia L. Kunt*	29.91	22.3	-
Pineapple[d]	*Ananas comosus L. Merril*	3.21	41.68	-
Grape rachi [h]	*Petit verdot*			0.035
	Syrah			0.042
Grape pomace [h]	*Chenin blanc*			0.128
	Syrah			0.031
Galic acid IC_{50} = mg.mL-1				
0.019				

[a] Dry weight;[b] Finco et al., (2012), [c] Cândido et al., (2015), [d] Isabelle et al. (2010), [e] Schulz et al., (2015), [f] Kang et al., (2012), [g] Neves et al, (2015), [h] Melo et al., 2015.

Summarily, the results suggest that white açaí has high scavenging capacity towards radical species with physiological relevance, standing out from fruit pulps commonly consumed. Since antioxidant properties interfere with diverse reactions related to oxidative stress leading to the development of various pathological conditions, here the great potential of white açaí as a functional food is shown, highlighting the necessity for further studies on the bioactivities of this product.

3.4 Effect on the formation of nitroso compounds

On the basis of epidemiologic studies, the International Agency for Research on Cancer panel considers the colorectal cancer risk associated with red and processed meat intake to be convincing and recommends limiting the consumption of red meat and avoiding the consumption of processed meat (IARC. 2015). On the other hand, research show the key role played by diet on reducing CRC risk, mainly by providing vitamins and polyphenols capable of blocking nitrosation reaction and therefore decrease endogenous formation NOC. Here, we tested the hypothesis that white açaí, which possesses polyphenols, might work as an inhibitor of the nitrosation reaction.

Figure 4 shows the contribution of different NOCs to the ATNC detected in control and intervened samples in both of the systems studied (A and B). Nitrosothiols were predominant, accounting for more than 60% of ATNC formed. Nitrosyl-haem represented another fraction of NO-contributing compounds (6-13%), whereas N-nitroso compounds not yet distinguishable, such as N- Nitrosopyrolidine, N-Nitrosourea, accounted for 6-19%. Nitrosothiols formation is mainly due to the reaction between nitrosable thiols groups present in hemoglobin and bovinum serum albumin (cysteine residues) and nitrosating species to give S-Nitrosocysteine, S-Nitrosoglutathione and S-Nitrosoalbumin. As for nitrosyl heme, it is produced from nitrosable groups of the prosthetic part (heme) of myoglobin and haemoglobin (Bonnett et al., 1975). These results are in line with previous studies on the *in vitro* formation of nitroso compounds under gastric conditions (Kuhnle et al., 2007, Pereira, Barbosa, Laranjinha, 2015), which have shown that nitrosothiols are preferably generated at this stage of the digestion, probably for possessing a higher constant reaction k (465000 $M^{-2}s^{-1}$) than that of nitrosamines formation (4600 $M^{-2}s^{-1}$) (Kuhnle et al., 2007, Pereira, Barbosa, Laranjinha, 2015).

Table 6 and Figure 5 show the amount of nitroso compounds formed during incubation of control and intervened samples in systems A and B. With respect to system A, the incubation with white açaí caused the ATNC to reduce notably, yielding nearly 70% inhibition. With regard nitrosothiols and nitrosyl heme, white açaí attained 66% and 56% inhibition, respectively. In addition, there was 91% inhibition of other nitroso compounds, which might comprise nitrosamines possessing carcinogenic properties.

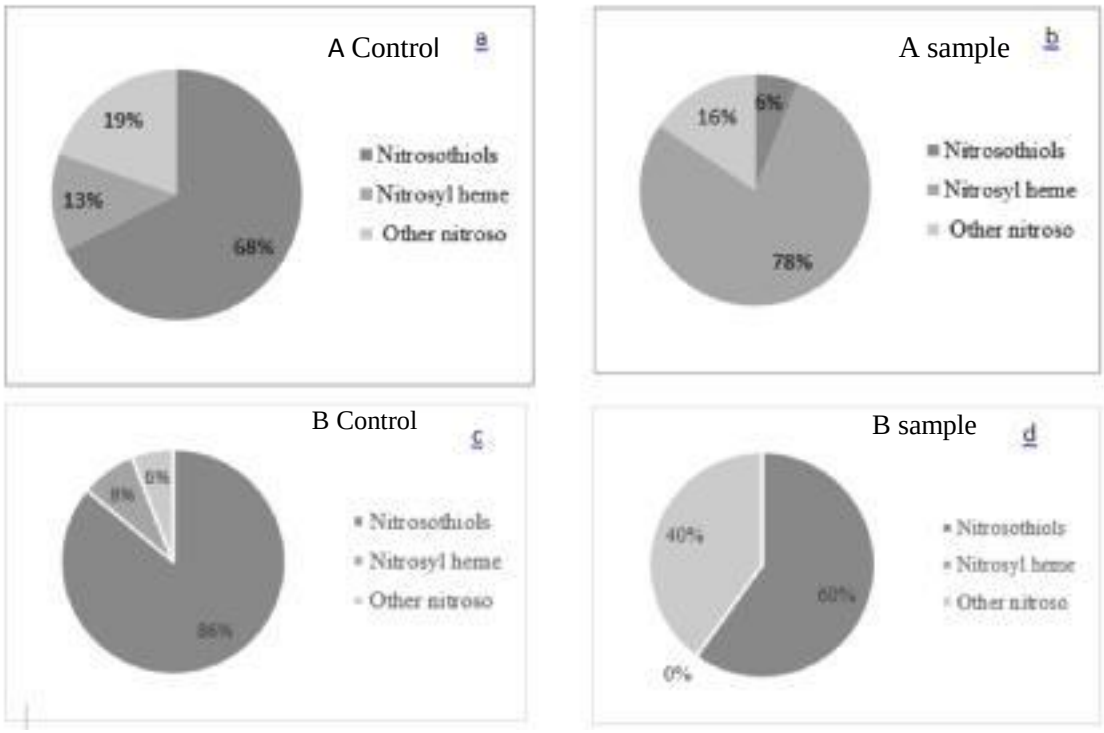

Figure 4. Composition of ATNC formed during *in vitro* incubation in the presence and absence of white açaí in both systems studied: (a) control of system A (myoglobin+BSA+haemoglobin) and (b) system A after intervention with white açaí; (c) control of system B (minced beef) and (d) system B after intervention with white açaí.

