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Marcela Bromberger Soquetta

# METODOLOGIAS NÃO CONVENCIONAIS NA EXTRAÇÃO DE COMPOSTOS BIOATIVOS DE CASCA DE BERGAMOTA

Santa Maria, RS
2019

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Tese apresentada ao Curso de Pós-graduação em Engenharia Química, da Universidade Federal de Santa Maria (UFSM, RS) como requisito parcial para obtenção do título de **Doutora em Engenharia Química.**

Orientadora: Dra. Raquel C. Kuhn
Coorientadora: Dra. Lisiane de Marsillac Terra

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**Aprovado em 8 de novembro de 2019:**

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Santa Maria, RS
2019

## DEDICATÓRIA

A minha família, em especial ao meu filho Theodoro Soquetta Abaide.

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“Os sonhos são projetados por nosso arquiteto interior, mas a realização está em nossas mãos”

Nietzsche

## RESUMO

## METODOLOGIAS NÃO CONVENCIONAIS NA EXTRAÇÃO DE COMPOSTOS BIOATIVOS DE CASCA DE BERGAMOTA

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As frutas citrus, produzidas em grandes quantidades no Rio Grande do Sul, vem recebendo destaque pelo alto teor de compostos bioativos que apresentam. Estes componentes podem ser extraídos a partir dos resíduos gerados no processamento destas frutas, possibilitando a utilização destes subprodutos, bem como a utilização dos extratos para enriquecimento nutricional, proteção e/ou pigmentação na indústria alimentícia. A extração convencional baseia-se no aquecimento e/ou agitação da matéria-prima com solventes orgânicos. As extrações não convencionais são consideradas promissoras, podendo reduzir ou eliminar o uso de solventes orgânicos e minimizar a degradação dos compostos, o tempo e o gasto de energia. A extração assistida por ultrassom (EAU) e a extração com fluido supercrítico (EFS), vêm se destacando nos últimos anos. Uma alternativa para a redução no uso de solventes orgânicos pode ser a utilização da água eletrolisada (AE). Portanto, o objetivo deste trabalho foi extrair compostos bioativos de casca de bergamota usando água eletrolisada como solvente na extração ultrassônica e co-solvente na extração com fluido supercrítico ($CO_2$). Primeiramente, foram realizadas as caracterizações físico-química e microbiológica, a determinação de compostos bioativos (fenólicos e flavonoides totais) e atividade antioxidante (DPPH, radical superóxido, hidroxil e FRAP) das variedades *Citrus deliciosa*, *Citrus reticulata* e *Citrus reticulata* Blanco e a comparação da extração convencional com etanol 80% e empregando os três tipos de AE, ácida (AEA), básica (AEB) e levemente ácida (AELA), como soluções extratoras. Após, foram testados os métodos de extração com ultrassom, fluido supercrítico e a combinação dos dois, usando água eltrolisada como solvente e como co-solvente (EFS), respectivamente. Conforme os resultados, a variedade C*itrus reticulata* apresentou maiores valores de fenólicos totais (3681,31 mgGAE (100g) $^{-1}$) e flavonoides totais (571,56 mgEQ (100g) $^{-1}$) em relação as demais variedades. Os três tipos de água eletrolisada apresentaram maior capacidade de extração de compostos bioativos do que o etanol 80 %, a variedade *Citrus reticulata* com AEB extraiu 25 % a mais de fenólicos totais e 50 % de flavonoides, já a AEA foi superior em 14 % na atividade antioxidante (FRAP). A EAU apresentou 87,5 % de redução no tempo do processo em relação a extração convencional. Dentre as respostas das extrações ultrassonicas combinadas com água eletrolisada, a maior extração de fenólicos (4324,32 mgGAE (100g) $^{-1}$) e FRAP (663,69 µmolTEAC (100g) $^{-1}$) foi utilizando EAU+AELA, enquanto que para flavonoides (691,76 mgEQ (100g) $^{-1}$) foi EAU+AEA. Na EFS-$CO_2$ a AEB foi o melhor co-solvente para todas as análises. Os resultados da extração combinada, ultrassom e $CO_2$ supercrítico, não apresentaram diferença significativa a extração somente com $CO_2$ supercrítico. Para fenólicos, o melhor resultado (5344,59 mgGAE (100g) $^{-1}$) foi obtido em 40 °C, razão extrato:co-solvente 1:40 (p/v) e pressão de 10 MPa, para flavonoides (1271,53 mgEQ (100g) $^{-1}$) foi em 80°C, 1:20 (p/v) e 10 MPa e para FRAP (854,85 µmolTEAC (100g) $^{-1}$) foi a 80°C, 1:40 (p/v) e 10 MPa. As extrações não convencionais foram eficientes na redução do tempo e eliminação de solventes orgânicos na extração de compostos bioativos de casca de bergamota. Sendo a extração com fluido supercrítico ($CO_2$) usando água eletrolisada básica como co-solvente o melhor método de extração de compostos bioativos de *Citrus reticulata*.



**Palavras-chaves:** *Citrus*. Fenólicos totais. Flavonoides totais. Atividade antioxidante. Ultrassom. Fluido supercrítico. Água eletrolisada.

## ABSTRACT

## UNCONVENTIONAL METHODOLOGIES IN EXTRACTION OFBIOACTIVE COMPOUNDS FROM BERGAMOT PEEL

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Citrus fruits, produced in large quantities in Rio Grande do Sul, have been highlighted by the high content of bioactive compounds they present. These components can be extracted from the residues generated in the processing of these fruits, allowing the use of these by-products, as well as the use of extracts for nutritional enrichment, protection and/or pigmentation in the food industry. Conventional extraction is based on heating and/or stirring the raw material with organic solvents. Unconventional extractions are considered promising and can reduce or eliminate the use of organic solvents and minimize compound degradation, time and energy expenditure. Ultrasound assisted extraction (UAE) and supercritical fluid extraction (SFE) have been highlighting in recent years. An alternative for reducing the use of organic solvents may be the use of electrolyzed water (EW). Therefore, the objective of this work was to extract bioactive compounds from bergamot peel using electrolyzed water as a solvent for ultrasonic extraction and co-solvent for supercritical fluid ($CO_2$) extraction. First, the physicochemical and microbiological characterization, the determination of bioactive compounds (total phenolic and total flavonoids) and antioxidant activity (DPPH, superoxide radical, hydroxyl and FRAP) of the varieties *Citrus deliciosa*, *Citrus reticulata* and *Citrus reticulata* Blanco were compared using 80 % ethanol and the three types of EW, acidic (AEW), basic (BEW) and slightly acidic (SAEW), as extractor solutions were performed. Subsequently, the extraction methods with ultrasound, supercritical fluid and their combination were tested using electrolyzed water as solvent and as co-solvent (SFE), respectively. According to the results, the *Citrus reticulata* variety presented higher values of total phenolics (3681.31 mgGAE (100g) $^{-1}$) and total flavonoids (571.56 mgEQ (100g) $^{-1}$) compared to the other varieties. The three types of electrolyzed water presented higher extraction capacity of bioactive compounds than 80 % ethanol, the *Citrus reticulata* variety with BEW extracted 25 % more from total phenolics and 50 % from flavonoids, while AEW was 14 % higher antioxidant activity (FRAP). The UAE showed 87.5 % reduction in process time compared to conventional extraction. Among the responses of ultrasonic extractions combined with electrolyzed water, the highest extraction of phenolics (4324.32 mgGAE (100) $^{-1}$) and FRAP (663.69 µmolTEAC (100g) $^{-1}$) was using UAE+SAEW, while for flavonoids (691, 76 mgEQ (100g) $^{-1}$) was UAE+AEW. At SFE-$CO_2$ the BEW was the best co-solvent for all analyzes. The results of combined extraction, ultrasound and supercritical $CO_2$ showed no significant difference compared to supercritical $CO_2$ extraction only. For phenolics, the best result (5344.59 mgGAE (100g) $^{-1}$) was obtained at 40 °C, extract: co-solvent ratio 1:40 (w/v) and 10 MPa pressure for flavonoids (1271.53 mgEQ (100g) $^{-1}$) was at 80 °C 1:20 (w/v) and 10 MPa and for FRAP (854.85 µmolTEAC (100g) $^{-1}$) it was at 80 ° C 1:40 (w/v) and 10 MPa. Unconventional extractions were efficient in reducing the time and elimination of organic solvents in the extraction of bergamot peel bioactive compounds. Extraction with supercritical fluid ($CO_2$) using basic electrolyzed water as co-solvent is the best method of extracting bioactive compounds from *Citrus reticulata*.



**Keywords:** *Citrus*. Total phenolics. Total Flavonoids. Antioxidant activity. Ultrasound. Supercritical Fluid. Electrolyzed water.

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## APRESENTAÇÃO



## ARTIGO 1



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## ARTIGO 4

## LISTA DE ABREVIATURAS E SIGLAS

| | |
|---|---|
| AE | Água Eletrolisada |
| AEA | Água Eletrolisada Ácida |
| AEB | Água Eletrolisada Básica |
| AELA | Água Eletrolisada Levemente Ácida |
| ANVISA | Agência Nacional de Vigilância Sanitária |
| CFS | Cromatografia de Fluido Supercrítico |
| CG | Cromatografia Gasosa |
| $CO_2$ | Dióxido de Carbono |
| $CO_2$-FS | Dióxido de carbono como fluido supercrítico |
| DH | Hidrogênio dissolvido |
| DNA | Ácido Desoxirribonucleico |
| DO | Oxigênio Dissolvido |
| DPPH | Método do sequestro do radical livre estável 2,2-difenil-1-picrilhidrazil |
| EAU | Extração Assistida por Ultrassom |
| EAU+EFS-$CO_2$ | Extração combinada, Ultrassom e $CO_2$ fluido supercrítico |
| EFS | Extração com Fluido Supercrítico |
| EFS-$CO_2$ | Extração usando $CO_2$ como supercrítico |
| EMBRAPA | Empresa Brasileira de Pesquisa Agropecuária |
| FAO | Food and Agriculture Organization |
| FRAP | Ferric Reducing Antioxidant Power |
| HCl | Ácido clorídrico |
| HOCl | Ácido Hipocloroso |
| NaCl | Cloreto de Sódio |
| NaOH | Hidróxido de Sódio |
| ORP | Oxidation Reduction Potencial |
| EFS-$CO_2$-AEB | Extração utilizando $CO_2$ como fluido supercrítico e água eletrolisada básica como co-solvente |
| EFS-$CO_2$-AD | Extração utilizando $CO_2$ como fluido supercrítico e água deionizada como co-solvente |

**SUMÁRIO**

# 1 INTRODUÇÃO

As frutas desempenham um papel importante, tanto economicamente, através da comercialização de seus produtos, como nutricionalmente pelo teor de fibras, vitaminas, minerais e compostos bioativos (SOQUETTA et al., 2016; KARAM et al., 2016). As frutas cítricas, pertencentes à família das Rutáceas e do gênero *Citrus*, são originárias das regiões tropicais e subtropicais da Ásia (BUBLITZ et al., 2015). Pertencem a este grupo as bergamotas, também conhecidas como mandarinas, mexericas e tangerinas, possuindo ampla importância na cultura mundial (BABAZADEH-DARJAZI, 2017; LAZAROTTO, 2018). Os extratos citrinos são ricos em compostos bioativos e os extratos de cascas possuem alto teor de flavonóis e ácidos fenólicos (ZHANG et al., 2014).

As cascas das frutas cítricas geralmente são descartadas como resíduos no meio ambiente. Estima-se que aproximadamente um terço de todas as frutas e verduras produzidas no mundo são perdidas durante a pós-colheita e não chegam ao consumidor (SAGAR et al., 2018). Devido ao seu baixo custo e fácil disponibilidade, principalmente no Rio Grande do Sul, estes resíduos são capazes de oferecer suplementos nutricionais de baixo custo. Estudos reconhecem a presença de compostos bioativos, como polifenóis, vitaminas, minerais, fibras alimentares, conteúdo de óleos essenciais e caratenoides, o que faz dos citrus um fruto que promove benefícios à saúde (RAFIQ et al., 2018).

Segundo a legislação específica, compostos bioativos, são aqueles encontrados nos alimentos, tanto de origem natural quanto sintética e que possuem ação metabólica ou fisiológica específica, desde que comprovada a sua segurança para o consumo humano (BRASIL, 2008).

Os compostos fenólicos têm sido associados a vários benefícios à saúde, como prevenção de doenças cardiovasculares, câncer, diabetes e obesidade. E têm sido efetivamente utilizados como ingredientes funcionais nos alimentos, pois podem impedir a oxidação lipídica e prevenir fungos e crescimento bacteriano (VU; SCARLETT, VUONG, 2018).

Para a análise e exploração destes constituintes é necessária sua extração a partir da matriz celular. Diferentes métodos têm sido utilizados, podendo ser classificados como convencionais e não convencionais (CASQUETE et al., 2015). Os métodos convencionais incluem a extração com solventes orgânicos com ou sem aquecimento e agitação (RODRIGUEZ-PEREZ et al., 2015). Já os não convencionais, como extração assistida por ultrassom (EAU) e extração com fluido supercrítico (EFS), têm como objetivo preservar o meio ambiente, reduzindo ou eliminando o uso de solventes orgânicos e gasto de energia. Estas

técnicas também minimizam a degradação dos compostos bioativos, usando temperaturas moderadas e menor tempo de extração (MUSTAFA; TURNER, 2011; DA SILVA; ROCHA-SANTOS; DUARTE, 2016; TIWARI, 2015).

A extração assistida por ultrassom (EAU) tem sido recomendada por ser um sistema simples e eficiente, que pode ser operado em grande escala. Esta técnica promove uma melhor extração devido ao aumento de transferência de massa entre o solvente e o material. O mecanismo do processo é basicamente a formação de bolhas e cavitação, formando poros que facilitam a entrada da solução extratora e arraste dos compostos solúveis (TOMŠIK et al., 2016).

A extração com fluido supercrítico (EFS) tem apresentado rendimentos elevados e melhor seletividade comparada com os métodos convencionais (PEREIRA et al., 2016). Além disso, tem a capacidade para extrair compostos termicamente lábeis sob condições de temperaturas moderadas (BRANDÃO et al., 2017). É uma operação de transferência de massa, na qual a convecção é o mecanismo de transporte, caracterizada pelas mudanças de temperatura e pressão que transformam o fluido supercrítico. O $CO_2$ é usualmente o solvente utilizado pelo baixo custo, disponibilidade e segurança. A extração depende da solubilidade dos compostos e para a extração de compostos polares, como compostos fenólicos e flavonoides, é necessário a utilização de co-solventes polares, como etanol e água, para aumentar os rendimentos de extração, visto que o $CO_2$ é apolar (TALMACIU et al., 2016).

Na mesma linha de preservação do ambiente e recuperação de componentes naturais, a escolha da solução extratora é imprescindível. Na procura de substituintes para os solventes orgânicos, a água eletrolisada, destacou-se como possibilidade, devido a suas aplicações em alimentos, como desinfecção e atividade antimicrobiana, sem indícios de impacto na saúde dos consumidores e ao meio ambiente (HAO; WANG, 2019). A água eletrolisada é reconhecida como segura e já é considerada como aditivo alimentar nos EAU, Japão e Coreia (XUAN et al., 2017). É produzida através de uma solução de cloreto de sódio diluída através de uma célula eletrolítica, dentro do qual o ânodo e o cátodo são separados por uma membrana, formando dois tipos de água, ácida e básica, as quais misturadas formam a água eletrolisada levemente ácida (GUENTZEL et al., 2008; HUANG et al., 2008; XIE et al., 2010).

Levando em consideração a obteção de melhores resultados das tecnologias EAU e EFS, mencionadas acima, e tendo em vista que não foram encontrados trabalhos na literatura utilizando água eletrolisada (AE) como solução extratora de compostos bioativos de bergamota, bem como de outras matrizes, esta pesquisa torna-se inovadora. Já que o uso de AE como

solvelte e co-solvente levará obtenção de extratos diferenciados, tendo em vista suas características peculiares como pH e ORP.

Com isso, as metodologias não convencionais, combinando água eletrolisada e as técnicas de ultrassom e fluido supercrítico potencializam a extração de componentes da matriz vegetal e contribuem com o meio ambiente, reduzindo o uso de energia e solventes orgânicos. Alem disso, este trabalho teve como foco o aproveitamento dos resíduos orgânicos provenientes de frutas. Visto que nos últimos anos houve a instituição de leis mais rigorosas pelos órgãos mundiais para gerenciamento de resíduos sólidos e a necessidade de uso racional dos recursos.

Esta pesquisa foi dividida em quatro artigos científicos. O primeiro abordou uma revisão da literatura dos métodos não convencionais de extração de compostos bioativos de frutas e vegetais. O segundo baseou-se na escolha da variedade de bergamota e comparação de solução extratora convencional (etanol 80%) e os três tipos de água eletrolisada (ácida, básica e levemente ácida). No terceiro, foi comparada a extração convencional com a assistida por ultrassom utilizando água eletrolisada como solvente. E o quarto artigo apresentou os resultados da comparação entre os diferentes métodos de extração através da aplicação do ultrassom, fluido supercrítico e a combinação destes, utilizando água eletrolisada selecionada como solvente e co-solvente.

# 2 OBJETIVOS

## 2.1 OBJETIVO GERAL

O objetivo geral deste trabalho foi extrair compostos bioativos a partir da casca de bergamota, usando água eletrolisada como solvente e co-solvente, comparando a aplicação de diferentes métodos de extração: convencional, ultrassom e $CO_2$ supercrítico.

## 2.2 OBJETIVOS ESPECÍFICOS

- Revisão das aplicações de extração de compostos bioativos de frutas e vegetais, utilizando fontes de energia não convencionais;
- Realizar a caracterização físico-química e microbiológica e a determinação de compostos bioativos das farinhas de casca de bergamota, das variedades, *Citrus deliciosa*, *Citrus reticulata* e *Citrus reticulata* Blanco;
- Avaliar a extração convencional de compostos bioativos da casca de bergamota utilizando a água eletrolisada ácida, básica e levemente ácida e comparação com a extração com etanol 80%;
- Avaliar a extração assistida por ultrassom com água eletrolisada (ácida, básica e levemente ácida) na extração de compostos bioativos de casca de *Citrus reticulata* (tangerina) avaliando a intensidade e ciclo de ultrassom através de um planejamento de experimentos;
- Comparar a extração, de compostos bioativos de *Citrus reticulata* (tangerina), com ultrassom, fluido supercrítico e a combinação destas, utilizando água eletrolisada (ácida, básica e levemente ácida);
- Avaliar, através de um planejamento experimental, a influência das variáveis pressão, temperatura e proporção (farinha de tangerina: água eletrolisada básica) na extração utilizando $CO_2$ supercrítico e água eletrolisada básica como co-solvente;

# 3 REVISÃO DE LITERATURA

## 3.1 BERGAMOTA

As bergamotas também são conhecidas como mandarinas, mexericas ou tangerinas, possuem ampla importância na cultura mundial. De acordo com dados da *Food and Agriculture Organization* (FAO), em 2011, a China foi a maior produtora de *Citrus* do mundo, deixando o Brasil em segundo lugar. Em 2017, o Rio Grande do Sul foi o segundo maior produtor de bergamota no Brasil (31%), produzindo em média 150.00 toneladas do fruto cítrico (EMBRAPA, 2017).

Existem duas classificações botânicas para *Citrus,* segundo Swingle (1948), todas as bergamotas são *Citrus reticulata,* já Tanaka (1954) dividiu o grupo em quatro: *Citrus unshiu:* Satsumas, precocidade e ausência de sementes (Okitsu, Owari e Miyagawa); *Citrus clementina*: sem sementes (Clemenules, Marisol, Fina); *Citrus reticulata*: Ponkan, Danci e Limão bergamota e *Citrus deliciosa*: sementes abundantes (Caí e Montenegrina) (Figura 1).

Figura 1- *Citrus deliciosa* (a), *Citrus reticulata* (b) e *Citrus reticulata* Blanco (c).

(a) (b) (c)

Fonte: (Acervo pessoal).

A *Citrus deliciosa* (Montenegrina) (Figura 1a) é conhecida popularmente no Rio Grande do Sul como bergamota do céu, é uma cultivar produtiva. Dependendo das condições climáticas e de cultivo, a produção anual média pode atingir 25 toneladas por hectare. Tem como características morfológicas: planta medianamente vigorosa, com copa de porte médio e de

crescimento lento principalmente quando enxertada sobre o Trifoliata; folhas pequenas lanceoladas e com coloração verde-escura; flores completas com grãos de pólen e sacos embrionários férteis e frutos de tamanho médio, com peso em torno de 100 g, casca lisa com espessura média de 3 mm contendo bastante óleo, casca de endocarpo de coloração laranja (DE OLIVEIRA et al., 2011).