With regard to system B, which aimed to simulate a more realistic situation by incubating minced beef instead of pure compounds (Figure 2b), ATNC, nitrosothiols and nitrosyl heme values for control and intervened samples had a higher magnitude than system A (more than 10 times higher). This might be explained by the fact that real beef samples contain much more nitrosable sites, such as amines, cysteine residues and haem compared to the pure compounds used in system A. The inhibition capacity of white açaí towards nitrosating reaction was confirmed in system B, as can be seen by the large dropping in ATNC levels when the sample was present, yielding 85% inhibition (Table 6). Nitrosothiols formation was hindered in 89% whereas nitrosyl haem attained 100% inhibition. On the other hand, in contrast to system A, no inhibition effect was verified for the others nitroso compounds since control and intervened samples had similar responses (0.49 µM vs 0.56 µM. respectively). It may be that other nitrosable compounds not present in system A and for which white açaí did not have any blocking activity for, contributed to the ATNC determined in system B.

Table 6. Nitroso compounds formed under simulated stomach conditions and inhibition

Compound	NO (µM)					
	System A[a]			*System B*[b]		
	Control	**White açaí**	**Inhibition (%)**	**Control**	**White açaí**	**Inhibition (%)**
ATNC[c]	0.67±0.05	0.20±0.02	69.87	8.76±0.03	1.37±0.13	84%
Nitrosothiols	0.48±0.03	0.16±0.02	66.17	7.55±0.20	0.81±0.09	89%
Nitrosylhaem	0.08±0.01	0.03±0.01	56.74	0.72±0.12	ND[e]	100%
Other NOCs	0.114	0.01±0.00	91.23	0.49±0.05	0.56±0.09	0%

a System A: Myoglobin (0.4 mg.mL-¹) + haemoglobin (0.13 mg.mL-¹) + bovinum serum albumin (0.05 mg.mL-¹) + gastric fluid pH 2.2

b System B: 1 g raw minced meat (5% fat) + gastric fluid pH

2.2 c Apparent Total Nitroso compounds

Nitrosothiols role as contributors to the development of CRC is still under debate, as they are molecules of natural biological occurrence and may exhibit transporting and signaling functions (Rychter et al., 2016). However, it is known that nitrosothiols are NO carriers and at higher pH might decompose and act as NO donors (Rychter et al., 2016). On this basis, it has been hypothesized that although not being carcinogenic itself, nitrosothiols can act as a nitrosating agent on further sites of the gastrointestinal tract (Kuhnle et al., 2007). Therefore, since the small and the large intestine are rich in amine residues, possibly nitrosothiols produced at early stages of digestion may contribute for endogenous NOC production later in the gastrointestinal tract (Lunn et al., 2007, Kuhnle et al., 2007).

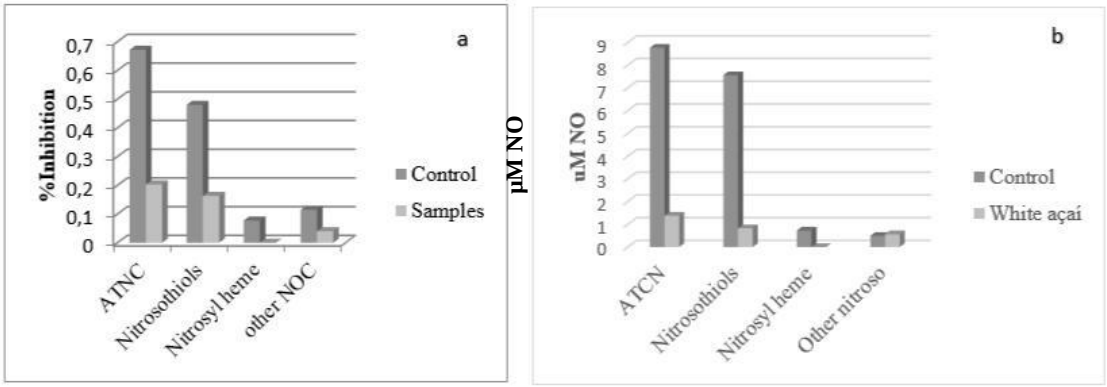

Figure 5. Contents of nitroso compounds formed during incubation of (a) system A (myoglobin + haemoglobin + bovinum serum albumin) and (b) system B: minced beef, both in the presence (samples) and absence (control) of white açaí.

In turn, nitrosyl heme was found to be main nitroso class found in feces of volunteers fed a high red meat diet (Kuhnle et al., 2007). There has been a consensus that heme is implicated in increasing CRC risk (Lunn et al., 2007, Bussche et al., 2014, Bastide et al., 2010, Bastide et al., 2015). One suggested mechanism is that heme catalyses endogenous nitrosation, since it can become readily nitrosylated and likewise nitrosothiols, act as nitrosating agent throughout the small and large intestine (Bonnett et al., 1975, Kuhnle et al., 2007). Santarelli et al (2010) found that nitrosylation of haeme was a key event in promoting an increase in ATNC in rats fed processed meat. Through the presence of such substances in the intestine, highly reactive alkylating agents such as diazoacetate can be formed to give the nitroso-compound-specific DNA adduct O_6CMeG, which has been found previously to increase in colonic cells of the colon in response to red meat intake (Lewin et al., 2006, Pierre et al., 2010).

Therefore, both *in vitro* systems studied indicated that red meat contains nitrosable constituents that upon the presence of nitrosating substances and a favourable reaction media yield nitroso compounds. Such a scenario may take place endogenously when they react with nitrite, e.g. from water, cured red meat, fruits and vegetables, nitrate-nitrite endogenous cycle, or NO donors. Alternatively, nitrosation reaction might occur due to curing process in processed meat, so that NOCs would be produced previously to consumption.

On the other hand, under the studied conditions, white açaí showed a noteworthy performance to inhibit nitrosation reaction by efficiently suppressing nitrosothiols and nitrosyl

heme formation, likely by participating in the redox chemistry in the simulated gastric environment. The active components exerting this effect are possibly polyphenols, which under acidic conditions usually react with nitrite more rapidly than most amino, reducing nitrite to NO and resulting in the formation of the corresponding phenoxyl radical (Bartsch, Ohshima, Pignateli, 1988, Pereira, Barbosa, Laranjinha, 2015). Hence, the results may indicate a possible protective mechanism of action of white açaí towards a high red meat diet, an effect that must be further investigated in *in vivo* assays. Moreover, they open up new possibilities for further research on the application of phenolic compounds-enriched extracts of white açaí for preventing NOCs formation during cured meat manufacturing.

4 Conclusion

This work provides the first data on the phenolic composition by LC-MS/MS and potential health benefits of white açaí pulp (*Euterpe oleracea)*. Twelve phenolic compounds were identified in the samples, 10 were both identified and quantified. The results showed a non-anthocyanin phenolic profile very similar to the purple type, containing vanilic acid as the major phenolic acid and orientin as the more relevant flavonoid. Moreover, white açaí had scavenging capacity towards oxygen reactive species with physiological relevance (ROO· and HOCl). Finally, results showed that white açaí pulp is capable of inhibiting nitrosation reaction in an *in vitro* gastric system, suggesting that this may be a possible protective mechanism of action of this fruit towards formation of nitroso compounds, an evidence that should be further investigated in *in vivo* assays.

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DISCUSSÃO GERAL

O consumo de frutas processadas tem aumentado continuamente ao longo dos anos devido ao conhecimento dos efeitos à saúde oriundos de uma dieta rica nesses alimentos, aliado a crescente demanda por produtos seguros, estáveis e práticos. Nesse contexto, a tecnologia de alta pressão hidrostática (APH) é um método de conservação emergente, desenvolvido como uma alternativa ao tratamento térmico com a premissa de promover maior preservação das características sensoriais, nutricionais (vitamina C, folatos) e de compostos antioxidantes (compostos fenólicos) dos alimentos, resultando em produtos com características mais próximas ao produto *in natura*. Em razão disso, tem sido amplamente empregada para a conservação de produtos à base de frutas, como sucos e polpas.

O Brasil destaca-se como o terceiro maior produtor mundial de frutas, sendo reconhecido mundialmente por sua rica flora nativa, portanto, tem um enorme potencial para a fabricação de produtos à base de frutas. Nesse sentido, a APH pode representar uma alternativa interessante para produzir, a partir das frutas presentes no país, alimentos seguros, com elevada qualidade sensorial, nutricional, funcional e com alto valor agregado, ajudando a aumentar os ganhos do País nesse setor e a alavancar a sua participação no mercado internacional.

Dentre as frutas com elevado potencial de mercado está o açaí (*Euterpe oleracea),* que atualmente é consumido no mundo inteiro na forma de bebida ou polpa congelada pasteurizada. Em 2015, constituiu o principal produto do extrativismo vegetal não-madeireiro, movimentando R$ 480 milhões com uma produção de cerca de 216 mil toneladas. Essa demanda nacional e internacional pelo fruto está relacionada com o fato de o açaí ser considerado uma superfruta devido as suas excelentes propriedades nutricionais e funcionais, contendo minerais, fibras, vitaminas, ácidos graxos insaturados, além de apresentar uma das maiores capacidades antioxidantes já registradas entre as frutas. O conteúdo de antocianinas, ácidos fenólicos, flavonas e tocoferóis do fruto tem sido associado aos efeitos à saúde verificados, portanto, a preservação dos mesmos no produto que chega ao consumidor é de elevada importância. Não menos importante, parâmetros como sabor, aroma e cor também devem ser preservados.

Nesse sentido, o efeito deletério do tratamento térmico a estas características dos alimentos já foi bem documentada (Hoffman-Ribani, Huber, Rodriguez-Amaya, 2009, Rawson et al., 2011). Assim, a alta pressão hidrostática pode representar uma técnica de pasteurização alternativa à térmica para a conservação da polpa de açaí, o principal produto derivado do fruto.

Estudos da APH para a conservação de produtos à base fruta têm indicado que esta técnica possui um efeito positivo para preservar compostos bioativos e nutricionais, porém os resultados variam muito em função da matriz. Nesse contexto, é essencial a realização de estudos que investiguem o efeito dessa nova tecnologia em cada matriz alimentícia e avaliem se de fato ela é vantajosa em relação ao método tradicional no que se refere a preservação dos compostos citados. Assim, este trabalho comparou o efeito destas duas técnicas de conservação nos compostos fenólicos antociânicos, não-antociânicos, tocoferóis e capacidade antioxidante contra espécies reativas do oxigênio e do nitrogênio da polpa de açaí roxo. Os resultados de ambos os tratamentos foram comparados aos obtidos para amostras controle, as quais não sofreram nenhum tratamento além do congelamento.