A *Citrus reticulata* (Figura 1b) popularmente conhecida como tangerina é nomeada como Nadorcott (EMBRAPA, 2016). Caracteriza-se pela casca fina e pela cor atrativa vermelho-alaranjada. Ela produz um suco bem doce, porém ácido. No Rio Grande do Sul a colheita é realizada de julho a agosto. Os frutos são pequenos e sensíveis a queimaduras do sol. A cultivar produz por ano, aproximadamente 30 toneladas por hectare, sendo essa uma das grandes vantagens do híbrido para o produtor (OLIVEIRA et al., 2013).

A variedade “Ponkan” (*Citrus reticulata* Blanco) (Fig. 1c) é uma *Citrus* não climatéria com elevado valor econômico (LEE, ZHONG; CHANG, 2015). É uma fruta de meia estação, apresenta frutos grandes, com paladar bastante agradável. Zhou (2010) detectou um total de 25 carotenoides incluindo alguns isômeros na casca de *Citrus reticulata* Blanco cv. Ponkan.

A casca de citrinos possui diversos metabólitos secundários, responsáveis por sua proteção contra fatores bióticos e abióticos, como terpenoides, carotenoides, cumarinas, furanocumarinas e flavonoides, principalmente flavononas e flavonas polimetoxiladas. As cascas já são utilizadas como medicamento tradicional para o tratamento estomacal, diaforese e expectorante na China e no Japão (ZHONG et al., 2016).

## 3.2 PROCESSAMENTO DE FRUTAS EM FARINHA

O aproveitamento integral dos alimentos caracteriza-se em uma prática saudável e ecologicamente correta. Muitas vezes, talos, folhas, cascas e sementes, provenientes de frutas, são mais nutritivos que as partes que se está acostumado a consumir (STEFANELLO; ROSA, 2012). A exploração de frutas como um todo amplia a diversidade de produtos que podem ser produzidos a partir de uma única cultura, causando benefícios econômicos significativos para os produtores e impactos positivos sobre o meio ambiente devido à redução ou eliminação completa de resíduos (LEÃO, 2017).

A transformação dos resíduos em farinha caracteriza-se em uma prática promissora, já que reduz o volume e a perecibilidade destes subprodutos, devido a diminuição de água livre, excluindo as reações químicas e microbiológicas. Conforme a ANVISA, as farinhas são definidas como produtos obtidos de partes comestíveis de uma ou mais espécies de cereais,

leguminosas, frutos, sementes, tubérculos e rizomas por moagem e outros processos seguros para a produção de alimentos (BRASIL, 2005).

A secagem é um processo utilizado para preservar a qualidade dos alimentos, de modo a desfavorecer o desenvolvimento de micro-organismos e eliminar quase totalmente suas atividades metabólicas (ZANATTA; SCHLABITZ; ETHUR, 2010). A qualidade de um pó de fruta é altamente dependente da secagem e moagem (KARAM et al., 2016). A desidratação concentra o conteúdo de compostos bioativos e fibras alimentares (SOQUETTA et al., 2016).

O desenvolvimento de ingredientes bioativos que são considerados aditivos naturais provenientes da extração de substâncias bioativas de fontes vegetais, devem atender a RDC n°243/2018, que define substâncias bioativas como nutrientes e não nutrientes que possuem ação metabólica ou fisiológica especifica, podendo ser usadas como antioxidantes naturais (BRASIL, 2018).

## 3.3 COMPOSTOS BIOATIVOS

Nas matrizes vegetais, o metabolismo se divide em primário e secundário. O primeiro é responsável pelos processos de fotossíntese, respiração e o transporte de solutos, os compostos envolvidos são os aminoácidos, os nucleotídeos, os lipídios, os carboidratos e a clorofila. No metabolismo secundário, são originados os compostos bioativos, substâncias que não possuem uma distribuição universal, pois não são necessários para todas as plantas. Existem três grupos deste metabolismo secundário: terpenos, compostos nitrogenados e compostos fenólicos (KRIS-ETHERTON et al., 2004; PEDRO et al., 2016).

Os compostos bioativos conferem características aos alimentos funcionais por apresentarem uma ou mais substâncias com funções biológicas e bioquímicas benéficas à saúde do homem, como influenciar as atividades celulares prevenindo o risco de doenças. Além de influenciar a qualidade sensorial, conferindo atributos como cor, textura, amargor e adstringência (DE SOUZA et al., 2012).

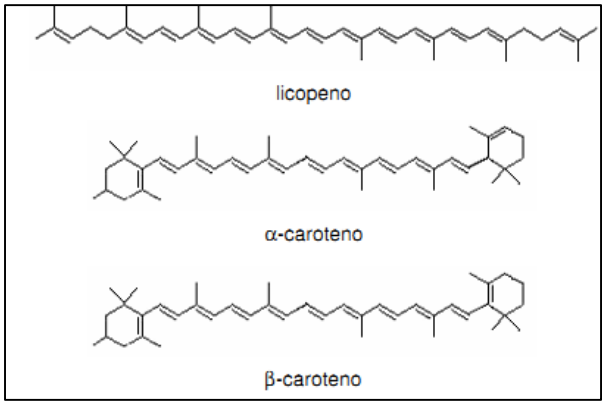
Pesquisas têm utilizado os compostos bioativos como aditivos alimentares naturais, com função antioxidante, por conter uma grande variedade de moléculas capazes de sequestrar radicais livres.

### 3.3.1 Carotenoides

Os carotenoides são uma classe de pigmentos naturais que são amplamente distribuídas em vegetais e frutas, e também usados como aditivos alimentares. Além de suas propriedades como corantes vermelho-amarelada, carotenoides possuem importantes funções e ações fisiológicas, principalmente pela provitamina A (VARGAS-MURGA et al., 2016).

Os carotenoides são pigmentos lipossolúveis intensamente coloridos sintetizados por plantas e micro-organismos e estão presentes em muitos alimentos, principalmente em frutas e vegetais. Existem diversos tipos de carotenoides, como: β-caroteno, α-caroteno, β-criptoxantina, licopeno, luteína, zeaxantina e neoxantina (AMBRÓSIO; CAMPOS; FARO, 2006). Alguns dos principais carotenoides, que possuem atividade antioxidante, encontrados na natureza podem ser observados na Figura 2.

Figura 2- Estruturas dos principais carotenoides, que possuem atividade antioxidante, encontrados em frutas e vegetais.




Fonte: Adaptado de Ambrósio; Campos; Faro, (2006).

Tais compostos são classificados em dois grupos: os carotenos, que são exclusivamente compostos por átomos de carbono e hidrogênio, e as xantofilas, que além destes dois elementos, possuem oxigênio em sua molécula, estando a astaxantina incluída neste grupo (COULTATE, 2004).

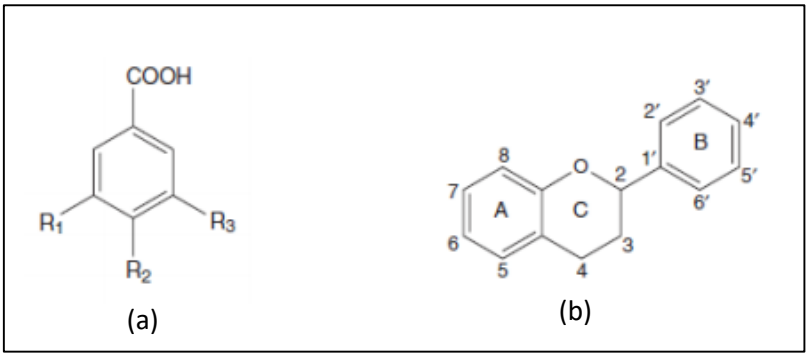
Os carotenoides têm aplicações comerciais como corantes naturais e antioxidantes. A manipulação é difícil devido as suas propriedades: carotenos sofrem degradação sob a influência de temperaturas moderadas, luz ou oxigênio, a aplicação nas indústrias de alimentos

e farmacêuticas exigem produtos muito puros e sem solvente e o uso de carotenos em produtos aquosos é dificultada pela sua baixa solubilidade em água (MATTEA et al., 2009).

### 3.3.2 Compostos fenólicos

Os compostos fenólicos são considerados um dos mais importantes grupos de compostos bioativos, seus mecanismos de ação consistem na habilidade de sequestrar espécies reativas de oxigênio e quelar íons metálicos. Podem ser divididos em dois grandes grupos, os flavonoides e os ácidos fenólicos (Figura 3). Estes componentes são substâncias aromáticas com um ou mais substituintes hidroxílicos, agem tanto na etapa de iniciação como na propagação da oxidação lipídica (KIM; LEE, 2003).

Figura 3 - Estrutura básica dos compostos fenólicos: ácido fenólico (a) e flavonoides (b).




Fonte: Adaptado de Kim; Lee, (2003).

Mais de 800 polifenóis podem ser encontrados na natureza, estando presentes principalmente em frutas e vegetais. As frutas, geralmente, contêm mais compostos fenólicos do que os vegetais, sendo o principal, o grupo dos flavonoides (KIM; LEE, 2003).

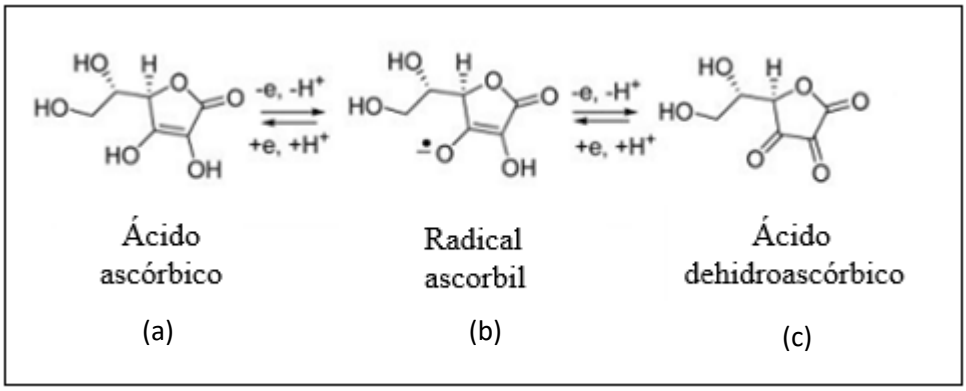
Os flavonoides possuem dois anéis aromáticos com pontes de três carbonos condensados num oxigênio formando um anel intermediário e podem ser divididos em seis grupos: flavanóis (catequinas), flavonóis (quercitina), flavonas (rutina), antocianinas (cianidina), isoflavonoides (genisteína) e flavononas (miricetina). Os ácidos fenólicos têm um anel benzênico, um grupamento carboxílico e um ou mais grupamentos de hidroxila e/ou metoxila na molécula e são divididos em três grupos: ácidos benzoicos, ácidos cinâmicos e cumarinas (PEREIRA; VIDAL; CONSTANT, 2009).

Os fenólicos das plantas atraem cada vez mais a atenção devido ao seu potencial antioxidante e seus efeitos marcados na prevenção de várias doenças associadas ao estresse oxidativo, como o câncer. Além de suas atividades antioxidantes, os polifenois também desempenham um papel importante na qualidade das frutas devido à sua contribuição para o sabor e a cor (AUBERT; CHALOT, 2018).

### 3.3.3 Vitamina C

A vitamina C ou ácido ascórbico é um composto orgânico essencial, também conhecido como antioxidante hidrossolúvel, envolvido em múltiplas funções biológicas. Suas ligações podem ser quebradas pelo oxigênio, bases alcalinas e temperaturas elevadas. Deste modo, existem duas formas, a reduzida e a oxidada (ácido desidroascórbico) (Figura 4). A reação de oxidação é um processo de duas fases em que se libertam dois átomos de hidrogênio que podem ser usados na redução de compostos biologicamente significativos. Uma vez formados, as formas podem ser novamente reduzidas a ácido ascórbico por vias enzimáticas assim como por compostos redutores presentes nos sistemas biológicos (CERQUEIRA; MEDEIROS; AUGUSTO, 2007).

Figura 4–Mecanismo oxidativo do ácido ascórbico (a) origina a forma reduzida, o radical ascorbil (b) que por sua vez gera a forma oxidada, o ácido dehidroascórbico (c).




Fonte: Adptado de Cerqueira; Medeiros; Augusto, (2007).

As duas formas podem ser encontradas em frutos, principalmente pertencentes à família dos citrinos, ambas possuem atividade antioxidante, por mecanismos de inativação de radicais livres, capacidade de quelar metais ou ainda regenerar antioxidantes fenólicos (BREWER, 2011).

Artigos da literatura documentam a aplicação desta vitamina C como benéfica a uma grande variedade de doenças, como infecções respiratórias virais, diabetes, cardiovasculares, entre outras (GRANGER; ECK, 2018). A deficiência de vitamina C resulta no enfraquecimento das estruturas de colágeno, causando dores nas articulações, fadiga, anemia e alterações de humor (VERRAX, CALDERON, 2008).

## 3.4 EXTRAÇÃO DE COMPOSTOS BIOATIVOS

A extração dos compostos bioativos depende de diversos fatores, dentre eles: escolha da técnica, polaridade do material e solvente, tempo e temperatura de extração. Um método ideal deve ser rápido, possuir rendimento de recuperação, sem degradação e os extratos devem ser facilmente separados a partir do solvente (PARNIAKOV et al., 2014; RODRÍGUEZ-PÉREZ et al., 2015).

Os métodos de extração podem ser classificados em convencionais e não convencionais. As extrações convencionais baseiam-se na utilização de solventes orgânicos com ou sem agitação e aquecimento, são exemplos, as técnicas: Soxhlet, maceração, agitação e hidrodestilação (TIWARI, 2015). Já as não convencionais, também são chamadas de tecnologias limpas ou verdes, pois reduzem ou eliminam a utilização de solventes orgânicos.

A técnica Soxhlet consiste em uma pequena quantidade da amostra seca, colocada num extrator por onde passa solvente. O processo é executado repetidamente até que a extração seja completa. Este sistema de extração requer muito tempo de extração e quantidades elevadas de solventes (HELENO et al., 2016).

A maceração consiste na moagem em partículas menores de modo a aumentar a área de superfície para uma boa mistura com o solvente. A agitação facilita a extração de duas maneiras: aumentando a difusão e removendo a solução concentrada da superfície da amostra (AZIMIR et al., 2013).

A hidrodestilação é realizada com água destilada e é aplicada para extração da fração volátil em alimentos, geralmente leva de 6 a 8 horas. Esta técnica envolve três processos físico-químicos principais: hidrodifusão, hidrólise e decomposição pelo calor (WU et al., 2011).

Os riscos de segurança, como toxicidade de alguns solventes, presença de resíduos de solventes nos extratos, juntamente com baixo rendimento, e elevado tempo, têm estimulado o desenvolvimento de outras tecnologias de extração, como as chamadas tecnologias limpas ou verdes, que podem minimizar ou eliminar o uso de solventes orgânicos. Estas técnicas são também conhecidas como técnicas de extração a frio, onde a estabilidade dos compostos extraídos não é afetada ao mesmo tempo em que a energia necessária para extração é reduzida (TIWARI, 2015). A aplicação dessas tecnologias também tem o objetivo de preservar o meio ambiente natural e seus recursos (MUSTAFA; TURNER, 2011).

## 3.5 EXTRAÇÃO NÃO CONVENCIONAL DE COMPOSTOS BIOATIVOS

O surgimento da química verde para fins de extração ocorreu nos anos 90, com o objetivo de reduzir o consumo de energia e substituir os solventes convencionais por alternativas menos prejudiciais ao meio ambiente (KHAW et al., 2017).

A química verde tem o desafio de tornar a tecnologia química mais sustentável do ponto de vista ambiental. Sendo levados em consideração diferentes fatores, como a toxicidade e carga ambiental de todos os produtos químicos utilizados nos processos de extração, bem como na produção destes produtos e na segurança de extração (CASTRO-PUYANA; MARIANA; PLAZA, 2017).

Alguns autores procuraram definir os principais pontos ou princípios elementares da química verde (NÁRAY-SZABÓ, 2018). Segundo Lenardão et al. (2003), basicamente há doze tópicos que precisam ser seguidos quando se pretende implementar a química verde. São eles: prevenção (evitar a produção de resíduos), economia de átomos (maximizar a incorporação de todos os materiais de partida no produto final), síntese de produtos menos perigosos (pouca ou nenhuma toxidade a saúde humana), desenho de produtos seguros (realizem a função desejada e ao mesmo tempo não sejam tóxicos), solventes e auxiliares mais seguros, busca pela eficiência de energia (impactos ambientais e econômicos reduzidos), uso de fontes renováveis de matéria-prima, evitar a formação de derivados, catálise (reagente tão seletivos quanto possível), desenho para a degradação (produtos de degradação inócuos e que não persistam no ambiente), análise em tempo real para a prevenção da poluição química, intrinsecamente segura para a prevenção de acidentes.

Além de reduzir ou eliminar o uso de solvetes e diminuir o tempo e energia do processo, as técnicas não tradicionais de extração podem reduzir a degradação térmica do extrato, preservando suas atividades-alvo (COVA et al., 2019). As tecnologias não convencionais que

têm sido utilizadas para extração de compostos bioativos de matriz vegetal são: com fluido supercrítico, com liquido pressurizado, assistida por ultrassom, assistida por micro-ondas, campo elétrico pulsado, descargas elétricas de alta tensão, alta pressão hidrostática e a combinação destas técnicas. Essas técnicas serão definidas na revisão bibliográfica apresentada no item 4.1 deste estudo.

Neste estudo foi realizada uma revisão sobre estas técnicas, no entanto foram utilizadas experimentalmente as técnicas de extração assistida por ultrassom e extração com fluido supercrítico, usando $CO_2$, descritas a seguir.

### 3.5.1 Extração com fluido supercrítico (EFS)

As primeiras patentes relacionadas à extração supercrítica surgiram somente no início do século XX. Na década de 1920 foram registradas algumas patentes relativas à extração do aroma do café com nitrogênio líquido. Na década de 1930 foram realizados estudos sobre novas tecnologias para o refino de petróleo em altas pressões, dos quais se originavam patentes sobre a utilização de fluidos supercríticos em tal área (MELO, 2005).

A partir do final da década de 1980 e início da década de 1990, os fluidos supercríticos vêm sendo aplicados em diversos setores da indústria. Aplicações na regeneração de carbono ativado, na separação de misturas etanol-água, no processamento e fracionamento de polímeros, em cromatografia, como meio solvente para reações químicas e enzimáticas, no projeto e desenvolvimento de surfactantes para emulsificar $CO_2/H_2O$, no processamento e produção de micro e nanopartículas de alimentos, fármacos e polímeros e na produção de membranas (MELO, 2005).

A condição supercrítica é atingida quando um fluido é submetido a condições acima da temperatura e da pressão críticas. Os fluidos supercríticos exibem propriedades de transporte desejáveis que melhoram a sua capacidade de adaptação. São mescladas algumas propriedades dos fluidos nas fases líquida e gasosa, como a alta densidade características da fase líquida e a baixa viscosidade, compressibilidade e alta difusividade associada à fase gasosa. A Tabela 1 mostra a mudança nas propriedades densidade, difusidade e viscosidade das fases de líquido e gás quando atingidas pressão e temperatura supercríticas.

Tabela 1 - Propriedades relativas das fases de líquido e gás submetidas a temperatura e pressão supercrítica.

| | Densidade x $10^2$ kg $(m)^{-3}$ | Difusividade x $10^{-3}$ $cm^2$ $(s)^{-1}$ | Viscosidade x $10^4$ Kg $(m.s)^{-1}$ |
|---|---|---|---|
| Líquido | 6-16 | <0,005 | 2-30 |
| Pc,Tc | 2-5 | 0,7 | 0,1-0,3 |
| 4 Pc, Tc | 4-9 | 0,2 | 0,3-0,9 |
| Gás | 0,006-0,02 | 0,1-0,4 | 0,1-0,3 |

Onde: Pc: Pressão crítica; Tc: Temperatura crítica; 4 Pc, Tc: Aumentando a pressão em quatro vezes.

Fonte: Adaptado de KHAW et al. (2017).

Com a Tabela 1 podemos observar que o aumento da pressão crítica em quatro vezes, a densidade pode ser aproximadamente duplicada, aproximando-se assim da densidade de um líquido mantendo a difusidade e viscosidade de um gás. Segundo Khaw et al. (2017) esta capacidade de variar a viscosidade também significa que a seletividade na extração dos compostos alvo pode ser obtida exclusivamente por extração supercrítica de fluido, dentro de um curto período de tempo.

A temperatura crítica é considerada como a temperatura máxima, na qual o gás pode ser convertido em líquido pelo aumento da pressão, e a pressão crítica como a pressão máxima na qual o líquido pode ser convertido em gás pelo aumento da temperatura (PASQUALI; BETTINI; GIORDANO, 2006).