Porém, para que tais parâmetros sejam avaliados, o primeiro passo é realizar a separação seletiva e extração exaustiva dos compostos de interesse da matriz. Dentre os fatores determinantes para a realização de uma extração bem sucedida, a composição do solvente extrator, o tempo de contato da amostra com o solvente, a proporção de amostra:volume de solvente, têm sido demonstrados como parâmetros importantes (Pompeu et al., 2009). Além disso, no caso da extração das antocianinas, o uso de solventes acidificados é recomendado para favorecer a estabilidade do íon flavílium, a forma da antocianina contendo duplas ligações conjugadas responsáveis pela absorção da luz em torno de 500 nm resultando na cor típica destes pigmentos (Cavalcanti et al., 2011, Borges et al., 2011). Sobre esse aspecto, é importante considerar que elevadas concentrações de ácido podem promover a hidrólise da estrutura antociânica, portanto, a concentração de ácido também constitui um fator importante a ser avaliado (Bochi et al., 2014). Nesse sentido, ferramentas estatísticas de otimização são úteis por viabilizar, com um reduzido número de experimentos em comparação ao método univariado, a investigação simultânea do efeito de diferentes variáveis sobre o sistema estudado, levando em consideração efeitos de interação (Ferreira et al.. 2009).

Assim, a composição do solvente extrator foi o primeiro componente investigado no estudo de otimização da extração dos compostos fenólicos antociânicos e não antociânicos da polpa de açaí. Através de um planejamento de misturas simplex centroide foi avaliado o efeito dos quatro principais solventes usados na literatura (acetona, metanol, água, etanol) no teor de compostos fenólicos totais (CFT) e antocianinas totais (AT). O uso de solventes orgânicos em processos de extração é necessário para promover a degradação da parede celular vegetal e facilitar a liberação dos compostos de interesse. Além disso, misturas de solventes são vantajosas porque modificam a polaridade do solvente puro, favorecendo a interação entre os

compostos alvo e as moléculas do solvente extrator (Metrouh-Amir et al., 2015). Assim, o estudo do efeito do solvente e de combinações de solventes através de ferramentas estatísticas é a melhor forma de avaliar efeitos isolados, sinérgicos e antagônicos, de forma a gerar modelos de regressão que expliquem o sistema estudado e possibilitem a determinação da composição da solução extratora com melhores rendimentos de extração. No presente estudo, os modelos de regressão gerados para as respostas avaliadas (CFT e AT) foram significativos e sem evidência de falta ajuste a 95% de confiança, indicando que são apropriados para realizar previsões dentro da região experimental estudada.

O metanol mostrou-se como o solvente puro mais eficiente para a extração de AT e CFT. Entretanto, os resultados indicaram que o uso de solventes binários, como etanol:água e acetona:água promoveram uma elevação significativa dos teores destes compostos em comparação aos valores obtidos pelos solventes isolados. Além disso, as concentrações alcançadas nestes ensaios foram similares as observadas para o metanol puro. Estes resultados revelam efeitos sinérgicos entre estes solventes, enfatizando a importância da mistura de solventes para a eficiência da extração. Em complemento, indicam que através de diferentes combinações de solventes é possível atingir resultados equivalentes, o que confere liberdade ao pesquisador para escolher de acordo com o que for mais conveniente para o contexto da pesquisa.

Embora o metanol puro e as combinações binárias citadas tenham resultado em elevados valores de CFT e AT em relação as demais, os resultados indicaram um efeito sinérgico significativo das combinações ternárias envolvendo acetona: metanol:água e acetona:etanol:água, as quais proporcionaram um aumento de 45% e 15%, respectivamente, para AT, e em média 16% para os CFT. Assim, se esta última fosse a única resposta sendo avaliada, é possível afirmar que haveria uma grande liberdade de escolha entre as combinações de solventes, sendo possível optar entre misturas binárias ou ternárias, utilizando metanol, etanol ou acetona. Entretanto, a mistura contendo metanol resultou em um coeficiente de regressão ternário mais elevado para ambas as respostas, especialmente para as antocianinas, indicando haver uma interação sinérgica mais elevada entre estes solventes. Ademais, o teor de antocianinas desta combinação foi significativamente mais elevado (p<0.5) do que aquele observado quando o etanol foi utilizado. Assim, a combinação acetona:metanol:água foi escolhida. Com base nisso, através da utilização do método de otimização simultânea de respostas proposto por Derringer e Suich (1980), foi possível determinar uma combinação de

solventes que utilizou o mínimo de solvente orgânico, mas manteve elevados rendimentos de extração de AT e CFT.

Em seguida, foram avaliados os efeitos do tempo de extração, da razão sólido/líquido (S/L) e da concentração de ácido sobre as mesmas respostas através de um planejamento composto central 2^3. Como resultado, nenhuma das variáveis influenciou a extração das antocianinas, provavelmente porque estas substâncias estão localizadas na região mais externa do fruto, o que permite a sua rápida solubilização no solvente de extração (Pompeu et al., 2009).

Por sua vez, os CFT foram significativamente afetados pelo tempo e pela razão S/L. O modelo de regressão gerado foi significativo e sem evidência de falta ajuste a 95% de confiança. Os resultados indicaram que o aumento do tempo de extração é positivo, porém até um certo ponto, a partir do qual a exposição da amostra às condições ambientes (oxigênio, luz) pode favorecer a sua degradação. Além disso, a razão S/L também foi uma variável importante, sendo observado que o uso de volumes de solvente na região extrema do planejamento (próximo a 30 mL) elevou a eficiência da extração. É provável que com volumes de solvente inferiores tenha ocorrido a saturação do solvente, o que explica os resultados observados para essa variável.

Os efeitos de interação entre razão S/L e tempo foram altamente significativos, e apresentaram um coeficiente de regressão negativo, o que indica que se elevados volumes de solvente extrator são utilizados, é possível reduzir significativamente o tempo de extração. Assim, através da combinação de ferramentas estatísticas, foi possível determinar a melhor condição de extração dos CFT e das AT da polpa de açaí (31:24:45 acetona:metanol:água v/v/v, razão S/L 1:54, 0.1% de ácido clorídrico, 6 minutos de extração).

Estas condições foram utilizadas para extrair compostos fenólicos e antocianinas totais das amostras, e também foram usadas para a determinação do conteúdo individual destes compostos. Para tanto, foi necessário o estabelecimento de métodos cromatográficos que permitissem a separação e quantificação dos mesmos. Neste trabalho, para a fração antociânica, foram monitoradas apenas as antocianinas majoritárias do açaí (cianidina-3-glicosídeo e cianidina-3-rutinosídeo). Já a fração não-antociânica, que envolve ácidos fenólicos e outros flavonoides, consistiu de um sistema mais complexo, compreendendo 12 compostos com características químicas diversificadas. Assim, tendo em vista que ferramentas estatísticas multivaridas vem sendo utilizadas com sucesso para a otimização de sistemas cromatográficos (Ferreira et al., 2007), um planejamento composto central 2^3 foi utilizado para avaliar o efeito

da concentração inicial e final de acetonitrila, e tempo de gradiente linear na resolução de 5 pares de picos de compostos fenólicos não- antociânicos que apresentaram maior dificuldade de separação. A otimização foi realizada em uma coluna C18 instalada em um cromatografo líquido de ultra-alta eficiência, o qual permite realizar separações cromatográficas com a mesma eficiência de um equipamento de alta eficiência, porém em menores tempos e utilizando menores volumes de solvente.

Os dados foram ajustados a modelos lineares e quadráticos e apenas um deles não foi significativo. Porém, todos os modelos apresentaram evidência de falta de ajuste a 95% de confiança, o que os tornaria inadequados para realizar previsões, inviabilizando a sua utilização para a otimização. É possível que a falta de ajuste tenha sido resultado dos baixos valores do erro puro, os quais são uma medida do erro experimental do planejamento. Provavelmente, estes baixos valores foram obtidos porque o erro puro foi determinado a partir apenas das replicatas do ponto central, já que replicatas genuinas não foram realizadas para os demais ensaios. Como o sistema cromatográfico é bastante estável, a diferença entre as resoluções nesses ensaios foi muito baixa e consequentemente o erro puro também. Assim, o quociente entre a média quadrática do erro puro e a média quadrática da falta de ajuste (valor de $F_{calculado}$) pode ter sido inflacionado, resultando na falta de ajuste observada. No entanto, o exame dos gráficos de resíduos indicou não haver indícios de heterocedasticidade, e o gráfico dos valores observados vs preditos mostrou boa concordância entre si. Além disso, os modelos foram eficientes para predizer as condições ótimas do sistema, indicando que de fato eles são adequados para realizar previsões.

O comportamento cromatográfico dos compostos indicou que o tempo de gradiente linear foi a variável mais importante para a separação dos pares de picos monitorados, sendo que um aumento neste parâmetro promoveu um aumento na resolução. Já a influência da concentração inicial e final de acetonitrila variou de acordo com o par de picos avaliado. A partir do uso da metodologia de otimização simultânea de Derringer e Suich (1980) foi possível determinar as condições cromatográficas ótimas para obter a separação dos 12 compostos fenólicos investigados (7.8% a 15.2% de acetonitrila em 8 minutos de gradiente linear). A qualidade do método foi avaliada e os resultados indicaram que o mesmo foi adequado para realizar a quantificação das amostras. Além disso, os compostos fenólicos não-antociânicos de 3 amostras comerciais de polpa de açaí foram determinados com sucesso, mostrando a adequabilidade do método para avaliar o efeito dos métodos de conservação.

Métodos cromatográficos para a separação e quantificação das antocianinas majoritárias e dos tocoferóis da polpa de açaí também foram otimizados e a qualidade dos mesmos também foi examinada. Todos mostraram-se adequados para realizar quantificações e assim foram utilizados para determinar o efeito dos métodos de conservação nestes compostos. Estes métodos, bem como as condições de extração descritas, foram aplicados para determinar o efeito da alta pressão hidrostática e da pasteurização térmica nos compostos bioativos de polpa de açaí roxo.

Para as antocianinas (totais e individuais), os resultados indicaram que ambos os métodos aplicados promoveram uma redução significativa do teor desses compostos em relação a amostra controle. Outros estudos verificaram resultados similares, os quais foram atribuídos a atividade enzimática residual presente nas amostras tratadas por alta pressão, uma vez que tem sido demonstrado que esta técnica não é capaz de promover 100% de inativação das enzimas (Terefe et al., 2013). Apenas as amostras tratadas a 600 MPa não diferiram das amostras controle.

Em relação ao tratamento térmico, dentre os fatores que afetam a estabilidade das antocianinas, a sensibilidade dessas substâncias ao calor tem sido bem documentada (Khanal, Howard, Prio, 2010). Portanto, é possível que o decréscimo observado esteja relacionado a degradação térmica das antocianinas (Terefe et al., 2013).

Comparando as duas técnicas de conservação, observou-se que apenas as amostras tratadas a 600 MPa apresentaram valores de AT significativamente maiores do que as amostras submetidas a pasteurização térmica (PT). Já para a cianidina-3-glicosídeo e cianidica-3-rutinosídeo, observou-se a ocorrência de perdas significativamente maiores destes compostos nas amostradas submetidas a PT em relação a APH. Independente da pressão aplicada, verificou-se que as amostras processadas por APH apresentaram valores de concentração de antocianinas mais próximos ao produto controle, indicando uma maior retenção destes compostos em comparação a PT (até 40% mais). Estes resultados estão de acordo com estudos anteriores que compararam as duas técnicas de conservação em frutas vermelhas como amora, ameixa, romã e morango (Patras et al., 2009, Ferrari, Maresca, Ciccarone, 2010, Gonzáles-Cerebrino et al., 2013). Além disso, não houve diferença estatística no teor de antocianinas entre as pressões aplicadas.

Os fenólicos não-antociânicos (CFNA) foram determinados utilizando o método cromatográfico otimizado e validado, observando-se que a PT não afetou significativamente o teor destes compostos, o que indica que estes são menos termo-sensíveis do que as antocianinas.