A solubilidade é dependente das forças intermoleculares das moléculas envolvidas, onde a interação soluto/solvente é afetada pela proximidade entre as moléculas, ou seja, pela densidade do solvente. Alterando as condições de temperatura e pressão, o fluido supercrítico modifica sua densidade, especificidade na proximidade do ponto crítico, onde para uma pequena variação de pressão ou temperatura promove grande alteração dessa propriedade, apresentando uma nova característica para o solvente (WEBER et al., 2012).

A baixa viscosidade faz com que o solvente se difunda facilmente na matriz sólida, e uma baixa tensão superficial, permitindo penetração rápida do solvente no sólido e, consequentemente, aumentando a eficiência da extração. Uma vez que a densidade está relacionada com a solubilidade, através da alteração da pressão de extração, a força do fluido pode ser modificada (POULIOT; CONWAY; LECLERC, 2014).

A EFS é uma operação de transferência de massa em que a convecção na fase de solvente supercrítico é geralmente o principal mecanismo de transporte (SILVA; MARTÍNEZ, 2014). A extração é rápida, seletiva, não requer limpeza adicional e pode ser realizada em pequenas quantidades de amostras (OROIAN; ESCRICHE, 2015). Outra grande vantagem é a possibilidade de associação direta com as técnicas cromatográficas analíticas tais como a cromatografia em fase gasosa (CG) e a cromatografia de fluido supercrítico (CFS) (DA SILVA, ROCHA-SANTOS; DUARTE, 2016).

A técnica pode ser resumida em dois passos: a solubilização dos compostos químicos presentes na matriz sólida e a sua separação em solvente supercrítico. O solvente flui através do leito compactado, solubiliza os compostos existentes na matriz. Após, o solvente sai do extrator e por redução da pressão e aumento da temperatura, torna-se um extrato isento de solvente (DA SILVA, ROCHA-SANTOS; DUARTE, 2016).

A extração supercrítica é utilizada principalmente para isolar compostos bioativos não polares (lipídios e carotenoides) devido aos solventes mais utilizados serem desta natureza. Outra aplicação importante é a extração de compostos bioativos termolábeis de plantas. Além da fragrância, a mistura de compostos confere várias bioatividades como antimicrobianas e antioxidante. Uma opção para a extração de compostos polares como os fenólicos e flavonoides é a adição de modificadores, chamados de co-solvente, como etanol, metanol, água, acetona (HERRERO et al., 2013).

A solubilidade de extratos no fluido supercrítico é função da densidade do solvente e da pressão de vapor do soluto. Com isso, diferentes fluidos supercríticos têm sido descritos em estudos, o $CO_2$ é comumente utilizado (DA SILVA; ROCHA-SANTOS; DUARTE, 2016).

#### *3.5.1.1 Dióxido de carbono como fluido supercrítico (EFS-$CO_2$)*

O dióxido de carbono ($CO_2$) é utilizado como fluido supercrítico por três razões principais: é inócuo para a saúde humana e ao meio ambiente, respeitando os critérios de sustentabilidade, sua temperatura crítica é moderada (31,2°C), questão fundamental para a preservação dos compostos bioativos e o extrato é preservado no contato com o ar e luz onde podem ocorrer reações de oxidação. Além disso, também é possível modular a potência do $CO_2$ para executar uma extração seletiva (BRUNNER, 2005).

O maior coeficiente de difusão e menor viscosidade de EFS-$CO_2$ permite a rápida penetração nos poros das matrizes complexas, aumentando assim a eficiência de extração. Os extratos obtidos após o uso desta técnica são altamente concentrados, pois o processo de

despressurização separa facilmente o $CO_2$, não deixando vestígio de solventes no produto final (CASTRO-PUYANA; MARIANA; PLAZA, 2017).

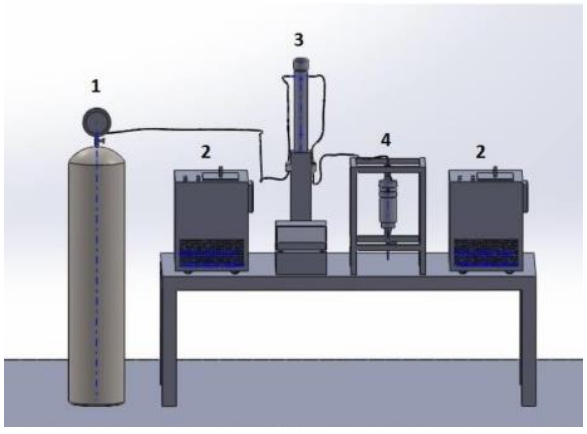
Entretanto, a extração de compostos polares requer a presença de solventes polares, utilizados como modificadores, devido à natureza não polar de $CO_2$ (SOLANA; MIROFCI; BERTUCCO, 2016; OROIAN; ESCRICHE, 2015; MENESES et al., 2015). Prado et al., (2013), descreveram que o $CO_2$ tem uma capacidade muito limitada para dissolver os compostos polares e de elevado peso molecular, e utiliza solvente para recuperar os compostos antioxidantes presentes no extrato de casca de manga.

### *3.5.1.2 Sistema de extração supercrítica*

Os sistemas de extração supercrítica de fluidos podem ser divididos em escala laboratorial, piloto ou industrial. O sistema em escala de laboratório visa produzir miligramas para gramas de extrato usando reatores de pequeno volume (50 a 300 mL). Onde são desejados quilogramas de extrato, é necessário um sistema com maiores volumes de reator, com capacidade para várias centenas de litros (KHAW et al., 2017).

Os componentes básicos de um sistema típico de fluido supercrítico de $CO_2$ são: uma bomba de $CO_2$ ou compressor, uma bomba modificadora em que é nesserário um solvente reator de extração e recipiente de fracionamento/coleta. Neste estudo utilizou-se um equipamento de escala laboratorial como mostrado na figura 5.

Figura 5 - Aparelho experimental para extração com $CO_2$.




Onde: Os dispositivos principais são: (1) reservatório de solvente; (2) banhos de resfriamento (à esquerda) e de aquecimento (direito); (3) bomba de alta pressão de seringa; (4) navio de extração.

Fonte: Adaptado de Scapin et al. (2017)

### 3.5.2 Extração assistida por ultrassom (EAU)

Ultrassom são ondas sonoras dispersas em um material, transmitindo energia. O nível e a energia transmitidas dependem da intensidade do ultrassom, que está relacionado com a potência por unidade de área do transmissor. A frequência destas ondas está acima da faixa de audição humana variando de 20 kHz a 100 MHz. Por ser uma onda mecânica, ela precisa de um meio para propagar, e a velocidade de propagação da onda depende das propriedades de cada meio.

Os efeitos que as ondas de ultrassom podem causar são diversos, o que aumenta a sua gama de utilização. A extração assistida por ultrassom é utilizada em diversas moléculas e biomateriais, incluindo polissacarídeos, óleos essenciais, proteínas, peptídeos, corantes, pigmentos e compostos bioativos. O uso desta técnica tem como vantagem não necessitar o incremento de temperatura para aumentar a difusão, uma vez que as ondas quebram a estrutura celular, melhorando a difusão (DOLATOWSKI; TASIAK, 2012).

Um dos fenômenos produzidos quando o ultrassom se propaga nos líquidos é chamado de cavitação. Neste processo ocorre a produção, crescimento e colapso de bolhas, levando a ruptura celular que aumenta a transferência de massa (AZMIR et al., 2013). O mecanismo de extração por ultrassom envolve dois tipos principais de fenômenos físicos, a difusão através da parede da célula e extração do conteúdo da célula após quebrar as paredes. A temperatura, a pressão, a frequência e o tempo de sonicação são os fatores que regulam a ação do ultrassom (TOMA et al., 2001).

#### *3.5.2.1 Mecanismo de extração*

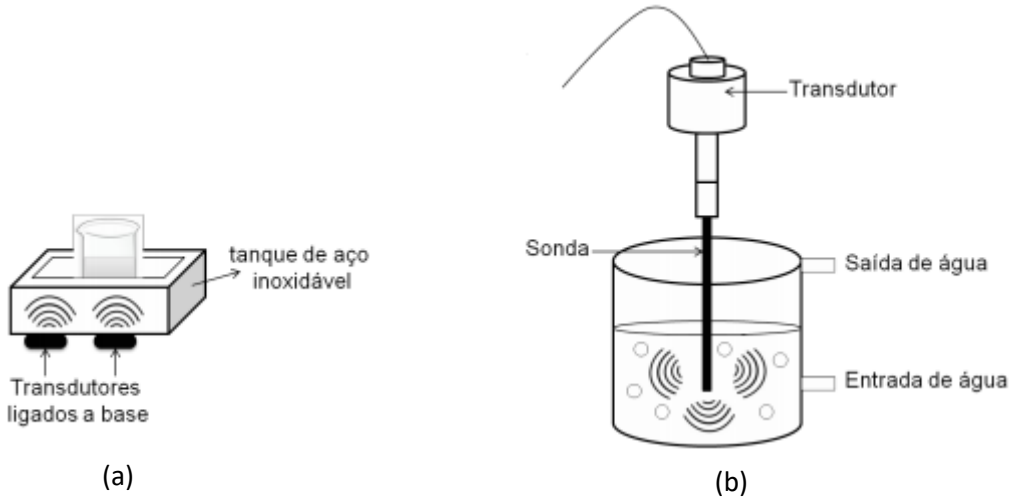
O sistema de produção de ondas contém basicamente um gerador, um transdutor e um sistema operacional. O gerador produz energia mecânica ou elétrica e o transdutor converte esta energia em energia sonora na frequência ultrasonica (CHEMAT; ZILLE-HUMA; KHAN, 2011).

O ultrassom é relativamente fácil de usar, versátil, flexível e requer baixo investimento em comparação com outras técnicas de extração. Existem basicamente dois tipos de equipamentos de ultrassom: a sonda (sonicação direta) e o banho (sonicação indireta) (TIWARI, 2015; BRIONES-LABARCA et al., 2015).

O banho de ultrassom (Figura 6a) é o mais utilizado, os transdutores estão presos na placa vibratória anexados ao fundo do vaso e as ondas devem ser transferidas através da água

até atingir a amostra. Há um descolamento longitudinal de partículas, resultando em sucessivos ciclos de expansão e compressão, funcionando de modo análogo a um pistão.

Figura 6 - Esquema básico de um sistema de banho de ultrassom (a) e sonda de ultrassom (b).




Fonte: Adaptado de CHEMAT et al., (2017).

Cada meio possui uma distância molecular crítica de suas partículas, abaixo deste valor crítico, as partículas se mantêm intactas, mas acima desta distância, o líquido rompe e pequenos vácuos são gerados dentro do líquido. Os vácuos gerados no meio são conhecidos como bolhas de cavitação que são as responsáveis pelo efeito ultrassônico. Quando o tamanho destas bolhas alcança um valor crítico, elas colapsam em um ciclo de compressão, liberando grandes quantidades de energia (CHEMAT et al., 2017).

Os sistemas de sonda (Figura 6b) têm alta intensidade cavitacional obtida para os menores volumes operacionais. A energia acústica é introduzida diretamente no líquido e a potência dissipada na mistura de reação pode ser tipicamente alterada, embora a frequência de irradiação permaneça constante. O sistema de sonda fornece intensidade aproximadamente 100 vezes maior (SOQUETTA et al., 2018).

Após testes preliminares e levando-se em consideração a maior intensidade e a possibilidade de avaliação dos ciclos de pulsos, neste trabalho utilizamos a sonda de ultrassom para a extração de compostos bioativos de bergamota. Devido a intensidade do ultrassom fornecido pelo sistema de sonda ao meio liquido ocorre um aumento rápido de temperatura no reator, com isso, utilizou-se o resfriamento do reator através de água resfriada.

## 3.6 ÁGUA ELETROLISADA (AE)

A água eletrolisada (AE) é um tipo especial de água, onde os íons foram dissociados, formando água eletrolisada ácida (AEA) e água eletrolisada básica (AEB). A AE surgiu com a descoberta do fenômeno da divisão eletrolítica de água, em hidrogênio e oxigênio, para o desenvolvimento de eletrólise, assim essa tecnologia tem crescido continuamente ao longo dos anos (SANTOS; SEQUEIRA; FIGUEIREDO, 2013).

O Japão foi o primeiro país a desenvolver e introduzir a AE no seu mercado em torno de 1980. A primeira forma utilizada na indústria de alimentos foi a água eletrolisada ácida, onde verificou-se que era útil para destruir bactérias e parasitas em peixe cru sem alterar as características sensoriais do peixe (HATI et al., 2012).

Atualmente a AE tem usos potenciais para a indústria de alimentos e tem sido objeto de muitas pesquisas, principalmente pela forte eficácia bactericida em vários micro-organismos, incluindo bactérias, fungos, vírus e outros (HUANG et al., 2008). A AE já foi aplicada diretamente em produtos como legumes, frutas, aves, carne, frutos do mar e peixes, não apenas como desinfetante e agente de limpeza, mas também por seu papel significativo na melhoria de qualidade e prevenção de doenças fisiológicas em frutas e vegetais (HAO; WANG, 2019). Além disso, também é proposta como desinfetante de superfícies de contato em alimentos, por exemplo, placas de corte, talheres, pratos, equipamentos (DING; LIAO, 2019).

Sun et al. (2012) utilizaram água eletrolisada ácida e básica para erradicar o biofilme formado por *Staphylococcus aureus*, onde concluíram que estas águas têm potencial bactericida e como agente removedor de biofilme. Kobayashi, Hara e Katsube (2006) extraíram componentes de folha de amoreira usando uma solução de etanol com água eletrolisada. O radical DPPH em AEA e AEB exibiram efeito de eliminação de radicais livres aumentado em comparação com a água da torneira. Kobayashi e Hara (2004) também investigaram as águas eletrolisada ácida e básica pela sua capacidade de mudar a cor de feijão (Sekihan).

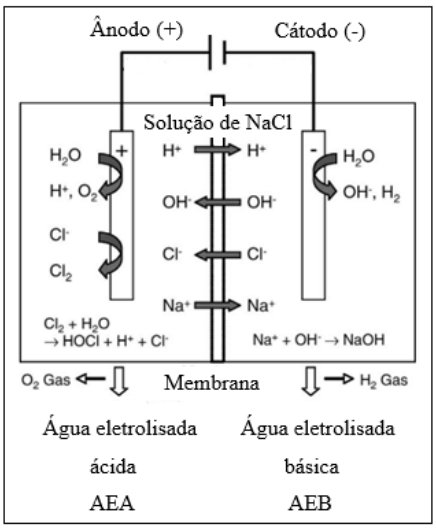
Alguns estudos também têm se concentrado na remoção de pesticidas em alimentos, especialmente em vegetais e frutas. Qi et al. (2018) estudaram a eficácia da água eletrolisada em degradar e remover três resíduos de pesticidas em produtos frescos. O efeito da AEA e AEB na degradação de diazinon, cyprodinil e fosmet em solução aquosa e em produtos frescos foram examinados. Hao et al. (2011) investigaram a eficácia na remoção de pesticidas (acefato, ometatoato e diclorovinil fosfato) em vegetais frescos. Os resultados mostraram que a AEA e AEB podem remover estes pesticidas sem perda na qualidade dos alimentos.

A aplicação de AE tem um conceito sustentável e verde e várias vantagens em relação aos sistemas convencionais, incluindo economia, facilidade de aplicação, produção no local e segurança, para os seres humanos e meio ambiente (RAHMAN; KHAN; OH, 2016). Já é considerada como um aditivo alimentar legítimo nos Estados Unidos e Japão (XUAN et al., 2017) sem alterar a sensorial e qualidade dos alimentos (DING; OH; LIU, 2019).

### 3.6.1 Mecanismo de obtenção

A AE é obtida a partir de uma solução salina, frequentemente cloreto de sódio (NaCl) ou ácido clorídrico (HCl) diluídos, através de uma unidade de eletrólise (Figura 7). O equipamento possui dois polos, ânodo (+) e cátodo (-), conectados através de uma fonte de alimentação externa e imersos em um eletrólito condutor. Uma corrente contínua é aplicada à unidade. Os elétrons fluem do terminal negativo da fonte de energia para o cátodo, onde são consumidos por íons de hidrogênio para formar átomos de hidrogênio. No processo geral de eletrólise da água, os íons de hidrogênio se movem em direção ao cátodo, enquanto os íons hidróxido se movem em direção ao ânodo. Uma membrana é utilizada para separar os dois compartimentos. Os receptores de gás são usados para coletar gases de hidrogênio e oxigênio, que são formados no cátodo e ânodo, respectivamente (SANTOS et al., 2013).

Figura 7- Célula eletrolítica utilizada para a obtenção de água eletrolisada ácida e básica a partir de uma solução de NaCl.




Fonte: (HRICOVA; STEPHAN; ZWEIFEL, 2008).

Este processo resulta em dois tipos de água: água eletrolisada ácida, do lado do ânodo, e água eletrolisada básica, do lado do cátodo. Os principais produtos formados no ânodo são $Cl_2$ dissolvido, hipocloroso (HOCl) e ácido clorídrico (HCl) e no cátodo, hidróxido de sódio (NaOH) e $H_2$ dissolvido (ATHAYDE et al., 2018). A mistura de AEA e AEB forma a água eletrolisada levemente ácida (AELA).

### 3.6.2 Propriedades da água eletrolisada

As propriedades básicas da água eletrolisada, incluindo a concentração de ACC (cloro disponível), nas formas $Cl_2$, -OCl e HOCl, pH e ORP (Oxidation Reduction Potential) influenciam diretamente sua eficácia (HANAOKA et al., 2004). Os parâmetros, corrente, taxa de fluxo, concentração de sal, eletrólito, temperatura da água, dureza e armazenamento interferem nestas variáveis. Existe uma correlação positiva entre o ACC e a concentração de eletrólito. Maior concentração de eletrólito pode aumentar a condutividade, o que pode aumentar a produção de cloro e aumentar sua capacidade bactericida (DING; OH; LIU, 2019).

Na Tabela 2 estão apresentadas as características de pH e ORP, de acordo com a solução salina aplicada no processo de eletrólise, dos três tipos de água eletrolisada, encontrados na literatura.

Neste estudo, utilizamos NaCl a 0,01%, para reduzir ao máximo a concentração de cloro, devido a utilização para alimentos, assim obtivemos uma solução básica com menor pH e maior ORP do que frequentemente encontramos na literatura. Com isso, podemos explicar que os parâmetros obtidos na AEB possuem diferenças dos demais estudos demostrados na Tabela 2.

Król et al. (2017) demostraram que a concentração de cloreto de sódio aumentou o efeito antibacteriano e foi positiva sobre atividade antioxidante, pois observaram valores maiores da capacidade antioxidante (DPPH) na obtenção de hidrosol de alginato de sódio.

O pH é o indicador que mostra a concentração de íons de hidrogênio. Quando a eletrólise é realizada em soluções eletrolíticas tais como NaCl, o pH da água reduzida produzida será medido pela hidrólise de carbonatos desses eletrólitos. Portanto o valor do pH depende da concentração inicial dos carbonatos e dos parâmetros da hidrólise (HANAOKA et al., 2004).

Tabela 2–Características de pH e ORP (mV), de acordo com a solução salina aplicada no processo de eletrolise, das águas eletrolisadas.

| Solução | pH | | | ORP (mV) | | | Referência |
|---|---|---|---|---|---|---|---|
| | AEA | AEB | AELA | AEA | AEB | AELA | |
| NaCl | 2,7 | 11,9 | | 863 | -746 | - | ZHU et al., 2016 |
| HCl 0,01 + NaCl 0,03% | - | - | 6,1 | - | - | 863 | LIAO et al., 2017 |
| HCl 0,15 + NaCl 0,6% | - | - | 6,3 | - | - | 867,4 | XUAN et al., 2017 |
| NaCl 0,03% | 2,61-2,87 | - | 5,92-6,04 | 1146-1185 | - | 938 -970 | ZHANG et al., 2018 |
| HCl 9% | - | - | 5,98 | - | - | 859 | WHANG et al., 2018 |
| NaCl 0,05% | 2,6 | 11,4 | 6,1 a 6,5 | 1134 a 1200 | -826 a 877 | 875 a 930 | ATHAYDE et al., 2017 |
| - | 2-3 | >10 | - | >1000 | <-700 | - | ATHAYDE et al., 2018 |
| - | 2-3 | 10-13 | 7-8 | >1100 | -800 a -900 | 750 | HRICOVA; STEPHAN; ZWEIFEL, 2008 |
| NaCl 1% | - | - | 6,5 - 8 | - | - | 800 a 900 | GUENTZEL, 2008 |
| NaCl 0,03% | 2,8 | 11,5 | 7,44 | 1126 a 1151 | -871 | 867 | QI; HUANG;HUNG, 2018 |
| NaCl 0,01% | 2,5 a 2,63 | 10,65 a 10,85 | 7,4 | 1104 a 1191 | 212 a 297 | 749 a 749 | Esta pesquisa |

Fonte: O autor (2019).