Pacheco-Palencia, Ducan, Talcott (2009) verificaram que o aquecimento da polpa de açaí a 85ºC por 1, 2, 30 e 60 min não implicou na redução do teor dos CFNA. As concentrações dos fenólicos submetios a TP também não diferiram das amostras processadas a 400, 450 e 600 MPa, mas foram significativamente menores do que aquelas processadas a 500 MPa. Por sua vez, em comparação ao controle, é possível observar que houve um aumento significativo no teor destes compostos para as amostras submetidas a 500 MPa (até 60% mais).

Em geral, tem sido demonstrado que o efeito da APH e do tratamento térmico sobre flavoides e ácidos fenólicos pode variar muito em função do composto, da matriz e das condições de processamento, sendo essencial a investigação de cada matriz alimentícia separadamente. Andrés et al. (2016) e Rodrigues-Roque et al. (2015) verificaram que o tratamento térmico provocou a degradação de alguns ácidos fenólicos e flavonoides de bebidas à base fruta, leite de vaca e soja (até 40% menos), enquanto outros não foram afetados por este tratamento.

O maior teor de CFNA verificado nas amostras pressurizadas pode estar relacionado ao aumento da extratibilidade dos compostos. A alta pressão pode provocar profundas modificações na estrutra da matriz alimentícia, causando a quebra da parede celular, o deslocamento de substâncias dentro das células, entre outras alterações que facilitam a liberação das substâncias no meio extra-celular e a interação das mesmas com o solvente extrator. Assim, a APH parece ter promovido um incremento no teor de CFNA. Embora tenha sido demonstrado que as antocianinas são as principais responsáveis pelas propriedades funcionais do açaí (Pacheco-Palência, Ducan, Talcott, 2009), a importância da fração não-antociânica já foi evidencida (Kang et al., 2011). Portanto, é possível que o aumento no teor destes compostos possa afetar positivamente as propriedades funcionais da polpa de açaí, como a capacidade antioxidante.

Embora alguns trabalhos tenham descrito os tocoferóis (α-, β-, γ-, δ-) como moléculas sensíveis à pasteurização térmica (Romeu-Nadal et al., 2008), não foram observadas diferenças significativas no teor desses compostos e na atividade da vitamina E entre a amostra controle, TP e APH. Estudos comparando o efeito da TP e APH nos tocoferóis são escassos e os resultados existentes são inconclusivos. Por exemplo, Moltó-Puigmarti et al. (2011) observaram que o teor de tocoferóis em leite humano não foi afetado significativamente pelo tratamento térmico (70ºC/30 min) nem pela alta pressão com condições similares as do presente trabalho (400/500/600 MPa/5 min). Já Cilla et al. (2012) reportaram uma diminuição no teor de tocoferois em formulações à base de fruta e leite de soja conservadas por alta pressão (400

MPa/5 min/36ºC) em relação a amostras controle e PT, enquanto Barba, Esteve, Frigola (2012), estudando uma formulação de leite com suco de laranja submetido a APH (100-400 MPa/9 min) e PT (90ºC/15 s), não encontraram diferença significativa entre a concentração de tocoferóis nas amostras contole e após aplicação de PT, mas observaram um aumento de cerca de 28% no teor de α-tocoferol e atividade de vitamina E em relação as amostras tratadas por alta pressão. Estes resultados indicam que o comportamento dos tocoferóis é fortemente dependente da matriz alimentícia e também das condições de processamento, e que mais estudos são necessários para estabelecer o efeito da APH sobre eles.

O estudo da capacidade antioxidante *in vitro* constitui uma medida importante para avaliar o potencial de uma determinada amostra no combate ao estresse oxidativo. Observou-se que todas as amostras apresentaram capacidade de sequestro das espécies reativas examinadas (ROO·, HOCl e H_2O_2), porém o método de conservação a que elas foram submetidas influenciou significativamente a extensão dessa propriedade. O mesmo comportamento foi observado para os CFT.

Para os CFT, não houve diferença significativa entre as amostras controle e as tratadas por APH, ao passo que uma diminuição significativa nos valores foi observado para as amostras PT, indicando que a APH foi mais eficiente para presevar os CFT nas amostras.

O tratamento térmico causou uma redução de 10% na capacidade de sequestro do radical peroxil (ROO·) em relação ao controle (não significativa), enquanto um aumento desta propriedade foi verificado com a elevação da pressão. As amotras processadas a 500 MPa e 600 MPa apresentaram um acréscimo de 28% e 16%, respectivamente, em relação ao controle, e foram significativamente maiores que todas as outras. Como houve boa correlação entre esta medida e o teor de CFNA, é possível que este aumento tenha sido causado pela elevação do teor destes compostos nas amostras. Outros estudos observaram tendências semelhantes de aumento da capacidade antioxidante acompanhado da elevação do teor de compostos fenólicos (Patras et al., 2009, Tadapenini et al., 2012).

Já para o HOCl, observou-se que as amostras controle apresentaram a maior capacidade inibitória (menor concentração de extrato necessária para atingir 50% de inibição da atividade do radical - IC50), sendo cerca de 3 vezes mais elevada do que a observada para PT. Embora tenham apresentado uma menor capacidade inibitória do que controle, as amostras tratadas por alta pressão mostraram menores valores de IC50 do que PT. Isto indica que a maior preservação das antocianinas nestas amostras pode ter contribuído significativamente para preservar esta propriedade da polpa de açaí, o que foi evidenciado pela correlação positiva entre

o teor destes compostos e a capacidade inibitória de HOCl. Por outro lado, o fato de 500 MPa ter resultado em polpas com teor mais elevado de CFNA não afetou esta propriedade, demonstrando uma baixa correlação entre os CFNA considerados neste estudo e a desativação desta espécie reativa. Para o H_2O_2, verificou-se que PT mostrou a maior capacidade inibitória dentre todas as amostras examinadas, e que no geral as amostras APH tiveram menores capacidades inibitórias do que a controle. Estes resultados indicam que os compostos antioxidantes considerados neste estudo não contribuíram para o sequestro desta espécie reativa. De fato, nenhuma correlação foi encontrada com o teor de compostos fenólicos (antociânicos e não antociânicos), resultado que já havia sido observado em estudos anteriores (Vissoto et al., 2013).