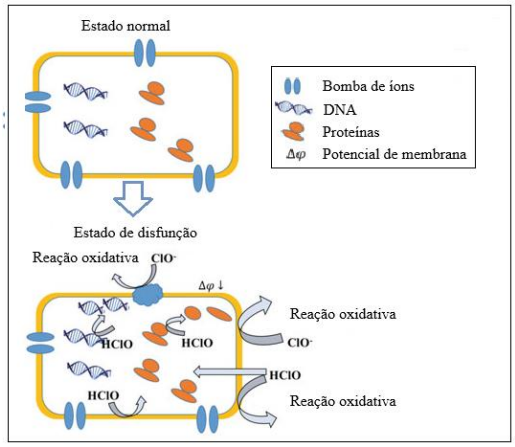
O potencial de oxidação-redução (ORP) é definido como a capacidade de um sistema bioquímico para receber ou doar elétrons. Quanto menor é o ORP, maior a possibilidade de doação de elétrons e a eliminação de radicais livres. Assim o ORP pode indicar componentes com propriedades antioxidantes no produto, que podem proteger naturalmente contra oxidação lipídica (KĘSKA; LIBERA; STADNIK, 2017). Os valores de ORP são afetados por todos os agentes oxidantes e redutores, não apenas ácidos e bases que influenciam a medição do pH (LATOCH; STASIAK, 2017).

Quando as soluções são eletrolisadas através da membrana, ocorre redução no cátodo e oxidação no ânodo. A oxidação de moléculas de água produz $H^+$ e $O_2$ no ânodo e $OH^-$ e $H_2$ no cátodo. Portanto, a água alcalina catódica (água reduzida) é abundante em hidrogênio dissolvido (DH), enquanto que a água ácida anódica (água oxidada) é abundante em oxigênio dissolvido (DO) (HANAOKA et al., 2004).

As propriedades das águas eletrolisadas são influenciadas diretamente pelo ambiente de armazenamento, principalmente o componente antimicrobiano HOCl que pode ser decomposto e a evaporação de $Cl_2$ (DING; OH; LIU, 2019).

Os pesquisadores Ding and Liao (2019) propuseram um diagrama dos mecanismos de inativação microbiana propostos sob os tratamentos de AE (Figura 8).

Figura 8- Diagrama de inativação microbiana através de água eletrolisada.




Onde: Bomba de íons ou bomba iônica é uma proteína transmembrana que, através da membrana plasmática, transfere íons através da membrana celular, contra os respectivos gradientes de concentração, para a região de potencial químico mais elevado, mediante a utilização de uma fonte de energia externa; Potencial elétrico de membrana ou potencial transmembrana ou voltagem da membrana é a diferença de potencial elétrico (voltagem) entre os meios intra e extracelular.

Fonte: (Ding and Liao, 2019)

Os mecanismos de eficácia da água eletrolisada ainda não é um consenso. Entretanto, é claro que a desinfecção é dependente do pH, ACC e ORP. O HClO produz radicais hidroxila que penetram as membranas celulares e incorre na ruptura de vias metabólicas chave (HAO; WANG, 2019). Os principais fatores que afetam a eficácia da remoção de resíduos de pesticidas são o pH (baixo), o ACC e o tempo de tratamento. A estrutura superficial de cada produto afeta a eficácia dos tratamentos (WANG; HAN, 2019).

### 3.6.3 Tipos de água eletrolisada

#### *3.6.3.1 Água eletrolisada ácida (AEA)*

A AEA é obtida no lado do ânodo da célula eletrolítica. Possui pH baixo, em média de 2,5 a 3,5, ORP alto, 1000 a 1200 mV, e cloro livre de 30 a 90 ppm. Várias investigações foram lançadas sobre o mecanismo de atuação desta solução. Como as células microbianas podem sobreviver sob um pH de 4-9, um pH baixo tende a destruir os compostos da parede celular (por exemplos polissacarídeos) e aumenta a permeabilidade, resultando na morte de micro-organismos. Além disso, as células microbianas mantem um potencial de membrana de -400 a 900 mV, sob condições físicas normais. Um ORP circundante mais alto pode perturbar a distribuição de íons nas superfícies das células interna e externa, o que leva ao rompimento de envelopes celulares e à perda de componentes intracelulares. Também contribuem para a atividade bactericida de AEA, as espécies de cloro ativo, como HClO, $Cl_2$, $ClO_2$, ClO-, (DING; LIAO, 2019). Aoki et al. (2016) descobriram que a AEA tem a capacidade de destruir micobactérias via proteínas citoplasmáticas desnaturantes após danificar as paredes celulares.

Com estas características esta solução tem forte atividade antimicrobiana contra uma variedade de micro-organismos e pode ter uma ampla gama de aplicações, tais como medicamentos (tratamento de feridas ou desinfecção de equipamentos e superfícies médicas), odontologia, agricultura, manejo de gado, aquicultura e indústrias alimentícia (HRICOVA, STEPHAN, ZWEIFEL, 2008). Na Tabela 3 estão apresentadas algumas pesquisas utilizando água eletrolisada ácida no tratamento de alimentos.

Tabela 3 – Usos de água eletrolisada ácida em alimentos.

| Propriedades | Metodologia | Resultado | Referencia |
|---|---|---|---|
| pH 2,2<br>ORP 1146<br>ACC 161,6 | Contagem de placas<br>100 µL suspenção fúngica<br>+ 900 µL AEA | Destruiu as estruturas celulares de *Aspergillus flavus* | XIONG et al. (2014) |
| pH 2,6<br>ORP 1130 | Imersão de morangos em AEA por 10 min | Redução de bactérias aeróbias em 1,6 log CFU $g^{-1}$ e coliformes em 2,4 log CFU $g^{-1}$ | KOSEKI et al. (2004) |
| pH 2,91<br>ORP 1131<br>ACC 201 | Imersão de maçã e laranja em AEA por 5 min | Redução de *L. innocua* em 0,7 a 1,7 log CFU $g^{-1}$ em maçãs e 0,5 a 1,1 log CFU $g^{-1}$ em laranjas | GRAÇA et al. (2010) |
| pH 2,6<br>ORP 1160 mV<br>ACC 56 | Imersão de maçãs em AEA | Redução de *Listeria monocytogenes* em 1,9 log CFU/82,5 $cm^2$ | KIM et al. (2001) |
| pH 2,4<br>ORP 1163 mV<br>ACC 47,12 | Tratamento de biofime com AEA | Redução de *Listeria monocytogenes* em 4,33 a 5,21 log CFU $g^{-1}$ | AYEBAH et al. (2005) |
| pH 2,3<br>ORP 1170<br>ACC 70 | Imersão de espinafre em AEA por 30 min. | Redução dos pesticidas Acephate em 74%, Omethoate em 62% e DDVP em 59% | HAO et al. (2011) |
| pH 2,8<br>ORP 1151<br>ACC 120 | Imersão de feijão em AEA por 15min | Redução do pesticida Diazinon em 66% | QI et al. (2018) |

Onde: AEA- Água Eletrolisada Ácida; ORP- mV; ACC- mg/L.
Fonte: O autor (2019).

O principal método de desinfecção de frutas e vegetais é a imersão dos frutos em AEA. A eficácia depende dos parâmetros de processamento, como tempo de enxágue, temperatura e a frequência do agitador rotativo. A pulverização também pode ser usada e o tamanho do orifício do disco em combinação com o tempo de duração afeta a eficácia da desinfecção (HAO; WANG, 2019).

As principais vantagens de utilizar AEA para inativação de bactérias são os menores impactos ambientais e a facilidade de transporte e armazenamento de produtos químicos potencialmente perigosos. Outro uso eficaz comprovado é a sua aplicação diretamente em produtos alimentares frescos para reduzir o número de micro-organismos ou agentes patogênicos presentes, podendo até substituir o uso de pesticidas (HATI et al., 2012).

*3.6.3.2 Água eletrolisada básica (AEB)*

A AEB, também conhecida como água redutora, possui um pH alto, em média, 10 a 13, e ORP baixo, -800 a -900 mV. Inicialmente era descartada como subproduto durante a produção da AEA. Entretanto, nos últimos anos, vários trabalhos têm sido publicados reportando a AEB com funcionalidades tipo: detergente, eficaz na lavagem de proteínas, gorduras e óleos, inativação de micro-organismos, devidos aos íons hidroxila e ORP negativo, e antioxidante, contra estresse oxidativo. Além disso, foi descrito que a AEB remove efetivamente os biofilmes de *Staphylococcus aureus* (HATI et al., 2012; DING; OH; LIU, 2019).

O pré-tratamento com AEB seguido de tratamento com AEA foi mais eficaz do que somente AEA. O pré-tratamento parece sensibilizar as superfícies das células bacterianas para o agente desinfetante (HRICOVA, STEPHAN, ZWEIFEL, 2008). A sinergia de ambas águas eletrolisadas pode reduzir a contagem de micro-organismos existentes em frutas e vegetais em 4-6 log, o que provou a forte capacidade de sua combinação (HAO; WANG, 2019).

Aplicada diretamente como descontaminante em alface cortada e carcaças de aves, apresentou resultado satisfatório (SUN et al., 2012). Ovissipour et al. (2016) verificaram que a AEB resultou em uma redução de 1 a 3 log de *E. coli, L. monocytogenes, Campylobacter jejuni, Aeromonas hydrophila* e *Vidrio parahaemolyticus* em suspensão. Os autores propuseram que a AEB auxilia na penetração de agentes ativos de cloro e danifica as paredes celulares, desestabilizando os compostos poliméricos extracelulares ao redor dos envelopes das células bacterianas.

Também possui um forte potencial de redução que é responsável pela diminuição de radicais livres, devido seu forte redutor ORP, podendo ser utilizada como antioxidante. Hanaoka et al. (2004) avaliaram os efeitos da água reduzida sobre o dano oxidativo do DNA. A AEB teve tendência de suprimir a quebra de DNA de cadeia simples, sugerindo que é capaz de proteger contra as quebras da fita de DNA, possivelmente por efeitos antioxidantes aumentados contra o radical aníon superóxido.

Miyashita et al. (1999) avaliaram a eficácia de uma solução catódica preparada pela eletrólise de uma solução de NaCl na inibição da oxidação. A diminuição do substrato não oxidado e a formação de peróxidos totais durante a oxidação indicam que a solução catódica inibiu completamente a oxidação de ambos os ésteres de etila, enquanto estes lipídios foram facilmente oxidados em uma solução de NaCl.

Miyashita et al. (2003) mostraram forte atividade antioxidante na oxidação de ácidos graxos poli-insaturados com solução catódica preparada pela eletrólise de NaCl. O efeito seria

em parte devido à capacidade de eliminação radical e/ou moléculas alcalinas formadas na solução catódica. Na Tabela 4 são apresentadas algumas aplicações da água eletrolisada básica em alimentos.

Tabela 4 – Aplicação da AEB em alimentos.

| Propriedades | Metodologia | Resultado | Referência |
|---|---|---|---|
| pH 10,8 a 11,6<br>ORP 856 a 774 | Ensaio de biofilme com coloração de safranina | Remoção de biofilme de *S. aureus* | SUN et al. (2012) |
| pH 11 a 11,2<br>ORP 830 a -850 | Imersão de cenouras trituradas em AEB | Redução da contagem bacteriana total, leveduras e fungos | RAHMAN, (2011) |
| pH 11,7<br>ORP − 662 | Mistura de óleos de plantas com AEB | Remoção de 100% de Aflatoxina B1 | FAN et al. (2013) |
| pH 11,6<br>ORP −860 | Imersão de espinafre em AEB | Redução dos pesticidas: Acephate em 86%, Omethoate em 75% e DDVP em 46% | HAO et al. (2011) |
| pH 10,1<br>ORP −541 | Imersão de feijão em AEB por 45 min. | Redução dos pesticidas: Isoprocarb em 75% e Chlorpyrifos em 54% | HAN et al. (2017) |

Fonte: O autor (2019).
Onde: AEB- Água Eletrolisada Básica; ORP- mV; ACC- mg/L.

### *3.6.3.3 Água eletrolisada ligeiramente ácida (AELA)*

A AELA é obtida misturando-se a AEA e AEB, resultando em um pH e ORP desejado. De acordo com Król et al. (2017) devido ao seu pH mais neutro que a AEA, apresenta vantagens aplicada a indústria de alimentos, devido ao menor efeito corrosivo, ao mesmo tempo em que possui amplo espectro de atividade de desinfecção, devido principalmente a presença de HClO (DING; OH; LIU, 2019). Na Tabela 5 estão apresentadas pesquisas abordando a aplicação da AELA em alimentos.

Tabela 5- Aplicação da água eletrolisada ligeiramente ácida em alimentos.

| Propriedades | Metodologia | Resultado | Referencia |
|---|---|---|---|
| pH 6,1<br>ACC 20 | Imersão de repolho roxo em AELA | Redução de 1,5 log UFC $g^{-1}$ de bactérias aeróbicas e 1,3 log UFC $g^{-1}$ de bolores e leveduras | KOIDE et al. (2009) |
| pH 8,7<br>ORP 721<br>ACC 89 | Alfaces inoculadas tratadas com AELA | Redução da população bacteriana em 1,5 log UFC $g^{-1}$ | ABADIAS et al. (2008) |
| pH 6,29<br>ORP 870 a 900<br>ACC 40 | Imersão de carne em solução AELA | Redução da população microbiana em 0,7 log UFC $g^{-1}$ | SHENG et al., (2018) |
| pH 7,0<br>ORP 1110<br>ACC 65 | Placas inoculadas com biofilme e AELA por 10 min. | Redução de 6,5 log UFC $g^{-1}$ do biofilme *Listeria monocytogenes* | SÁNCHEZ et al. (2012) |
| pH 7,5<br>ACC 30 a 200 | Placas inoculadas com biofilme e AELA por 1 min | Redução de 100% do biofilme de *Candida albicans* e *Streptococcus mutans* | OZAKI et al. (2012) |
| pH 5,74<br>ORP 832 a 855<br>ACC 150 | Placas inoculadas com biofilme e AELA 20 min | Redução de 3,8 log UFC $g^{-1}$ do biofilme | HUSSAIN et al. (2018) |
| pH 6,03<br>ORP 820<br>ACC 66 | Imersão de tomate por 15 min | Redução de 60% do pesticida Parathion | WUYUN (2011) |
| pH 6,32<br>ACC 11,3 | Imersão de alho-poró por 15 min | Redução de 78% do pesticida Dimethoate | HU et al. (2016) |

Fonte: O autor (2019).
Onde: AELA- Água Eletrolisada levemente ácida; ORP- mV; ACC- mg / L.

O uso de AELA como meio de desinfecção ganhou popularidade com os investigadores no processamento de alimentos e preservação. Descobriu-se que a AELA possui capacidade antimicrobiana contra bactérias e fungos relacionados a frutas e vegetais. No geral, pode reduzir a população microbiana em 2 a 3 log UFC de bactérias ou fungos ligados a frutas, maior do que o nível de redução causado pelo tratamento convencional com cloro (HAO; WANG, 2019).

Rahman, Ding e Oh (2010) investigaram a AELA para inativar os agentes patogênicos nas folhas de espinafre como sanitizante. Os resultados demonstraram redução de microflora patogênica, sendo um sanitizante promissor para lavar vegetais sem poluição ambiental.

Hao et al. (2013) investigaram a eficiência de limpeza com AELA para inativar os micro-organismos no ambiente de reprodução de aves. Os resultados indicaram que a concentração de cloro dissolvido de 250 mg/L, pH de 6,19 e ORP de 974 mV inativou 100 % de bactérias e fungos em materiais sólidos (poeiras, fezes, pluma e alimentação).

Forghani e Oh (2013) examinaram a atividade microbiana de água eletrolisada levemente ácida (pH 5,2 a 5,5, ORP 500 a 600 mv e concentração de cloro disponível) em

repolho chinês, alface, folha de sésamo e espinafre. Foram observadas reduções microbianas de bolores e leveduras e contagem total de bactérias.

*3.6.3.4 Água eletrolisada neutra (AEN)*

Ainda esta controverso na literatura o uso de AELA e AEN, devido o pH semelhantes. No entanto pesquisadores tem definido a AEN com pH de 7 a 8 e ORP de 750 a 900 mV, obtida através da mistura entre AEA e AEB (RAMAN, KHAN, OH, 2016).

## 4. ARTIGOS CIENTÍFICOS

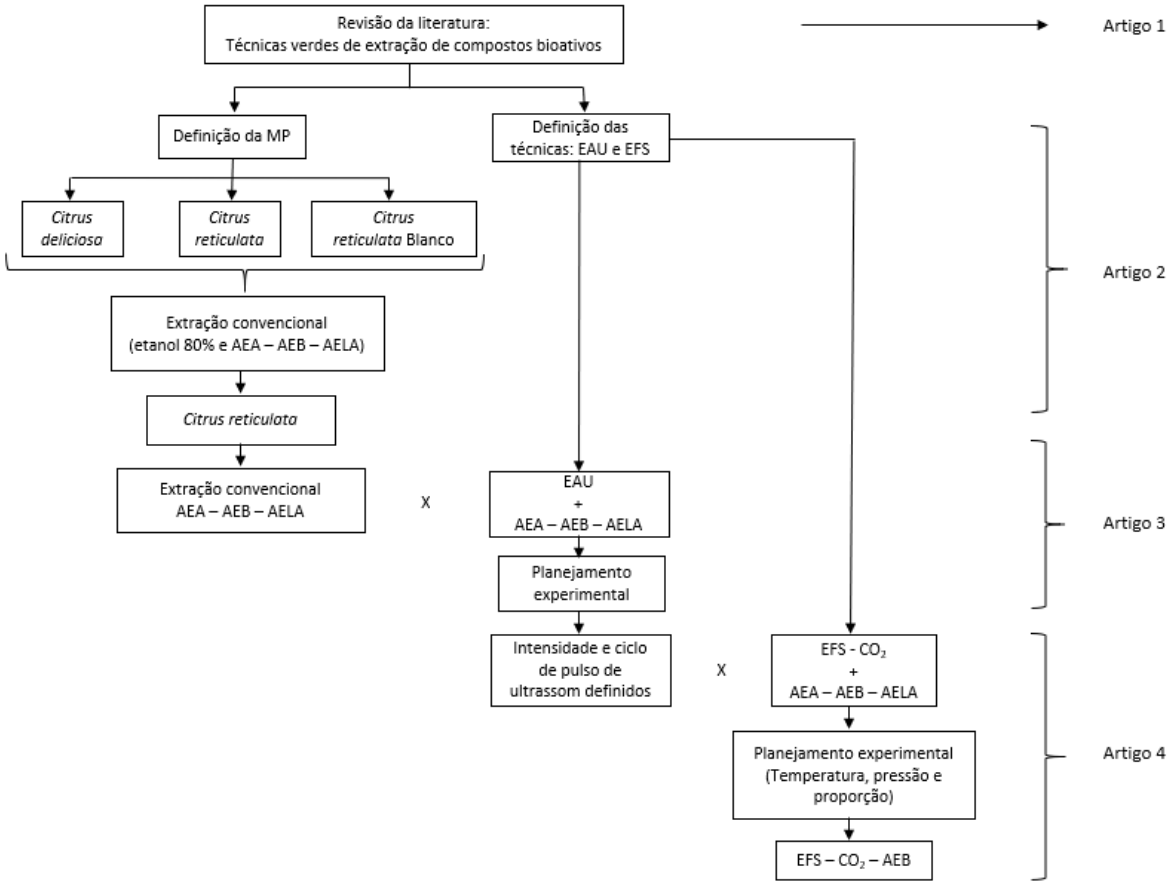
Esta tese foi dividida em quatro artigos científicos, como é mostrado no fluxograma da Figura 9.

No primeiro artigo foi feita uma revisão com as extrações não convencionais de compostos bioativos de frutas, para assim definir os equipamentos e matéria-prima utilizadas para a realização do projeto.

O segundo artigo descreve a parte de caracterização da matéria-prima das variedades de *Citrus deliciosa* (bergamota do céu), *Citrus reticulata* (tangerina) e *Citrus reticulata* Blanco (Ponkan). Neste artigo foram comparadas as concentrações de compostos bioativos utilizando etanol 80% (solvente convencional) comparando e água eletrolisada como solventes na extração convencional. De acordo com os resultados obtidos, selecionou-se uma variedade, *Citrus reticulata*, para desenvolvimento do terceiro artigo onde aplicou-se a extração assistida com ultrassom, utilizando somente a água eletrolisada como solvente, comparando com a extração convencional.

No quarto artigo, foram comparadas as extrações com fluido supercrítico, assistida por ultrassom e a combinação das duas, para a variedade selecionada, *Citrus reticulata*, utilizando água eletrolisada como co-solvente e solvente, respectivamente. A seguir estão apresentados os quatro artigos.

Figura 9- Fluxograma das etapas deste projeto de pesquisa.




Onde: MP – Matéria-prima; EAU – Extração Assistida por Ultrassom; EFS – Extração com Fluido Supercritico; EFS-$CO_2$ Extração com $CO_2$ como Fluido Supercrítico; - AEA – Água Eletrolisada Ácida; AEB – Água Eletrolisada Básica; AELA: Água Eletrolisada Levemente Ácida; Extração com CO2 como Fluido Supercritico utilizando Água Eletrolisada Básica.

Fonte: Autor, (2019).

## 4.1 ARTIGO 1: Green technologies for the extraction of bioactive compounds in fruits and vegetables

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# Green technologies for the extraction of bioactive compounds in fruits and vegetables

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## ABSTRACT

Bioactive compounds are extracted from natural sources and they have beneficial effects on human health. Fruits and vegetables are rich in carotenoids, phenolic compounds, Vitamin C, among others. Extraction processes for these compounds depend on several factors such as the technique that is used, the raw material, and the organic solvent. Conventional techniques generally require large amounts of organic solvents, high energy expenditure, and are time consuming, which has generated interest in new technologies that are referred to as clean or green technologies. These can reduce or eliminate the use of toxic solvents, and thus preserve the natural environment and its resources. The aim of this review is to discuss recent techniques used to extract bioactive compounds from natural sources, in order to reduce the economic and ecological impact of these processes.

**Keywords:** Clean technologies; phytochemicals; supercritical extraction; pressurized liquid; assisted-ultrasound; microwave

## 1 INTRODUCTION

Fruits and vegetables are fundamental foods for human health because they provide different flavors and are associated with improved quality of life. The consumption of vegetables and fruit is inversely associated with the development of cardiovascular diseases, and tends to be linked with protection from the major diet-related chronic diseases (Fardet & Boirie, 2014; da Silva, Barreira, & Oliveira, 2016).

Factors such as protection against diseases are related to the components of fruits and vegetables, particularly phenolic compounds, carotenoids, and vitamins, which are products of the secondary metabolism of plants (Sricharoen, Limchoowong, Techawongstien, & Chanthai, 2016). Bioactive compounds are found in foods, both of natural and synthetic origin, and they have specific metabolic or physiological actions provided that their safety for human

consumption is proven (BRASIL, 2002). The levels of bioactive compounds in fruits depend on factors such as the cultivar, the growing conditions, storage, and transport conditions (Bennett et al., 2011).

Bioactive compounds can be used as food additives due to their antioxidant properties. Antioxidants are substances that reduce oxidative stress in foods. Synthetic antioxidants are widely used because of their stability and their widespread availability; however, they are related to mutagenic and carcinogenic effects and this has led to the search for antioxidants extracted from plant matrices (Soquetta et al., 2016).

The extraction of bioactive compounds depends on several factors, such as the extraction technique, raw materials, and the extraction solvent that are used (Tiwari, 2015). The techniques can be classified into conventional or non-conventional. Conventional techniques require the use of organic solvents, temperature, and agitation. Examples of this type of technique include Soxhlet, maceration, and hydrodistillation. Modern techniques, or non-conventional techniques, are green or clean techniques due to reduced use of energy and the implementation of organic solvent, which are beneficial in relation to the environment (Rodriguez Perez et al., 2015).

Many studies have discussed the use of green technologies in relation to food processing (Barba, Zhu, Koubaa, de Souza Sant'Ana, & Orlien, 2016; Boussetta; Vorobiev, 2014; Chemat et al., 2017a; Chemat et al., 2017b; Mustafa, Turner, 2011; Soliva-Fortuny, Balasa, Knorr, & Martín-Belloso, 2009). However, it was not possible to find a review in the literature which covers the various techniques in the same scientific paper, and which also suggests different extraction techniques according to the target biocompounds.

The aim of this paper was to discuss applications using non-conventional energy sources, such as supercritical extraction, pressurized liquid, assisted ultrasound, microwave, pulsed electric field, electric high-voltage discharges, and high hydrostatic pressure, in the extraction of bioactive compounds from fruit and vegetables.

## 2 CONVENTIONAL TECHNIQUES

The main conventional extraction techniques in relation to bioactive compounds are Soxhlet, maceration, and hydrodistillation.

The Soxhlet technique involves a small amount of dry sample, which is placed on the equipment where the solvent passes through. The process is performed repeatedly until the extraction is complete. This extraction system is optimized and the literature provides a vast

amount of practical examples of favorable conditions (Cravotto et al., 2011; Xhaxhiu, Korpa, Mele, & Kota, 2013). However, this technique requires extensive extraction time and large amounts of solvent (Heleno et al., 2016).

Maceration consists of grinding the sample into smaller particles so as to increase the surface area for a good mixture with the solvent. The agitation in the maceration process makes extraction easier in two ways: by increasing the diffusion and by removing the concentrated solution from the surface of the sample. This process has been used for a long time to obtain essential oils and bioactive compounds (Azmir et al., 2013).

Ćujić et al. (2016) used the conventional method to extract polyphenols from dried chokeberry (*Aronia melanocarpa*) fruit. The effects of various parameters in the extraction of total phenolics and anthocyanins were studied. The solvents, particle size, solid-solvent ratio, and extraction time were investigated as independent variables in two factor levels. The aforementioned study indicated that the steeping was effective and was a simple technique for the extraction of bioactive compounds from chokeberry fruit.

Hydrodistillation is performed with distilled water and is used to extract the volatile fraction in foods; this method usually takes 6–8 h and organic solvents are not involved. This technique involves three main physicochemical processes: hydrodiffusion, hydrolysis, and decomposition by heat. High temperatures during extraction can degrade compounds, which limits the use of this technique (Wu, Wang, Liu, Zou, & Chen, 2015). Hydrodistillation is a very complete process: volatile organic compounds and non-volatile organic compounds can be extracted and physically separated in one step. The volatile organic compounds are stripped from the matrix by azeotropic distillation; they are then condensed, collected, and separated in a Florentine flask. The soluble non-volatile organic compounds are extracted in the boiling water, which is in contact with the matrix inside the alembic. However, hydrodistillation consumes high levels of energy and is time consuming (Petigny et al., 2014).

The efficiency of conventional extraction methods depends on the choice of solvent and the polarity of the compound, since solvents of different polarities are needed for identification and isolation. The polarities of compounds vary and it is difficult to develop a single method for the efficient extraction of all compounds. A good solvent provides low toxicity, a low boiling point, quick mass transfer, preservative action, and the inability to make the complex extract dissociate. The yield and the amount of the extract obtained also depend on several other factors such as the type of extract, the temperature, and the extraction time (Silva, Rock-Santos and Duarte, 2016).

# 3 GREEN TECHNOLOGIES

Security risks, such as the toxicity of solvents and the presence of solvent residues in the extracts, together with low yield, have stimulated the development of other extraction technologies, such as clean or green technologies, which can minimize or eliminate the use of organic solvents. These techniques are also known as cold extraction techniques, where the stability of the extracted compounds is not affected and the energy required for extraction is reduced (Tiwari, 2015).

According to Jacotet-Navarro et al. (2016) the objective of these green extraction processes is to achieve a faster extraction rate, more effective energy use, increased mass and heat transfer, reduced equipment size, and a reduction in the number of processing steps.

The application of these technologies is also intended to preserve the natural environment and its resources (Mustafa & Turner, 2011; Silva et al., 2016).

Some authors have sought to define the main points or elementary principles of green chemistry. Basically, the following 12 factors need to be considered in the implementation of green chemistry: prevention (avoiding waste); economy of atoms (maximizing the incorporation of all the starting materials in the final product); the synthesis of less hazardous products (little or no toxicity in relation to human health); safe product design (which performs the desired function and at the same time is non-toxic); safer solvents and auxiliaries; the search for energy efficiency (reduced environmental and economic impacts); the use of raw materials from renewable sources; the prevention of the formation of derivatives; catalysis (reagent as selective as possible); design for degradation (innocuous degradation products which do not persist in the environment); real-time analysis for the prevention of pollution; and chemistry that is intrinsically safe in order to prevent accidents (Lenardão, Freitag, Dabdoub, Batista, & Silveira, 2003).

A number of new alternatives to conventional techniques have been proposed to extract target compounds from various matrices (Table 1).

Table 1- Green technologies for the extraction of bioactive compounds in fruits and vegetables.

| Technique | Concept | References |
|---|---|---|
| Supercritical fluid (SFE) | Supercritical extraction is characterized by changes in temperature and pressure which transform the gas in the supercritical fluid. | Silva, Rocha-Santos and Duarte, 2016. |
| Pressurized liquid (PLE) | The extraction occurs at elevated pressures, so the solvent may remain in liquid state even when taken in temperatures much of their boiling points. | Machado et al., 2015 |
| Ultrasound assisted (UAE) | Ultrasound is a sound wave of 20 kHz to 100 MHz. This process produces a phenomenon called cavitation, which means that the production, growth and collapse of the bubbles to form pores that facilitate the leaching of organic compounds and inorganic plant matrix. | Azmir et al., 2013; Vinatoru, 2001; Rajha et al, 2015 |
| Microwave assisted (MAE) | Microwaves are electromagnetic fields in the range of 300 MHz to 300 GHz. The solvent penetrates into the solid matrix by diffusion and the solute is dissolved to reach a concentration limited by the solid characteristics. | Angiolillo et al., 2015; Li et al., 2013. |
| Pulsed electric field (PEF) | The material is placed between two electrodes. The pulse amplitude varies from 100-300 V / cm to 20-80 kV / cm. The treatment is conducted at room temperature or slightly higher. | Barba et al., 2016; Parniakov, et al., 2014. |
| High voltage electrical discharges (HVED) | It is an effective method to damage the cell structure and the extraction of valuable cellular compounds. The first step is the formation and propagation of a coil of a needle electrode and the formation of gaseous cavities. The second stage occurs when the streamer reaches the electrode plate (phase decomposition). | Brianceau et al., 2016; Barba, Boussetta and Vorobiev, 2015 |
| High hydrostatic pressure (HHP) | This technology applied very high pressures (100-1000 MPa) at 0 ° C to less than 100 ° C for a short period of time. | Andrés et al., 2016; Labarca-Briones et al., 2015. |

Fonte: Autor.

Table 2 shows how the extraction of bioactive compounds using green technologies has been studied.

Table 2 - Extraction of bioactive compounds through green Technologies.

| Bioactive compound | Green technology | Fruits and vegetables | Variables | References |
|---|---|---|---|---|
| **Total polyphenols** | SFE | Hazelnut, coffee and grape wastes | Supercritical fluid ($CO_2$), cosolvent (ethanol), temperature (40–60 °C) and pressure (350–500 bar) | Manna, Bugnone & Banchero, 2015 |
| | PLE | Apple | Pre-heating period, 5 min; solvent flush volume, 60% of the extraction cell volume; number of extraction cycles, 1; purge, 90 s using pressurized nitrogen (150 p.s.i.) | Alonso-Salces et al., 2001 |
| | UAE | Chicory | Temperature (20–60 °C), ethanol content in the solvent (0–60% (vol.) in ethanol–water mixtures) and ultrasound power (0–100 W) | Pradal et al., 2016 |
| | MAE | *Citrus sinensis* peels | Microwave power (300–600 W), extraction time (90–240 s), solvent-to-solid ratio (15–30 mL $g^{-1}$) and acetone in water concentration (20–80%, v/v) | Nayak et al., 2015 |
| | PEF | Opuntia dillenii cactus fruit | PEF in a batch treatment chamber with two parallel stainless steel electrodes, applying 56 exponential decay pulses at maximum electric field strength of 3 kV/cm resulting in a total energy input of 5 kJ/kg fruit mash. | Moussa-Ayoub et al, 2016 |
| | HVED | Grape seeds | The pulse energy Wp was in the range 3–10 J (and the corresponding specific pulse energy was in the range 41.8–139.4 J/kg). Electric arc treatment consisted in applying up to 1800 successive pulses with a repetition rate of 2 Hz. | Boussetta, Lesaint & Vorobiev, 2013 |
| | HPP | Red Fruit | The processing conditions were set at 500 MPa, 50°C, 10 min for the mousse samples and 400 MPa, 25 °C and 5 min for the pomegranate juice samples. | Ferrari, Maresca & Ciccarone, 2011 |

SFE- Supercritical fluid; PLE- Pressurized liquid; UAE- Ultrasound assisted; MAE- Microwave assisted; PEF- Pulsed electric field; HVED- High voltage electrical discharges; HPP- High hydrostatic pressure.

Continuação

Table 2 - Extraction of bioactive compounds through green Technologies.

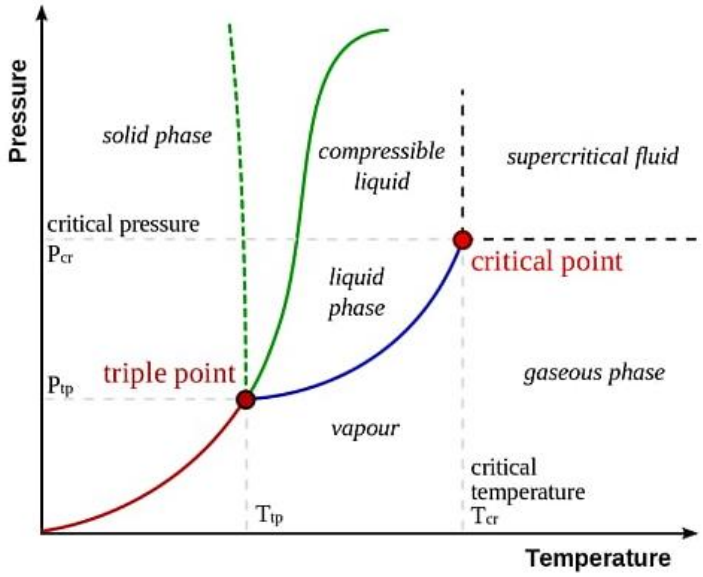
| Bioactive compound | Green technology | Fruits and vegetables | Variables | References |
|---|---|---|---|---|
| **Flavonoids** | SFE | Persimmon | Supercritical fluid ($CO_2$), Temperature (40-60°C), Pressure (100-300bar), Ethanol (5-25%), Flow rate (1-3mL/min),Time (30-110 min) | Zaghdoudi et al, 2016 |
| | PLE | Cherry seeds | Ethanol, Temperature (40-80°C), static time (2-10 min), number of cycles (1-5 n°), and flush volume (60-140%) | Oliveira et al, 2014 |
| | UAE | Grapefruit | An ultrasound bath with temperature control and working at 40 ± 2 kHz of frequency was used for the experiments. | Garcia-Castello et al., 2015 |
| | MAE | Carrot peels | A domestic microwave oven, which is capable of operating at a maximum input power of 850W at a frequency of 2450MHz | Hiranvarachat & Devahastin, 2014 |
| | PEF | Tomato juice | Electric field strength (0.4–2 kV/cm) and number of pulses (5–30) applied | Vallverdú-Queralt et al, 2013 |
| | HHP | Tomato waste | Pressure range of 100–800 MPa for 1–30 min. Temperature prior to pressurization was 25 °C and was elevated during compression, due to adiabatic heating, approximately 3 °C/100 MPa. Solvent to solid ratios of 10:1 mL:g, 6:1 mL:g and 4:1 mL:g were examined | Strati, Gogou & Oreopoulou, 2015 |

SFE- Supercritical fluid; PLE- Pressurized liquid; UAE- Ultrasound assisted; MAE- Microwave assisted; PEF- Pulsed electric field; HVED- High voltage electrical discharges; HPP- High hydrostatic pressure.

## 3.1 SUPERCRITICAL FLUID EXTRACTION

Supercritical extraction is characterized by changes in temperature and pressure, which transforms the gas in the supercritical fluid, where the gas and liquid phases are indistinguishable. As shown in Figure 1, the critical temperature is considered to be the maximum temperature at which gas can be converted into liquid by increasing the pressure, and the critical pressure is the maximum pressure at which the liquid can be converted to gas by increasing the temperature.

Figure 1- Diagram of the critical point.




Fonte: SILVA; MARTÍNEZ, (2014).

It is a mass transfer operation, in which the convection in the supercritical solvent phase is generally the main transport mechanism (Silva & Martínez, 2014). The extraction is rapid, selective, and does not require further cleaning; furthermore, it can be performed on small amounts of sample (Oroian & Escriche, 2015). Another great advantage is the possibility of direct association with analytical chromatographic techniques such as gas chromatography (GC) and supercritical fluid chromatography (SFC) (Silva-Santos Rocha and Duarte, 2016).

This technique can be summarized in two steps: the solubilization of the chemical compounds present in the solid matrix, followed by their separation in the supercritical solvent. The solvent flows through the packed bed and solubilizes compounds that are present in the matrix. The solvent subsequently leaves the extractor and by a reduction in pressure and an increase in temperature it becomes a solvent-free extract (Silva et al., 2016).

Supercritical fluids exhibit desirable transport properties that enhance their ability to adapt. Compared to liquid solvent, which is used in conventional extraction processes, supercritical fluids have low viscosity, spreading more easily within the solid matrix, as well as low surface tension, which allows rapid penetration of the solvent into the solid and, consequently, increased extraction efficiency. Since density is related to solubility, by changing the extraction pressure the force of the fluid can be modified (Pouliot, Conway, & Leclerc, 2014).

Supercritical extraction is primarily used to isolate nonpolar bioactive compounds (carotenoids and lipids) due to the fact that the solvents used in this technique are of this nature.

One option for the extraction of polar compounds, such as flavonoids, is the addition of modifiers such as ethanol, methanol, water, and acetone (Herrero, CastroPuyana, Mendiola, & Ibañez, 2013).

The solubility of the extracts depends on the density of the solvent. Thus, the following different supercritical fluids have been described in studies: $CO_2$; propane; cooking gas (LPG); ethane; ethene; methanol; nitrous oxide; n-butene; n-pentene; sulfur hexafluoride; and water (Silva, RochaSantos and Duarte, 2016).

### 3.1.1 $CO_2$ as a supercritical fluid

Carbon dioxide ($CO_2$) is used as a supercritical fluid for three main reasons: it is harmless to human health and the environment, thereby respecting sustainability factors; its critical temperature is moderate (31.2 °C), which is a fundamental issue for the preservation of bioactive compounds in extracts; and the extract is preserved from contact with the air and light, where oxidation reactions may occur. Furthermore, it is also possible to modulate the power of $CO_2$ in order to perform a selective extraction (Silva-Santos; Rocha and Duarte, 2016, Brunner, 2005).

The extraction of polar compounds, such as most phenolic compounds, requires the presence of polar solvents used as modifiers due to the non-polar nature of $CO_2$ (Meneses, Caputo, Scognamiglio, Reverchon, & Adami, 2015; Oroian & Escriche, 2015; Solana Mirofci and Bertucco, 2016).

The use of $CO_2$ is an alternative to the extraction of natural herbal antioxidants (Meneses et al., 2015; Vargas Table 1. et al., 2013), fruits (Song et al., 2016; Viganó et al., 2016), vegetables (Bagheri et al., 2016), microorganisms (Guedes et al., 2013; Yen, Yang, Chen, & Chang, 2015), among others.

Cavalcanti, Albuquerque, and Meireles (2015) conducted a study regarding the technical and economic feasibility of cupuaçu butter extraction using $CO_2$ as the supercritical fluid. Cupuaçu butter obtained from extraction can be a valuable ingredient in foods, pharmaceuticals, and cosmetics, due to the high levels of alpha, gamma, and delta tocopherols that it contains, as well as high levels of unsaturated (oleic and linoleic) acids compared to saturated acids (palmitic and stearic). The optimum conditions in relation to the extraction kinetics, chemical composition, and manufacturing costs were 30–35 MPa and 50°C.

Supercritical $CO_2$ has been commonly used for the extraction of bioactive compounds. Guedes et al. (2013) used supercritical fluid to extract carotenoids and a, b and c chlorophylls

from Microalga Scenedesmus obliquus for subsequent use in food processing. The highest yields were obtained at 250 bars. The most suitable temperature to obtain the highest yields for chlorophyll was 40°C and for carotenoids it was 60°C.

Malaman, Moraes, West, Ferreira, and Oliveira (2011) used $CO_2$ as the supercritical fluid to extract bioactive compounds in Brazilian cherry. The extractions were performed under different temperature and pressure conditions. The extracts showed high concentrations of phenolic compounds and the authors highlighted the flavor intensity, mainly in the extract which was performed at 50 °C independent of pressure.