Assim, em vista dos resultados obtidos aliados a análise de componentes principais aplicada aos dados, é possível afirmar que a APH foi eficiente para resultar em uma polpa de açaí com elevada qualidade nutricional e funcional. Compostos antioxidantes e a capacidade de sequestro de espécies reativas do oxigênio foram mais efetivamente preservados em relação a PT, causando inclusive um aumento significativo de alguns desses parâmetros em relação a polpa controle. Além disso, os tocoferóis, a atividade de vitamina E e a capacidade inibitória de formação de compostos nitrosos não foram afetados pela alta pressão. Dessa forma, a APH é uma técnica promissora a ser utilizada para a conservação deste produto. Levando em consideração as maiores concentrações de CFNA e a maior capacidade antioxidante pelo método ORAC, o processo realizado a 500MPa foi o mais vantajoso dentre todas as condições testadas, o qual, além destas características, resultou em polpas com bons teores de tocoferóis, vitamina E e capacidade antioxidante contra as demais espécies reativas.

No que se refere a pasteurização térmica, observou-se que esta de fato promoveu uma maior degradação de importantes compostos bioativos do açaí do, como as antocianinas, e resultou em uma menor capacidade antioxidante contra espécies reativas de relevância fisiológica. Entretanto, tocoferóis, vitamina E e CFNA não foram drasticamente afetados pelo tratamento térmico, bem como a capacidade antioxidante frente à H_2O_2 e a propriedade de inibição da formação de compostos nitrosos, indicando que esta ténica também resulta em um produto com boa qualidade nutricional e funcional.

Apesar de o açaí roxo ser o mais popular, esta espécie é composta por outros tipos ou variedades que se diferenciam em relação ao tamanho, características da palmeira e principalmente em relação à coloração do fruto. Dentre elas, o açaí branco, um tipo de açaí que possui uma polpa esverdeada quando maduro, constitui a variedade mais consumida depois do

açaí roxo. Apesar disso, estudos concernentes a sua composição e potenciais efeitos à saúde são praticamente inexistentes. Assim, tendo em vista preencher esta lacuna, o presente trabalho também investigou os compostos fenólicos, a capacidade antioxidante contra espécies reativas do oxigênio e a capacidade da polpa de inibir a reação de nitrosação sob condições gástricas simuladas *in vitro*, a qual pode levar à formação de compostos relacionados com o desenvolvimento de câncer gastrointestinal.

A extração da amostra de açaí branco utilizando o método previamente estabelecido para o açaí roxo demonstrou um teor de AT não mensuráveis pela metodologia do pH diferencial, portanto, optou-se por utilizar a combinação etanol:água para realizar as extrações. Um planejamento composto central 2^2, medindo o efeito do tempo de extração e porcentagem de etanol no teor de CFT foi realizado para encontrar o melhor tempo e concentração de etanol. Os resultados do planejamento indicaram que o tempo não foi significativo para a eficiência da extração, enquanto o aumento da concentração do etanol mostrou ser vantajosa apenas até certo nível (50%), a partir da qual começou a decrescer. Pompeu et al. (2009) obtiveram comportamento semelhante para os CFT de açaí roxo, com concentrações de etanol acima de 80% promovendo diminuição da eficiência de extração. Assim, estabeleceu-se um tempo de extração de 10 minutos e um solvente extrator composto por 35:65 água:etanol v/v.

A análise do perfil de compostos fenólicos foi realizada por cromatografia líquida de ultra-alta eficiência acoplada a uma fonte de eletrospray no modo negativo e a um triplo-quadrupolo operando no modo MRM (multiple reacation monitoring), o qual constitui uma método bastante sensível e seletivo. O perfil de compostos fenólicos do açaí branco foi bastante similar ao do açaí roxo estudado neste trabalho e de estudos anteriores (Pacheco-Palencia, Ducan, Talcott, 2009, Gordon et al., 2012), com ácido vanílico, orientina e isoorientina sendo os compostos fenólicos majoritários. No entanto, o ácido clorogênico e a catequina não foram detectados no açaí roxo utilizado neste estudo, enquanto a taxifolina foi monitorada apenas para o açaí branco. Gordon et al. (2012) e Garzon et al. (2017) encontraram ácido clorogênico em açaí roxo da Amazônia brasileira e colombiana, respectivamente, enquanto a catequina foi detectada em concentrações abaixo do limite de quantificação nestes trabalhos. Pacheco-Palencia, Duncan, Talcott (2009) encontraram níveis quantificáveis de catequina em amostras de duas espécies de açaí roxo (*Euterpe oleracea* e *Euterpe edulis).*

Considerando os resultados obtidos neste estudo, o teor dos compostos fenólicos foi similar em ambos os tipos de açaí, com exceção do ácido siríngico, que foi muito maior no açaí roxo, e dos teores dos flavonoides orientina e isoorientina, os quais foram mais elevados

para o açaí branco. A potente capacidade antioxidante *in vitro* da orietina e isoorientina isoladas de açaí roxo já foi demonstrada por Kang et al. 2010, portanto, as propriedades bioativas associadas a fração não antociânica do açaí roxo (Kang et al, 2011, Kang et al., 2010) podem estar presentes no açaí branco.