Sodeifian, Ghorbandoost, Sajadian, and Ardestani (2015) performed a supercritical fluid extraction (SFE) with carbon dioxide to obtain oil from Pistacia khinjuk Stocks fruit. Response surface methodology (RSM) was used to optimize the process and to evaluate the effect of different operating parameters, including pressure (12– 24 MPa), temperature (35–55 °C), flow rate (2–6 g/min), and extraction time (0–300 min.) on the total oil yield. The relationship between the yield of P. khinjuk fruit oil and the extraction variables was determined by a secondorder polynominal equation (Eq. 1) applying a central composite design (CCD).

$$\text{Oil yield} = \frac{\text{Amount of extracted oil g}}{\text{Amount of total oil in initial ground sample g}} \text{x } 100\% \quad (1)$$

Romo-Hualde, Yetano-Cunchillos, González-Ferrero, SáizAbajo, and González-Navarro (2012) studied various different parameters that can affect the extraction yield, using $CO_2$ as the supercritical fluid. The highest yield of vitamin E (97 %) and provitamin A (68.1 %) in red pepper (*Capsicum annum L.*) was found at a temperature of 60 °C, 24 Pa and using a particle size of 0.2–0.5 mm.

$CO_2$ is also a suitable solvent for the extraction of bioactive compounds from biological substrates but it has limited ability to dissolve high molecular weight compounds such as carotenoids (Araus, del Valle, Robert, & Juan, 2012).

Babova, Occhipinti, Capuzzo, and Maffei (2016) investigated the use of $CO_2$ as supercritical fluid for the selective extraction of antioxidants from blueberry. The supercritical treatment allowed the selective extraction of the compounds.

### 3.1.2 Propane as a supercritical fluid

Extraction with propane has been shown to have significant benefits, even when compared to extraction with $CO_2$, such as higher yields, higher solubility of non-polar compounds, selectivity, as well as shorter extraction times and reduced solvent consumption (Correa, Mesomo, Pianoski, Torres, & Corazza, 2016; Hamdan, Daood, Toth-Markus, & Illés, 2008).

Zanqui et al. (2015) extracted flaxseed oil using n-propane as supercritical fluid under different pressures and temperatures. The yield was 28 % at temperatures ranging from 30 to 45 °C and pressures of 8–12 MPa.

Da Silva et al. (2015) evaluated the influence of temperature and pressure on perilla oil extraction using compressed n-propane gas. The experiments were performed using a temperature range of 40–80 °C and pressures of 8–16 MPa at a constant flow rate of 1.0 $cm^3$/min n-propane. The extraction yields showed satisfactory extraction and higher stability compared to the classical method.

Santos et al. (2015) studied crambe seed (*Crambe abyssinica*) oil extraction using supercritical propane as solvent. The authors observed that temperature had a significant effect on the extraction, and the highest yield was obtained using 353 °K and 16 MPa (32.8 % in weight).

Pessoa et al. (2015) studied the pequi pulp oil extraction process using supercritical propane at pressures of 5–15 MPa and temperatures ranging from 303.15 to 333.15 °K. The highest yield (43 % in weight) was for conditions of 15 MPa and 333.15 °K.

Pederssetti et al. (2011) investigated canola seed oil extraction using carbon dioxide and propane tablets as solvents. The extractions were performed at temperatures of 40, 50, 60, and 20 °C and pressures 22.5 and 25 MPa for carbon, and temperatures of 30, 40, and 60 °C and pressures of 8, 10, and 12 MPa for propane. The results indicated that the pressure and temperature were important variables for the efficiency of extraction. Extraction with propane proved much faster than with carbon dioxide.

Corso et al. (2010) evaluated sesame seed oil extraction using carbon dioxide and propane tablets as solvents. The extractions were performed at temperatures of 313–333 °K and pressures of 19–25 MPa for carbon dioxide and temperatures of 303–33 °K and pressures of 2–12 MPa for propane. The results indicated that the solvent and the density were important variables in the extraction with $CO_2$, while the temperature was most important in relation to

propane. The extraction with propane was faster in than carbon dioxide; the extract characteristics were similar.

Nimet et al. (2011) compared sunflower seed oil extraction using propane and $CO_2$ as the supercritical fluid. The best yield was obtained used propane as a solvent, and the extracts showed high concentrations of vitamin E.

### 3.1.3 Compressed liquefied petroleum gas as supercritical fluid

Liquefied petroleum gas (LPG) is characterized by a mixture of propane and n-butane, and it has been used as fuel for heating appliances and for cooking food (Silva et al., 2015).

Silva et al. (2015) evaluated the use of LPG to treat some enzymes high pressure to increase their catalytic power; LPG increased the processing speed.

Soares et al. (2015) used $CO_2$ and compressed LPG to obtain rice bran oil and concluded that the fatty acid profiles were similar. However, a kinetic analysis demonstrated that LPG decreased the mass of solvent/feed and extraction time. The authors concluded that LPG is promising, considering that reducing the analysis time reduces the energy required for the re-compression of the solvent.

Dal Prá et al. (2016) published an article in which they described the use of solvents (including LGP) to extract biocompounds from palm (*Elaeis guineensis*).

Using LPG avoids the main recurring problem in terpene extraction and offers a series of advantages regarding ecological and technical issues. Therefore, this method offers a solution that can be applied for the recovery of terpenes in industrial processes and on a laboratory scale (Bier et al., 2016).

## 3.2 EXTRACTION WITH PRESSURIZED LIQUID

This technique uses a separation process that involves the transfer of solutes from a solid matrix. Liquid solvents, at elevated temperatures and pressure, are used, which produces a reduction in the surface tension of the solvent, which in turn facilitates the penetration of the solvent into the pores of the matrix. The process disrupts the matrix, which increases the mass transfer of the analyte from the solvent sample (Garcia-Mendoza, Paula, Paviani, Cabral, & Martinez-Correa, 2015). The solvents are chosen based on the solubility characteristics of the desired solute. The versatility of pressurized solvents is excellent due to the physicochemical properties of the solvent, including the density, diffusivity, viscosity, and dielectric constant,

which can be controlled by varying the temperature and the pressure of the extraction system (Pronyk & Mazza, 2009).

Extraction using pressurized liquid is an attractive technique because it allows rapid extraction and reduced solvent consumption; it has been successfully employed for the extraction of anthocyanins from various plants (Santos, Veggi, & Meireles, 2012).

Machado et al. (2015) obtained an extract with antioxidant compounds from cranberry waste using extraction with pressurized liquid. They analyzed different solvents (water, acidified water: 2.5, ethanol) and temperature (60, 80, and 100 °C). The best condition was compared with conventional extraction (Soxhlet and maceration). The best results were obtained by extraction with pressurized liquid at a temperature of 100 °C, using water and ethanol as solvent. The authors proved that the use of this technology is promising in terms of recovering bioactive compounds from fruit.

Xu et al. (2016) used the pressurized water methodology to extract polysaccharides from gooseberry and to investigate the antioxidant activity. The optimum conditions were: 51 min, pressure of 1.6 MPa, and a temperature of 52 °C. The study provided a new and efficient extraction method for polysaccharides from gooseberry.

Machado et al. (2015) obtained extracts from blackberry residue using pressurized liquid extraction. The influence of the solvent (acidified water pH = 2.5 and ethanol + 50 % water) was evaluated, as well as the temperature. The authors proved that this is a promising technique.

Bajer, Bajerová, Kremr, Eisner, and Ventura (2015) applied the method of extraction with pressurized hot water in peppers to optimize the yield of bioactive compounds. An optimization study was performed using water as solvent at a constant pressure of 20 MPa, temperatures of 120–240 °C and time from 5 to 60 min. The study was compared to the Soxhlet method and was approximately 113 % relatively efficient in comparison. The authors emphasized that the only disadvantage of this method is that it requires sophisticated instrumentation because it requires greater application of pressure and extraction temperatures.

## 3.3 ULTRASOUND-ASSISTED EXTRACTION

Ultrasound is a special kind of sound wave that ranges from 20 kHz to 100 MHz. Ultrasound-assisted extraction produces a phenomenon known as cavitation, which entails the production, growth and collapse of bubbles (Azmir et al., 2013). UAE is an effective extraction technique for a wide range of analytes from different types of samples. Ultrasounds have effects

that accelerate heat and mass transfer via the disruption of plant cell walls, leading to improved release of the target compounds from several natural sources (Roselló-Soto et al., 2015).

Extraction using ultrasound involves two main types of physical phenomena; diffusion through the cell wall, and rinsing the cell content after breaking the walls. The temperature, pressure, frequency, and sonication time are all factors which regulate the action of ultrasound (Rajha et al., 2015; Vinatoru, 2001).

Ultrasound is relatively easy to use; it is versatile, flexible, and requires low investment compared with other extraction techniques. Ultrasound has been used to extract molecules and various biomaterials, including polysaccharides, essential oils, proteins, peptides, dyes, pigments, and bioactive compounds (Briones-Labarca et al., 2015; Tiwari, 2015).

This phenomenon can be indirect or direct. When ultrasound is applied directly to the medium without any barrier, such as a probe system, it provides an intensity that is approximately 100 times higher. For indirect sonication, such as using an ultrasonic water bath, the waves have to be transferred through the water until they reach the sample (Kek, Chin, & Yusof, 2013).

The application of ultrasound energy has been considered to be a promising alternative for the extraction of bioactive compounds; it increases the mass transfer coefficient, accelerates the kinetics, and increases the final yield (Riera et al., 2004).

Xu, Li, and Sun (2015) reported on the impact of ultrasound-assisted extraction of natural antioxidants from the *Eucommia oliver* plant using distilled water as solvent. The use of ultrasound has improved the effectiveness of traditional treatments, providing higher yields and the selectivity of natural antioxidants.

D'Alessandro, Dimitrov and Nikov (2014) validated the extraction of bioactive compounds from black chokeberry fruit. They studied the influence of the extraction time (0–240 min), the composition of the solvent (ethanol-water) and the levels of ultrasound energy (0–100 W). According to these authors, ultrasound is a suitable technology for the extraction of phenolic compounds (total anthocyanins and polyphenols).

Sivakumar, Ilanhtiraiyan, Ilayaraja, Ashly, and Hariharan (2014) studied the influence of ultrasound in Avaram shell (*Cassia auriculata*) for the extraction of tannins. The results showed an improvement of 160 % when using ultrasound at 100 W in comparison with magnetic stirring, suggesting that this was strongly connected to improved mass transfer in the leaching of tannins.

Khan, Abert-Vian, Fabiano-Tixier, Dangles, and Chemat (2010) reported on the extraction of polyphenols, especially flavonoids, from orange peel (*Citrus sinensis L.*), using

ethanol as a solvent of food grade. The best conditions were 40 ° C, power of 150 W and a ratio of 4:1 (ethanol: water). The authors found an increase in the yield of the compounds and antioxidant activity of the extracts obtained by ultrasound, and confirmed the suitability of this technique for preparing fruit extracts.

Rodríguez-Pérez, Quirantes-Piné, Fernández-Gutiérrez, and Segura-Carretero (2015) used ultrasound to determine the best way to extract bioactive compounds from Moringa oleifera. They concluded that using ultrasound increased the yield of the extracts and the content of bioactive compounds.

Rabelo, Machado, Martínez, and Hubinger (2016) used the ultrasound-assisted method to extract phenolic compounds from artichoke residues. The highest yields were observed for the extracts with high ethanol content (50 %), ultrasonic power of 240 W, and 10 min. sonication.

Meullemiestre, Petitcolas, Maache-Rezzoug, Chemat, and Rezzoug (2016) evaluated the impact of ultrasound on the extraction of phenolic compounds from waste pine. The optimum extraction conditions were 40 °C, with ultrasonic intensity of 0.67 W/cm$^2$, and a time of 43 min.

Corbin et al. (2015) extracted phenolic compounds from pine seeds using ultrasound. The method was proven to be very effective for reducing the trapping of phenolic compounds. The optimal conditions were using supplemented water as solvent with 0.2 N sodium hydroxide, a 60 min. extraction time, 25 °C temperature, and an ultrasonic frequency of 30 KHz. In comparison with conventional maceration this technique resulted in a 30 % increase in the content of phenolic compounds.

De Paula et al. (2016) used ultrasound-assisted extraction regarding bioactive compounds from dried fruit of Azadirachta indica A. Juss (*Meliaceae*). The results showed that the optimum conditions were a concentration of 75–80 % ethanol, 30 °C temperature and a material-solvent ratio of 0.55 gmL$^{-1}$.

The authors suggest that ultrasound assisted extraction is a more efficient extraction process because it is simple, fast, and inexpensive. Wang et al. (2015) investigated the optimum conditions for the ultrasound-assisted extraction polysaccharides, as well as the antioxidant activity of pears (*Pyrus sinkiangensis*, subfamily Maloideae). The highest yield (5.16 %) was obtained at 70 °C, with a power of 230 W, and a water ratio of 13:1 mL/g. The technique was efficient and the authors concluded that this fruit has potential for applications in the food industry.

Hammi, Jdey, Abdelly, Majdoub, and Ksouri (2015) also used ultrasound-assisted extraction regarding bioactive compounds from *Zizyphus lotus* fruit. The optimum operating conditions were as follows: 50 % ethanol concentration, extraction time of 25 min., temperature of 63 °C, and a solvent: material ratio of 67 mL/g. The authors' conclusion was that this methodology was suitable for the extraction of bioactive compounds from fruit.

Chen, You, Abbasi, Fu, and Liu (2015) used ultrasound to extract polysaccharides with antioxidant activity from black mulberry fruit. The most favorable conditions were with a water: material ratio of 40:25, a temperature of 69 °C, time of 75 min., and ultrasound power of 190 W. The maximum yield was 3.13 %, showing that the ultrasound technique was effective.

## 3.4 MICROWAVE ASSISTED EXTRACTION

Microwaves are electromagnetic fields in the range of 300 MHz to 300 GHz with two oscillating fields which are perpendicular, such as electric field and magnetic field frequencies. The solvent penetrates inside the solid matrix by diffusion and the solute is dissolved to reach a concentration that is limited by the solid's characteristics (Angiolillo, Del Nobile and Conte, 2015).

Microwaves are a non-contact heat source that can provide more effective heating, accelerating the transfer of energy and reducing the thermal gradient. Several classes of compounds, such as essential oils, antioxidants, pigments, flavorings, and other organic compounds, can be efficiently separated using this method (Li et al., 2013).

According to Leadbeater (2014), the use of microwave equipment is a flourishing technology because it is possible to have access to higher temperatures easily, safely, and in a reproducible manner; the reaction time can be reduced; the yield can be increased; and the purity can be improved, in comparison to conventional heating methods. This technique can be performed either with or without the addition of any solvent (Oroian & Escriche, 2015).

Simha, Mathew, and Ganesapillai (2016) investigated the effectiveness of microwave-assisted extraction (MAE) to recover bioactive compounds from the pharmaceutically significant medicinal plants Cymbopogon citratus and Adathoda vasica.

Li et al. (2013) provide an overview of the techniques that are available to extract bioactive compounds using microwaves without solvents. They showed that this can be an alternative to other techniques, with the advantages of reduced time, energy consumption, use of solvents, and $CO_2$ emissions.

Grigoras, Lazar, and Elfakir (2012) performed a comparative study of conventional methods; maceration; and extraction using pressurized liquid, ultrasound, and microwave. The microwave-assisted methodology provided the highest concentration of bioactive compounds in the apple extract.

Krishnan and Rajan (2016) used the microwave-assisted technique to extract flavonoids from the Terminalia bellerica plant. The microwave equipment (R-219T (S)/ (W), SHARP, Japan) was fitted with a timer to control the duration of the irradiation cycles. The maximum yield of flavonoids was 25.21 mg/g using water as solvent, with a ratio of 40 mL/g and a temperature of 100 °C. The authors concluded that the extraction technique using microwaves was a suitable method for flavonoid extraction, recovering 82.74 %, whereas the conventional method only recovered 63.75 %.

Inoue, Tsubaki, Ogawa, Onishi, and Azuma (2010) isolated hesperidin from the skin of Citrus unshiu fruit using microwave-assisted extraction. The microwave that was used was at 1 kW and 2.45 GHz of power. The optimal parameters were a temperature of 140 °C and a time of 8 min. Under these conditions 86.8 % (47.7 mg/g) of hesperidin was isolated. The process was efficient and it can be considered to be a simple application.

Thirugnanasambandham & Sivakumar, In Press optimized the extraction of betalains from pitaya (*Dragon fruit*) using microwave-assisted extraction. The microwave that was used (VCX 400, Sonics) had a power of 100 W. The optimum conditions were: a temperature of 35 °C, a sample weight of 20 g, and 8 min. treatment time. This extraction method was found to be efficient for bioactive compounds in fruits.

Simic et al. (2016) optimized microwave-assisted extraction of phenolic compounds from chokeberries using response surface methodology. The parameters that were examined were: microwave power (300, 450, and 600 W), ethane concentrations (25 %, 50 %, and 75 %) and extraction time (5, 10, and 15 min). The highest yield (420.1 equivalents mg gallic acid/100 g of plant material) was at a concentration of 53.6 %, power of 300 W, and time of 5 min.

Seixas et al. (2014) extracted pectin from passion fruit skin by heating that was induced by microwave. The results indicated that the exposure time and the microwave power significantly affected the yield of pectin with nitric and tartaric acid. The highest yield was obtained using the longest time (9 min) and the highest power (628 W). The method was efficient for the extraction of pectins in fruits.

Maran, Sivakumar, Thirugnanasambandham, and Sridhar (2014) also extracted pectin from Citrullus lanatus shells. The parameters that were studied were the microwave power (160–480 W), irradiation time (60–180 s), and liquid-solid ratio (1:10–1: 30 g/ml). The results

showed that all the process variables had a significant effect. The best yield (25.79 %) was obtained with a power of 477 W, time of 128 s, and a solid: liquid ratio of 1:20, 20.3 g/ml. The extraction method was satisfactory.

Hydrodiffusion microwave is a green technology that does not use solvents, which results in increased permeability and fabric softening. It is an extraction method that is economic, efficient, and environmentally friendly (Oroian & Escriche, 2015).

Petigny et al. (2014) presented a study from lab to pilot scale for the extraction and separation of volatile and nonvolatile compounds from boldo leaves using microwave. The experimental conditions (microwave power and time of extraction) were optimized by using an experimental plan design. These authors concluded that the reduced cost of extraction provided by this proposed MAE method was based on reduced consumption of energy and time because hydrodistillation required 30 min to start the azeotropic distillation, whereas MAE only required 5 min. This proved the efficiency of heating energy delivered in the matrix. This extraction method, combined with this new microwave apparatus, indicates potential for industrial use for day-today operations; however, it could also be used in a process to create equipment using continuous extraction.

## 3.5 PULSED ELECTRIC FIELD ASSISTED EXTRACTION

The principle of pulsed electric field extraction is to induce the electroporation of the cell membrane, thereby increasing the extraction yield. An electric potential passes through the cell membrane and separates molecules according to their charge. This repulsion forms pores, increasing their permeability (Azmir et al., 2013; Rajha et al., 2015). Among the different applications of PEF, food preservation and the recovery of intracellular valuable compounds from plant food materials, food wastes, and by-products have been the most widely studied. PEF is a useful tool to selectively recover valuable compounds from different fruit and vegetable tissues from an economic and sustainable point of view, mainly due to its ability to soften and disrupt cell membranes, thus facilitating the release of intracellular compounds (Roselló-Soto et al., 2015).

Soliva-Fortuny et al. (2009) studied the system typically used for the treatment of pumpable fluids, which consists of a PEF generation unit, which is in turn composed of a high voltage generator and a pulse generator, a treatment chamber, a suitable product handling system and a set of monitoring and controlling devices.

The effectiveness of the treatment depends on the process parameters, including the intensity field, input energy, pulse number, temperature, and material properties (Azmir et al., 2013).

Scientific studies and recent practice have shown that the pulsed electric field technique is compatible with the concept of green extraction techniques since it uses renewable plant resources and alternative solvents, such as water or agri-solvents (ethanol and methyl esters of fatty acids from vegetable oils), reduced energy consumption and unit operations, as well as producing extracts of high quality and purity (Parniakov et al., 2014).

The application of the pulsed electric field technique in water has been shown to improve the extraction of compounds from different raw materials, as well as increasing the rate of extracted compounds, reducing the temperature and reducing the level of solvents (Bousseta and Vorobiev, 2014).

This technique can facilitate the selective recovery of valuable compounds without deteriorating the treated matrix, thus favoring the separation and purification of subsequent stages (Barba et al., 2015b).

Electrically pulsed and high-voltage discharges can be useful technologies for the recovery of food waste and byproducts (Oroian & Escriche, 2015). Methods assisted by pulsed electrical energy can increase productivity and the quality of the extracted compounds, thus decreasing the time and temperature of the extraction operations (Parniakov et al., 2014).

Luengo, Alvarez and Raso (2013) used the pulsed electric field method to extract of polyphenols and flavonoids from orange peel. A time of 60 μs (20 pulses of 3μs) resulted in the highest cell disintegration index. In comparison with the untreated sample, the yield of phenolic compounds using pulsed electric fields for 1, 3, 5, and 7 kV/cm increased by 20 %, 129 %, 153 %, and 159 %, as well as increasing antioxidant activity by 51 %, 94 %, 148 %, and 192 %, respectively. The results demonstrated the potential of the pulsed electric field technique as a method of extraction of bioactive compounds, reducing the extraction times and not requiring the use of organic solvents.

Xue and Farid (2015) studied the effects of continuous treatment in relation to the extraction of bioactive compounds using pulsed electric field in the range of 12.4–38.4 kV/cm. The optimal yields were estimated at 38.4 kV/cm and a temperature of 85 °C. The increased yield of bioactive compounds compared with conventional treatment was clear. The data obtained indicated that the permeabilization of the membrane, which assists the extraction of compounds, occurs within a short period of time.

Leong, Burritt and Okey (2016) assessed the release of anthocyanins in grape juices after pulsed electric field treatment. The treatment chamber consisted of two stainless steel electrodes and the optimal variables were: a pulse of 20 mS, a frequency of 50 Hz and an electric field strength of 1.5 kV/cm. Compared with the untreated grape juice, PEF helped the release of anthocyanins, increased the content of bioactive compounds and vitamin C, as well as improving the antioxidant activity. The technique was shown to be efficient in providing a better phytochemical composition of the extracts, as well as the ability to protect cells from oxidative stress.

Segovia, Luengo, Corral-Pérez, Raso, and Almajano (2015) reported on the extraction of polyphenols from Borago officinalis leaves using pulsed electric field treatment. The equipment used generated wave pulses with a maximum frequency of 300 Hz; the maximum output voltage was 30 kV and the current was 200 A. The polyphenol and ORAC values were increased between 1.3 and 6.6 % and from 2.0 to 13.7 %, respectively. The authors concluded that the procedure increased the antioxidant capacity of the extracts and reduced the extraction time.

Jaeger, Schulz, Lu, and Knorr (2012) used the pulsed electric field technique in relation to apple juice. The yield increased according to the field intensity. The overall composition, polyphenol content and antioxidant capacity were similar to conventional treatments, but there was a reduction in process time.

Elez-Martinez and Martín-Belloso (2007) subjected orange juice to high-intensity pulsed electric field treatment. The vitamin C content was higher than in conventionally pasteurized juices. This technique is effective in the recovery and protection of bioactive compounds.

In some cases, the application of electric fields at room temperature is not sufficient to damage the cells. The application of pulsed ohmic heating consists of increasing the temperature through ionic movements in the sequence for applying treatment (Barba et al., 2016).

## 3.6 HIGH-VOLTAGE ELECTRICAL DISCHARGES

In this technique, energy is introduced directly into an aqueous solution through a plasma channel formed by a current of high-voltage electrical discharge between two submerged electrodes (Barba et al., 2015). The intensity of the electric field is able to induce an avalanche of electrons that are responsible for starting the spread of the positive streamer for

the negative electrode. Secondary phenomena, such as bubble cavitation, turbulence, and pressure shock waves, contribute to the improvement of cell damage, facilitating the release of compounds and the extraction of biomolecules from the cytoplasm of the cells (Rajha et al., 2015).

It is necessary to optimize the extraction parameters for each product. The nature of the raw materials significantly affects the efficiency of the treatment, as was demonstrated in the study by Barba et al. (2015).

Authors who work with this type of apparatus have noted that during the electrical discharges cavitation bubbles are produced, which resembles the ultrasound technique (Vinatoru, 2001).

Boussetta et al. (2011) applied the technique of highvoltage electrical discharge to extract phenolic antioxidants from grape pomace. The HVED experiments were performed in a laboratory treatment chamber connected to a pulsed high-voltage power supply. The technique increased the yield and improved the extraction kinetics. The best electrical treatment parameters were an 80 kJ/kg power source, an electrode aperture of 5 mm, and a liquid-solid ratio of 5.

In a comparative study between conventional techniques, pulsed electric field and high voltage discharge, Parniakov et al. (2014) concluded that the latter showed better extraction efficiency to recover high value compounds. However, the electrical discharges can produce chemical electrolysis and free radicals, which can react with valuable compounds and antioxidants, reducing their beneficial properties.

## 3.7 HIGH HYDROSTATIC PRESSURE

High hydrostatic pressure (HHP) has been developed as an alternative to thermal processes, with the aim of obtaining microbiologically safe food products and avoiding undesirable changes in the sensory, physicochemical, and nutritional properties of foods (Escobedo-Avellaneda et al., 2011). This technology operates under pressures generally ranging from 100 to 1,000 MPa (Briones-Labarca et al., 2015). It is widely known as an alternative to conventional heating treatments (Tao et al., 2016) and is considered to be a green technology. It is recognized by the Food and Drug Administration in the United States, since it only requires electric power and does not generate waste (Andrés et al., 2016).

The use of HHP improves mass transfer rates and increases the secondary metabolite diffusion according to phase transitions (Oroian & Escriche, 2015).

High pressures generate the deprotonation of the charged groups and disrupt hydrophobic bonds and salt bridges, resulting in changes in form and the denaturation of proteins. Additional solvent can enter the cells and further compounds can permeate the cell membrane, increasing the yield (Briones-Labarca et al., 2015).

In a comparative study between conventional, ultrasound and high hydrostatic pressure extraction techniques, Briones-Labarca et al. (2015), found that the latter was more effective than the other techniques in the extraction of bioactive compounds from papaya (Vasconcellea pubescens).

George, Selvan, and Rastogi (2016) studied high pressure treatment in the extraction of anthocyanins from apple. The high pressure treatment resulted in increased humidity and solid mass transfer due to the cell permeabilization, which was revealed via microstructure analysis. The authors also suggested that HHP resulted in greater infusion of the bioactive compounds compared with infusion at atmospheric pressure.

Andrés et al. (2016) used the high pressure technique to isolate bean protein. The treatment was carried out at 70– 90 °C and at hydrostatic pressures of 200, 400 and 600 MPa. The functional properties were improved compared with the less energetic treatments (70°C and 200 MPa).

High hydrostatic pressure enhances the extraction of phenolic compounds from oak chips. The phenolic compounds and antioxidant activity of wine increased after processing in the presence of oak chips (Tao et al., 2016).

Andrés et al. (2016) evaluated the physical and chemical properties, as well as the bioactive compounds from a soy smoothie treated with high hydrostatic pressure. They observed a higher antioxidant capacity using the latter technique.

## 3.8 COMBINATION TECHNIQUES

Rapid breakthroughs have occurred in the development and improvement of extraction methods. The combination of sample preparation and analytical techniques is a strategy that is primarily utilized to save energy and resources (Mustafa & Turner, 2011).

Extraction using ultrasound can be coupled with other techniques such as extraction heat-reflux, supercritical $CO_2$, and microwave. Yang and Wei (2015) developed an efficient method to extract bioactive compounds from Rabdosia rubescens by combining heat reflux extraction (conventional, solvent: ethanol) and ultrasound-assisted extraction (40 kHz frequency and power of 185 W, with agitation). The authors concluded that the combination of

extraction techniques reduced the processing time and increased the yield of bioactive compounds.

Corrales, Toepfl, Butz, Knorr, and Tauscher (2008) measured the extraction of anthocyanins from grape by-products using the methods of ultrasound, and a combination of pulsed electric field and high hydrostatic pressure. The authors concluded that the combination of effective extraction technologies and low-cost raw materials represents an environmental and economical alternative to conventional extraction methods, which require large amounts of organic solvents and long extraction times.

Garcia-Mendoza et al. (2015) used two sequential steps for the extraction of bioactive compounds from mango skin (Mangifera indica L.). First, supercritical $CO_2$ was used, followed by ethanol under pressure, both at 30 MPa and a temperature of 40 °C. The results of this study demonstrated that a two-stage extraction process allows the recovery of bioactive compounds. The extracts showed significant antioxidant activity and potential application in the food industry.

Dias et al. (2016) evaluated the effects of the extraction of bioactive compounds from red pepper (Capsicum baccatum L. var. Pendulum) through ultrasound and supercritical fluid techniques. The processes were tested at pressures of 15–25 MPa, temperatures of 40–60 °C, ultrasound powers from 200 to 600 W, for 40–80 min. The authors found a higher content of phenolic compounds when using these techniques.

Parniakov, Barba, Grimi, Lebovka, and Vorobiev (2016) used pulsed electric field and high voltage techniques to recover of bioactive compounds from mango. The techniques were performed using a 40 kV – 10 kA pulse generator (Polytechnic University Tomsk, Russian Federation) in the treatment chamber and two types of electrodes. The results of this study demonstrated the feasibility of a combination of the pulsed electric field and high voltage techniques to recover antioxidants and proteins from mango skin.

## 4 CONCLUSION

The extraction of bioactive compounds involves complex mechanisms and it can be accomplished by various techniques. Seeking to improve the extraction yields, reduce processing time and reduce environmental damage caused by toxic solvents, it has been proven that the replacement of conventional techniques by green technologies is promising. Studies have also suggested that a combination of methods can possibly improve these processes.

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### 4.1.1 Texto de conexão entre artigos 1 e 2.

O artigo 1 teve como objetivo discutir técnicas de extração não convencional de compostos bioativos de frutas e vegetais. A partir deste estudo selecionaram-se as técnicas que foram utilizadas nos artigos posteriores: extração assistida por ultrassom e extração com fluido supercrítico, de acordo com a disponibilidade de equipamentos e matéria-prima escolhida, bergamota, que é de grande consumo no Rio Grande do Sul.

No artigo 2, realizou-se uma caracterização das variedades de bergamota mais conhecidas do Rio Grande do Sul e a extração convencional de compostos bioativos utilizando etanol 80 % e água eletrolisada ácida, básica e levemente ácida como solventes.

## 4.2 ARTIGO 2: Evaluation of electrolyzed functional water as an alternative solvent for the extraction of bioactive compounds from *Citrus* peel

Artigo submetido para a Green Processing and Synthesis

# Evaluation of electrolyzed functional water as an alternative solvent for the extraction of bioactive compounds from *Citrus* peel

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## ABSTRACT

The aim of this study was to investigate the use of electrolyzed water for the extraction of bioactive compounds from different varieties of bergamot peel. After obtaining the extracts, using 80% alcohol and electrolyzed water, acidic (AEW), basic (BEW) and neutral (NEW) the content of phenolic compounds, flavonoids and antioxidant capacity was determined through the hydroxyl, superoxide, DPPH (2,2-diphenyl-1-picrylhydrazyl radical), and FRAP (Ferric Reducing Antioxidant Power). The extracts obtained with electrolyzed water presented higher compounds bioactive than the extracted with 80% ethanol. The BEW (pH 10.5 to 11) presented the best results for phenolics (3681.31 mgGAE (100 g)$^{1}$) e flavonoids (571.57 mgEQ (100 g)$^{-1}$) in *Citrus reticulata* variety e DPPH (86.88 %) e superoxide radical (82.74 %) in *Citrus reticulata* Blanco. The NEW presented the best antioxidant capacity by hydroxyl radical (68.83 %) and FRAP (914.50 µmol TEAC (100g)$^{-1}$) in *Citrus reticulata* Blanco. Therefore, these results suggest the use of electrolytic functional waters (BEW and NEW) as extractive solutions of bioactive compounds, as an alternative reduce the use of organic solvents in food by-products.

**Keywords:** bergamot fruit; phytochemicals; acid electrolyzed water; basic electrolyzed water; slightly acidic electrolyzed water.

## PRACTICAL APPLICATIONS

This study examined the potential of electrolyzed water as a phytochemical extracting solution with a view to eliminating the use of solvents. This is the first published study regarding to this issue, together with the use of fruit waste, and both factors are important in terms of environmental protection. The discovery of the nutritional potential of fruit residues has led to studies regarding the use of these residues, such as obtaining bioactive compounds to be applied in food products. Extraction is conventionally performed through the use of organic solvents and/or high cost equipment in industries. These solvents can be toxic to the environment and to humans. Electrolyzed water is currently used for microbiological control, and it can possibly be applied in foods, beeng non-toxic and low-cost. In addition, it is very important to select the best type of bergamot to take advantage of the proposed method.

## 1 INTRODUCTION

Fruits are important dietary sources of bioactive compounds and they have been extensively studied. Citrus fruits are one of the world's leading fruit crops with a global production of more than 100 million metric tonnes per year. In addition to providing sucrose, pectin and vitamin C, citrus fruits contain a number of phytochemicals that can potentially improve health [1].

The levels of bioactive compounds in fruits depend on factors such as the cultivar, the growing conditions (soil, fertilizers, and cultivation technique), storage and transport conditions, and transport technology [2].

Research is currently focused on the recovery of food industry waste or by-products because of the environmental problems associated with their disposal and also because of their high potential that they offer for the development of new products, such as the extraction of bioactive compounds [3].

Extraction in plant species is the first important step in the recovery and purification of active ingredients. Solvent extraction is often used and the extraction yield depends on certain conditions (time, temperature, and ratio) and solvent polarity. Ethanol, methanol, acetone, hexane, ether and chloroform are solvents commonly used in extraction, but they are toxic to the environment and humans [4]. Arivalagn et al. [5] used the "native" solvents of 80 % methanol, 80 % ethanol and 80 % acetone for the extraction of coconut meal.

The search for better and solvent-free products, in addition to concerns about environmental risks, has led to alternative processes, as green thecnologies: Supercritical fluids [6], Pressurized liquid [7], Ultrasound assisted [8], Microwave assisted [9], Pulsed electric [10], High voltage electrical [11].

Electrolyzed water (EW) is a newly discovered form of water that can be acidic (AEW) or basic (BEW). It is produced in specific equipment, where an electrolyte (sodium chloride) is added to an electrolytic cell. The blend of AEW and BEW forms neutral electrolyzed water (NEW). The salt solution used generates an electrical solution between the poles that generates changes in the concentration of water. EW is cost effective, environmentally friendly, and its use is an emerging technology with considerable potential [12].

According to ANVISA (Ordinance No.398), functional food is characterized as any food or ingredient that, in addition to its basic nutritional functions, when it is consumed as part of the usual diet it produces metabolic and/or physiological effects and/or beneficial effects on health, and must be safe for consumption without medical supervision [13].

There are studies in regarding the use of AEW for the sterilization and preservation of products, and the use of BEW to remove pesticide residues [14]. Morita et al. [15] investigated AEW (pH <2.7, ORP (Oxidation Reduction Potential) > 1100 mV and free chlorine concentration of 20-60 ppm) in mouthwash for the treatment and prevention of periodontal disease, as well as the gastrointestinal tract. Brychcy et al. [16] evaluated the physicochemical properties of AEW incorporated in chitosan films, which provided better integration of the coated product and a multidirectional protective effect. Yoon et al. [17] studied the ingestion of BEW in mice in order to explore the possibility of excreting melanin from the body.

However, no studies were found regarding the use of different types of electrolyzed water as extractive solution for to bioactive compounds and/or plant-based phytochemicals. Thefore the main objective of this study was to investigate functional electrolyzed water as a solution to extract bioactive compounds present in different varieties of bergamot peel.

## 2 MATERIALS AND METHODS

### 2.1. BERGAMOT PEEL

Three varieties of bergamots (Table 1), *Citrus deliciosa*; *Citrus reticulata* (tangerine) and *Citrus reticulata Blanco* (ponkan), were supplied by a rural producer (Tupanciretã, Rio Grande do Sul, Brazil, 29° 04' 51" S, 53° 50' 09" W). The fruits were carefully transported in foam-wrapped cartons immediately after harvesting. In the laboratory they were selected for the absence of defects, pests, and rot, and their surfaces sanitized with sodium hypochlorite (100 mg. $L^{-1}$) for 10 min. The fresh varieties were compared in relation to color, weight, ° Brix and humidity.

To obtain dried and ground samples, the fruits were manually peeled. The peels were stored in aluminum trays, dried in an air circulation oven at 35 ± 5 °C for 72 h, crushed in an analytical mill cooled to 4 °C (Quimis, model Q298A21, Brazil), and subsequently sieved (mesh 38). The powder was vacuum packed and stored in a freezer (-18 °C) until analysis.

### 2.2 FUNCTIONAL ELECTROLYZED WATER

The AEW and BEW were obtained from NaCl 0.01 % (w/v) (Dinâmica®, Brazil), using filtered and deionized water (Permution®, Brazil). The later was produced using electrolysis at 18 °C in an electrolyzer bench (Envirolyte®, Estonia). The NEW was obtained by mixing AEW and BEW at the same ratio 1:1 (v/v). Its physicochemical characteristics (pH, redox potential, and free chlorine) were determined.

The pH was measured with a direct electrode reading (Digimed®, DME-CV1, Brazil). The ORP (mV) was measured with a platinum electrode (Digimed®, DMR-CP1, Brazil). The free chlorine concentration (CCL) was evaluated according to the methodology of Smeww [18], with potassium iodide (KI) reacting in acid medium (pH 4), and the results were expressed in mg/L of $Cl_2$. The results are in Table 1.

Table 1 - Physico chemical characteristics of AEW, BEW and NEW.

| | AEW | BEW | NEW |
|---|---|---|---|
| pH | 3.7±0.1 | 10.4±0.3 | 7.6±0.3 |
| ORP (mV) | 700 | 150 | 297 |
| CCL (mg/L) | 1 | - | - |

AEW – Acidic electrolyzed water; BEW – Basic electrolyzed water; NEW - Neutral electrolyzed water.

## 2.3 PHYSICOCHEMICAL ANALYSES OF BERGAMOT PEEL POWDER

The proximate analysis of the powders followed the methodology described by the AOAC [19] for moisture (967.03), ash (942.05), crude protein (981.10), ethereal extract (920.39), and crude fiber analyses (985.29). The caloric value was calculated by multiplying the results of lipids, proteins, and carbohydrates by their respective caloric values: 9,4 and 4 kcal conversion factors [20].

The pH was measured using a Digimed digital potentiometer (Model DM-23, São Paulo, Brazil). The powder was mixed in distilled water at a ratio of 1:10 [19]. To determine the water activity ($a_w$), on average 3 g of powder was used at 25 °C using AquaLab equipment (Decagon Devices lnc, USA). The total acidity of the powders was measured by titration in a 0.1 N sodium hydroxide solution using phenolphthalein as an indicator. The total sugars were determined from 2 g of sample and titrated with Fehling solution. The calculation of the amount of soluble solids was performed in a digital refractometer, using the powder with distilled water [19].

The carotenoids were extracted according to the method described by Soquetta et al. [21]. A mixture of 0.2 g of bergamot peel powder and 7 mL of acetone was homogenized in a homogenizer (Ultra Turrax) to complete depigmentation (five times). The extract was transferred to a separatory funnel containing water, 15 mL of ethyl ether and 30 mL of petroleum ether. The saponification of the extract was then performed by shaking the extract with a KOH solution (10 %) in methanol in the dark for 16 h. After washing with water for total alkali, the extract was concentrated with the aid of a vacuum pump. For the UV-VIS spectrophotometer reading (UV-2600, Shimaszu), which was performed at 451 nm absorbance, the extract was diluted with petroleum ether. The results were calculated by the mathematical expression described by Gross [22], considering an absorption coefficient of 2592.

The determination of vitamin C followed the methodology described by Soquetta et al. [21]. 1 g of bergamot peel powder added to 50 mL of 1 % oxalic acid and an aliquot of 10 mL

was taken for titrated using the 2,6-dichlorophenolindophenol indicator. The result was calculated using Equation 1:

$$\text{Ascorbic acid}\,\frac{\text{mg}}{100\text{g}} = \frac{100 \text{ x } (\text{Vi}-\text{Mvb})\text{x F}}{\text{Vs}} \quad (1)$$

Where: Vi: volume of indophenol spent on sample titration; Vs: volume of sample used in titration; Vc: volume of solution spent on titration of ascorbic acid; MVb: mean of the volume of the indophenol solution spent on titration of the blank; F: factor of the indophenol solution in mg of ascorbic acid /mL (F=Va/(Vc-MVb)).

## 2.4 MICROBIOLOGICAL ANALYSES OF BERGAMOT PEEL POWDER

The methodology outlined in Normative Ruling No. 62 [23] was used for the microbiological evaluation of the powder. The analyses indicated for fruit flour contained in Resolution N$^{o}$. 12 were also used [24]. Portions of 25 g of bergamot peel powder were added to 225 mL of 0.1% peptone water and the dilutions were used for the microbiological analyses. The total coliform counts were performed at 35 °C in VRB (violet red bile) agar. The coliforms were measured at 45 °C in EC broth and the coagulase positive *Staphylococcus* was measured in Baird-Parker agar at 36 °C for 48 h. The *Salmonella* spp test was submitted to selective enrichment in tetrathionate brilliant green broth and Rappaport-Vassiliadis broth (24 h/42.5 °C), followed by isolation on agar plates containing SS and Rajhan agar. The *Clostridium* sulfite reducing count was performed in SPS medium with the plates incubated in anaerobic jars and the count of total mesophilic aerobic bacteria was performed using standard agar medium culture using depth plates (37 °C/48 h). The *Bacillus cereus* analysis was performed in surface plates, using polymyxin egg yolk agar [24].