A capacidade antioxidante do extrato de açaí branco também foi investigada, verificando-se que este apresentou propriedades redutoras (Folin Ciocalteu) e de sequestro de espécies reativas do oxigênio. A capacidade redutora do extrato e a sua eficiência de sequestro do radical ROO· (ORAC) foram muito menores do que as verificadas para o açaí roxo. Entretanto, os valores de ORAC e CFT observados para o açaí branco são muito superiores aos reportados para muitas frutas tropicais e subtropicais comumente consumidas no mundo. Além disso, os extratos apresentaram boa capacidade de inibição (IC50) da espécie reativa HOCl, porém, ela foi cerca de duas vezes menos potente do que o açaí roxo (controle). É possível que tais discrepâncias estejam relacionadas as diferenças no teor de antocianioninas entre estas amostras, as quais têm sido relatadas como as principais responsáveis pelas propriedades antioxidantes do açaí (Pacheco-Palencia, Duncan, Talcott, 2009).

Em contraste ao açaí roxo, não foi observada atividade inibitória dos extratos de açaí branco contra H_2O_2, indicando que os compostos fenólicos considerados não atuam na desativação desta espécie reativa. Uma vez que não foi observada nenhuma correlação entre os compostos fenólicos e o sequestro do H_2O_2 no açaí roxo, estes resultados confirmam que outras substâncias são determinantes no combate a esta espécie reativa.

As nitrosaminas são compostos nitrosos (NOCs) reconhecidos como potentes agentes cancerígenos em mamíferos (Pierre et al., 2009). A formação dessas substâncias pode ocorrer endogenamente sob as condições ácidas do estômago e contribuir para o desenvolvimento de tumores gastrointestinais (câncer de estômago, cólon, reto). Particularmente, o consumo de carne vermelha (fresca e curada) tem sido associado ao favorecimento da formação endógena dessas substâncias, uma vez que estes alimentos fornecem as substâncias nitrosantes e nitrosáveis necessárias para a ocorrência da reação de nitrosação (aminas, grupo heme, nitrito). Entretanto, os compostos antioxidantes são capazes de reagir rapidamente com espécies nitrosantes, as quais atuam como espécies reativas de nitrogênio, impedindo a formação de NOCs. Assim, no presente trabalho, foi investigada a capacidade do açaí branco e roxo para inibir a reação de nitrosação.

Para o sistema *in vitro* incluindo mioglobina, hemoglobina e albumina de sérum bovino como fonte de compostos nitrosáveis, observou-se que ambos os tipos de açaí foram

capazes de inibir a reação em uma extensão similar, sugerindo que as antocianinas não intereferiram na eficiência de sequestro das espécies nitrosantes.

Assim, os dados mostraram que o açaí branco pode ser considerado como uma boa fonte de compostos antioxidantes para a dieta. Além disso, a capacidade de inibição da reação de nitrosação sugere um potencial mecanismo de modulação do açaí (branco e roxo) na formação de nitrosaminas, efeito que deve ser investigado mais a fundo em estudos futuros.

Quanto ao efeito dos métodos de conservação, avaliado apenas para o açaí roxo, verificou-se que as amostras PT foram significativamente maiores do que a controle, porém não diferiram daquelas tratadas por APH. Por sua vez, em geral, a capacidade de inibição das amostras processadas por alta pressão não diferiram da controle. Portanto, estes resultados sugerem que outros compostos além dos fenólicos podem ter atuado para inibir a reação de nitrosação.

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CONCLUSÃO GERAL

O estudo do efeito do solvente indicou que o metanol é um importante solvente para a extração das antocianinas da polpa de açaí, ao passo que para a extração de compostos não-antociânicos este solvente pode ser substituído por etanol.

A razão sólido/líquido e o tempo mostraram-se como as variáveis mais importantes para a extração destes compostos.

As técnicas estatísticas multivariadas utilizadas foram eficientes para otimizar a extração de compostos fenólicos de açaí roxo e açaí branco, assim como foram úteis para determinar as condições cromatográficas ótimas para separação e quantificação de compostos fenólicos não antociânicos em ambas as matrizes.

A alta pressão hidrostática (APH) mostrou-se uma técnica emergente de conservação capaz de produzir polpa de açaí com elevada qualidade nutricional, e de promover uma maior retenção de compostos bioativos e propriedades funcionais do que PT.

Apesar de o tratamento térmico ter promovido perdas de antocianinas e ter apresentado uma menor capacidade de inibição da espécie reativa HOCl, os resultados dos demais parâmetros sugerem que esta ténica também resulta em um produto com boa qualidade nutricional e funcional.

A alta pressão é uma técnica de conservação em expansão, com um mercado em pleno crescimento. Portanto, o uso da alta pressão hidrostática para a conservação da polpa de açaí e de produtos derivados pode agregar valor aos mesmos, elevando a sua importância e representatividade no mercado internacional.

O açaí branco mostrou-se uma potencial fonte de compostos fenólicos não-antociânicos com atividade antioxidante contra espécies reativas do oxigênio de relevância fisiológica.

O açaí branco e o açaí roxo apresentaram excelente capacidade de inibição da reação de nitrosação, o que pode sinalizar um potencial mecanismo de proteção destes produtos contra a formação endógena de substâncias cancerígenas. As amostras de açaí roxo tratadas termicamente mostraram maior capacidade inibitória do que a controle, e a alta pressão não afetou esta propriedade em comparação aos demais tratamentos.

Para o futuro, sugere-se investigar os potenciais efeitos à saúde do açaí branco. Além disso, investigar se a alta pressão influencia na bioacessibilidade e biodisponibilidade dos compostos bioativos da polpa de açaí roxo. Ademais, examinar se a melhor retenção ou

elevação do teor extraível dos compostos estudados em relação ao produto tratado termicamente reflete-se em benefícios para a saúde constitui um campo de pesquisa importante. Finalmente, verificar se as condições favoráveis para a manutenção de um produto com potencial funcional mais elevado (no presente caso 500 – 600 MPa/5 min/ 20º) também produzem uma polpa de açaí bioquímica e microbiologicamente estável.

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