## 2.5 PREPARATION OF EXTRACTS

The conventional extracts was performed from 5 g of powder with 50 mL of 80 % ethyl alcohol at a ratio of 1:10 (w/v). This mixture was taken to a shaker (New Brunswick $^{TM}$, Innova$^{®}$ 44/44 R model) for 2 h using a rotation of 200 rpm at 25 °C. The extracts were centrifuged and filtered through filter paper. The supernatant was added in amber flasks and the procedure was repeated with the residue. The extracts were stored in a freezer (-18 °C) until the determination of bioactive compounds and antioxidant capacity.

The same procedure was carried out using AEW (pH 3.6 to 3.8), BEW (pH 10.1 to 10.7), and NEW (pH 7.3 to7.9) as solvent.

## 2.6 TOTAL PHENOLIC COMPOUNDS

The methodology described by Singleton et al. [25] was used to estimate the total phenolic compounds, using Folin-Ciocalteau reagent. The extracts were diluted at a ratio of 1:50 (v/v) in extractive solution. Subsequently, an aliquot (0.2 mL) of the solution was mixed with 1 mL of 2 N Folin, and 0.8 mL of 7.5 % sodium carbonate ($Na_2CO_3$) solution was added. After incubation at 25 °C for 2 h, the absorbance was measured at 765 nm using a UV-VIS spectrophotometer (UV-2600, Shimaszu). For the quantification, a calibration curve was performed using gallic acid at concentrations of 0 to 70 mg. $L^{-1}$. The values were expressed as mg of gallic acid in 100 g of bergamot peel powder.

## 2.7 FLAVONOIDS

The determination of total flavonoids followed the methodology described by Zhishen et al. [26], with modifications. The extract was diluted in distilled water at a ratio of 1:10 (v/v). A 250 μL aliquot of the diluted sample was homogenized with 1250 μL of distilled water and 75 μL of 5 % sodium nitrite ($NaNO_2$) (w/v). After 5 min, 150 μL of aluminum trichloride ($AlCl_3$) was added to 10 % (w/v), and after 1 min 500 μL of 1 M sodium hydroxide (NaOH) was added. The absorbance was read in a UV-VIS spectrophotometer (UV-2600, Shimaszu) at 510 nm. For the calculation, a calibration curve was performed using quercetin in concentrations of 0 to 80 mg. $L^{-1}$. The values were expressed as mg quercetin in 100 g of powder.

## 2.8 DPPH RADICAL SCAVENGING CAPACITY

This method measures the antioxidant capacity by reducing the stable radical DPPH (2,2-diphenyl-1-picrylhydrazyl) by the action of the antioxidants present in the sample. The technique described by Brand-Willams et al. [27] was used, which consisted of incubating (30 min) 5 mL of a 0.1mM DPPH methanolic solution with 5 mL of solutions containing the following increasing concentrations of extract (item 2.5) 0.3; 0.6; 1.25; 2.5; 5.0; 10; 15; 20; 25; 30; 35 and 40 mg/mL. A control solution (1% DPPH methanol solution) and a blank solution

(methanol) were conducted simultaneously. After incubation, the samples were measured at 517 nm using a UV-VIS spectrophotometer (UV-2600, Shimaszu). The percentage of antioxidant capacity was calculated by the percentage of DPPH radical uptake according to Equation 2:

$$AA\% = 100 - \left[\frac{\text{Abs.sample} - \text{Abs.white}}{\text{Abs.control}} x100\right] \quad (2)$$

From the percentage curve of DPPH ($y = 2.3994x + 9.1657$, $R^2 = 0.9618$) versus the sample concentration it was possible to obtain the amount of antioxidant necessary to decrease the initial concentration of DPPH by 50% ($IC_{50}$) in a fixed reaction time.

## 2.9 HYDROXYL RADICAL SCAVENGING CAPACITY ($OH^-$)

The hydroxyl radical scavenging ability was measured according to Halliwell, Gutteridge and Aruoma [28] with modifications. 100 μL of EDTA, 100 μL of extract, 100 μL of $FeCl_3 6H_2O$, 100 μL of 2-deoxy-D-ribose, 100 μL of $H_2O_2$, 100 μL of ascorbic acid, reducing $Fe^{3+}$ to $Fe^{2+}$, and 400 μL of phosphate buffer (pH 7.4) were mired for the sample preparation and control. For the blanks, the sample and the control were used following the same preparation, with the difference that the extract was replaced by 100 μL of solvent, in which the sample was diluted. The blends were taken to a 37 °C bath for 1 h and then, 1 mL of 10% TCA and 1 mL of 1% TBA were added and left in a 70 °C bath for 15 min. They ware then cooled in a freezer for 5 min and completed with 2 mL of deionized water. The absorbance was measured at 532 nm using a UV-VIS spectrophotometer (UV-2600, Shimadzu) and the hydroxyl radical activity was calculated using Equation 3:

$$HRSC\ \% = 100 - \left[\frac{A_A - A_{BA}}{A_C - A_{BC}} X100\right] \quad (3)$$

Where: $A_A$: antioxidant activity of each sample; $A_{BA}$: antioxidant activity of sample blank; $A_C$: antioxidant activity of control; $A_{BC}$: antioxidant activity of control blank.

## 2.10 SUPEROXIDE RADICAL SCAVENGING CAPACITY ($O_2^-$)

The ability of the supernatant to sequester the superoxide radical was assessed using the 2-deoxy-D-ribose method proposed by Zhao eu al [29], with some modifications. The reaction solution consisted of 100 μL of supernatant; 100 μL of EDTA solution (1 mM); 100 μl of ferric chloride hexahydrate solution (FeCl3.6H2O) (1 mM); 100 μL of 2-deoxy-d-ribose (36 mM); 100 μL of hydrogen peroxide solution (10 mM); and 100 μL of L-ascorbic acid solution (1 mM) in phosphate buffer (0.025 M) (pH 7.4). The volume of the reaction solution was quenched with phosphate buffer to a volume of 1.0 mL and maintained at 37 °C in a water bath for 1 h. At the end of the reaction time, 1.0 mL of trichloroacetic solution (TCA) (10%) and 1.0 mL of thiobarbituric acid solution (TBA) (1 %) were added in phosphate buffer. The samples were placed in a water bath at about 70 °C for 15 min and then cooled in a refrigerator to room temperature. Finally, deionized water was added to the samples to complete a volume of 5 mL. The absorbance of the samples was determined at 532 nm using a UV-VIS spectrophotometer (UV-2600, Shimaszu). The antiradical activity (AAOH) of the supernatant, or percentage of superoxide inhibition, was calculated according to Equation 4.

$$\text{SRSC } \% = \left[1 - \frac{A - A_B}{A_C - A_{BC}}\right] X\ 100 \qquad (4)$$

Where: A, $A_B$, $A_C$ and $A_{BC}$ correspond to the absorbance values of the sample, the blank, the control and the blank of the control, respectively.

## 2.11 FERRIC REDUCING ANTIOXIDANT POWER (FRAP)

To determine the iron reduction power of the extracts (item 2.5), the method described by Benzie and Strain [30] was used. The FRAP reagent (Fe (III) -TPTZ solution) was obtained from a combination of 25 mL of 0.3 M acetate buffer; 2.5 mL of a 10 mM TPTZ (tripyridyltriazine) solution (3.12 g of TPTZ in 1 L of 40 mM HCl); and 2.5 ml of a 20 mM aqueous solution of ferric chloride. In a test tube, 200 μL of the sample, which had been previously diluted in distilled water (1:25), and 1.8 ml of the FRAP reagent were mired and kept in a water bath (37 °C/30 min). The FRAP reagent was used as a blank. The absorbance was measured at 593 nm using a UV-VIS spectrophotometer (UV-2600, Shimadzu). TEAC (trolox equivalent antioxidant capacity) (range 0 to 25 μM) was used for the calibration curve ($y = 0.0602x - 0.0684$, $R^2 = 0.9934$). The results were expressed as μmol TEAC $(100g)^{-1}$ of bergamot peel powder.

## 3 RESULTS AND DISCUSSION

### 3.1 PHYSICOCHEMICAL ANALYSES OF FRESH BERGAMOT

The three varieties of bergamot were similar in color and geographical origin (Table 2), but the average weight of the fresh fruits was significantly lower for the *Citrus deliciosa* variety (85.5 g). In relation to the total soluble solids (° Brix), *Citrus reticulata* presented the highest value (16.05) but it differed ($p < 0.05$) only from the *Citrus deliciosa* variety (10.6). The moisture of the dried fresh fruits differed statistically beeng of $65.92^{a} \pm 2.06$ %, $59.65^{c} \pm 1.04$ % and $64.07^{b} \pm 1.90$ % for *Citrus deliciosa, Citrus reticulata*, and *Citrus reticulata* Blanco, respectively. Gerhardt et al. [31] found a value of 69.93% for ponkan, which was similar to the value found in this study.

Table 2 - Characteristics of the bergamot cultivars studied.

| Scientific name | *Citrus deliciosa* | *Citrus reticulata* | *Citrus reticulata* Blanco |
|---|---|---|---|
| Popular name in Brazil | Bergamot | Tangerine | Ponkan |
| Geographical origin | Asian continent | Asian continent | Asian continent |
| Peel color | Orange | Orange | Orange |
| Average weight (g) | $85.5^{b} \pm 21.2$ | $135.4^{a} \pm 9.0$ | $141.5^{a} \pm 9.0$ |
| °Brix - °B | $10.6^{b} \pm 2.0$ | $16.0^{a} \pm 0.7$ | $13.5^{ab} \pm 2.0$ |
| Moisture % | $65.9^{a} \pm 2.0$ | $59.6^{c} \pm 1.0$ | $64.0^{b} \pm 1.9$ |

Means ± standard deviation of analyses in triplicate.[abc]Means on the same line with the same superscript letters do not differ significantly by Tukey's test ($p<0.05$).

### 3.2 PHYSICOCHEMICAL ANALYSIS OF BERGAMOT PEEL POWDER

The values for protein, lipids, acidity and $a_w$ did not differ ($p > 0.05$) between the three varieties (Table 3), while the values for moisture, ash, crude fiber, and pH differed ($p<0.05$).

The moisture content ranged from 9.79 to 12.27 %, with the *Citrus deliciosa* variety having the highest value ($p<0.05$). These values are in accordance with current Brazilian legislation, which stipulates a maximum of 15 % moisture for fruit powder [32].

Table 3 - Physical chemical analysis of the bergamot peel flour of three varieties.

| | Analysis | *Citrus deliciosa* | *Citrus reticulata* | *Citrus reticulata* Blanco |
|---|---|---|---|---|
| Proximate analysis (%) | Moisture | $12.27^{a}$±0.18 | $9.79^{c}$±0.05 | $11.96^{b}$±0.02 |
| | Ash | $2.86^{b}$±0.01 | $3.92^{a}$±0.01 | $2.17^{c}$±0.02 |
| | Proteins | $5.62^{a}$±0.41 | $4.82^{a}$±0.09 | $5.07^{a}$±0.56 |
| | Total dietary fiber | $10.05^{b}$±0.14 | $10.79^{a}$±0.13 | $6.60^{c}$±0.12 |
| | Lipids | $1.57^{a}$±0.10 | $1.37^{a}$±0.03 | $1.34^{a}$±0.33 |
| | Carbohydrates | $67.62^{c}$±0.34 | $69.29^{b}$±0.26 | $72.84^{a}$±0.35 |
| Physical chemical analysis | Total sugars (%) | $35.11^{b}$±0.37 | $31.18^{c}$±0.40 | $47.25^{a}$±0.34 |
| | Energetic value ($Kcal.100^{-1}$) | $307.11^{b}$±1.13 | $308.83^{b}$±0.60 | $323.77^{a}$±1.40 |
| | Acidez % (v/p) | $16.60^{a}$±2.80 | $17.33^{a}$±1.52 | $15.55^{a}$±0.57 |
| | $A_w$ | $0.388^{a}$±0.009 | $0.387^{a}$±0.002 | $0.378^{a}$±0.004 |
| | pH | $5.45^{a}$±0.01 | $5.31^{b}$±0.01 | $4.99^{c}$±0.01 |
| | Total Carotenoids ($mg.100g^{-1}$) | $13.02^{b}$±1.01 | $18.05^{a}$±2.44 | $18.17^{a}$±1.44 |
| | Vitamin C ($mg.100g^{-1}$) | $98.44^{c}$±0.00 | $187.90^{a}$±2.66 | $181.9^{b}$±1.51 |

$^{abc}$ Means on the same line with the same superscript letters do not differ significantly by Tukey's test ($p < 0.05$). Means ± standard deviation of analyses in triplicate.

Low moisture content contributes to a greater conservation of products because it reduces the amount of water available for the proliferation of microorganisms and chemical reactions [33]. Fu et al. [34] found a similar moisture content (12.4 %) in the husks dried of *Citrus Reticulata* Blanco. The ash content varied from 2.17 to 3.92 % and the three varieties differed ($p<0.05$) from each other (Table 3). These values are in accordance with Brazilian legislation, which stipulates a minimum level of 2 % for ash in fruit powder. Gondim et al. [35] noted that fruit peels generally have higher nutrient content than their respective edible parts.

The bergamot peel powder that was analyzed in the present study can be considered to be easily conserved because for most foods deterioration reactions occur at levels of water activity $a_w > 0.65$, and the $a_w$ of the powders varied from 0.378 to 0.388 (Table 3). Fu et al. [34] found a similar value for $a_w$ (0.50) in *Citrus reticulata* Blanco peel.

Values of pH ranging from 4.99 to 5.45 also influence the durability and quality of powders, since more acidic products are more stable in relation to deterioration [36].

The total carotenoids values ranged from 13.02 mg $(100g)^{-1}$ to 18.17 mg $(100g)^{-1}$. The *Citrus deliciosa* variety presented the lowest value ($p < 0.05$), which differed significantly from the other samples, which in turn were similar to each other. The values for vitamin C, ranged from 98.44 mg $(100g)^{-1}$ to 187.9 mg $(100g)^{-1}$; the highest value was for *Citrus reticulata* and the lowest was for *Citrus deliciosa*.

## 3.3 MICROBIOLOGICAL ANALYSES

The results of the microbiological analysis of the bergamot peel powder are shown in Table 4. The found values complied with ANVISA legislation 12/2001 for powder obtained from sprayed dried fruits [24]. An absence of *Bacillus cereus* and *Salmonella* sp was observed. The powder showed low counts of *Staphylococcus aureus*, *Clostridium sulfite reducer*, coliforms at 35 °C and 45 °C, molds and yeasts, and mesophilic bacteria. These results indicate that the handling and storage conditions were adequate [37].

Table 4-Count of microorganisms in the flours of bergamot peels of different varieties.

| $CFU.g^{-1}$ | Maximum limit | *Citrus deliciosa* | *Citrus reticulata* | *Citrus reticulata* Blanco |
|---|---|---|---|---|
| Coliforms 35°C | - | $1.1 \times 10^3$ | $2.4 \times 10^3$ | $2.4 \times 10^2$ |
| Coliforms 45°C | $10^2$ | $< 10^1$ | $< 10^1$ | $< 10^1$ |
| *Aerobic mesophiles* | $5x10^5$ | $9.93 \times 10^2$ | $4.36 \times 10^2$ | $9.13 \times 10^2$ |
| *Bacillus cereus* | n.d | n.d | n.d | n.d |
| *Staphylococcus aureus* | $<10^1$ | $< 10^1$ | $< 10^1$ | $< 10^1$ |
| *Clostridium* sulphite reducer | 2x10 | $<10^1$ | $<10^1$ | $<10^1$ |
| *Salmonella sp* (in 25g) | n.d | n.d | n.d | n.d |
| Mold and yeast | - | $3.3 \times 10^1$ | $2.3 \times 10^1$ | $<10^1$ |

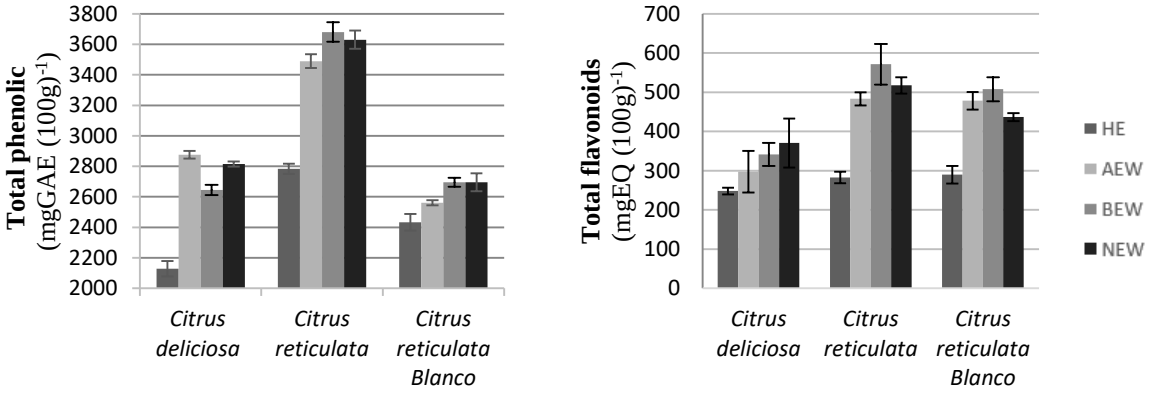
*n.d* – Absence in 25g.
Fonte: (Brasil, 1978)

## 3.4 TOTAL PHENOLIC AND FLAVONOIDS OF EXTRACTS

The choice of 80 % ethanol in the conventional extraction was made due to various studies that have been carried out with this solvent to determine bioactive compounds in citrus fruits [4, 38, 39]. No studies were found in the literature using functional electrolytic water for this purpose.

Figure 1 shows values of total phenolic compounds and total flavonoids from bergamot bark extracts of different varieties obtained by conventional extraction using hydroalcolic solvent and functional electrolyzed water (AEW, BEW and NEW).

Figure 1 - Content of total phenolic compounds (a) and total flavonoids (b) of bergamot peel from varieties *Citrus deliciosa*, *Citrus reticulata* and *Citrus reticulata* Blanco




HE- Hydroalcoholic Extract; BEW – Basic electrolyzed water; AEW – Acidic electrolyzed water; NEW - Neutral electrolyzed water.

The values of total phenolic compounds found in this study ranged from 2127.9 mg GAE (100 g)$^{-1}$ to 3681.3 mg GAE (100 g)$^{-1}$ (Fig. 1); the lowest value was for the *Citrus deliciosa* variety using the hydroalcoholic extract (HE) and the highest value was for the *Citrus reticulata* variety using basic electrolyzed water (BEW). Regardless of the type of solvent used, the highest concentrations of phenolics were found in the tangerine variety, especially the extracts with electrolyzed water. The BEW provided greater extraction of these compounds, presenting a difference of 25 % in relation to the extract obtained with 80 % ethanol (conventional).

Zahoor et al. [4] verified similar values for total phenolics in *Citrus reticulata* Blanco and *Citrus reticulata* of 2.297 mg GAE (100g)$^{-1}$ and 2.820 mg GAE (100g)$^{-1}$ respectively, using

80 % ethanol as solvent. In both studies, a ratio of 1:10 (v/v) was used, but in the aforementioned study by Zahoor et al. the extracts were obtained after 8 hours at 30 °C. In the present study the extracts were obtained after 2 hours at 25 °C, thereby reducing the energy expenditure and processing time. Chen et al. [38] used 70 % ethanol for extraction and found total phenolic values of 1,559.9 to 1,895.0 mg GAE $(100g)^{-1}$ for *Citrus reticulata* Blanco cv. Ougan from different locations, used the ultrasound-assisted method for 1 h at 25 °C. Values lower than those found in this study.

The total flavonoid values for the present study ranged from 248.03 mg $(100g)^{-1}$ to 571.56 mg $(100g)^{-1}$ (Figure 1). The value obtained with BEW was 50 % higher than the conventional extract (80 % alcohol) in *Citrus reticulata* variety. Chen et al. [38] extracted bioactive compounds with 70 % ethanol and found total flavonoid values from 467.1 mg RE $(100g)^{-1}$ to 581.7 mgRE $(100g)^{-1}$ for *Citrus reticulata* Blanco cv. Ougan from different locations.

*Citrus reticulata* (tangerine) showed the best results in relation to phenolics and flavonoids. These differences in the content of bioactive compounds among the bergamot varieties can be explained by the climatic conditions of the crops, as explained in a study by Soquetta et al. [21].

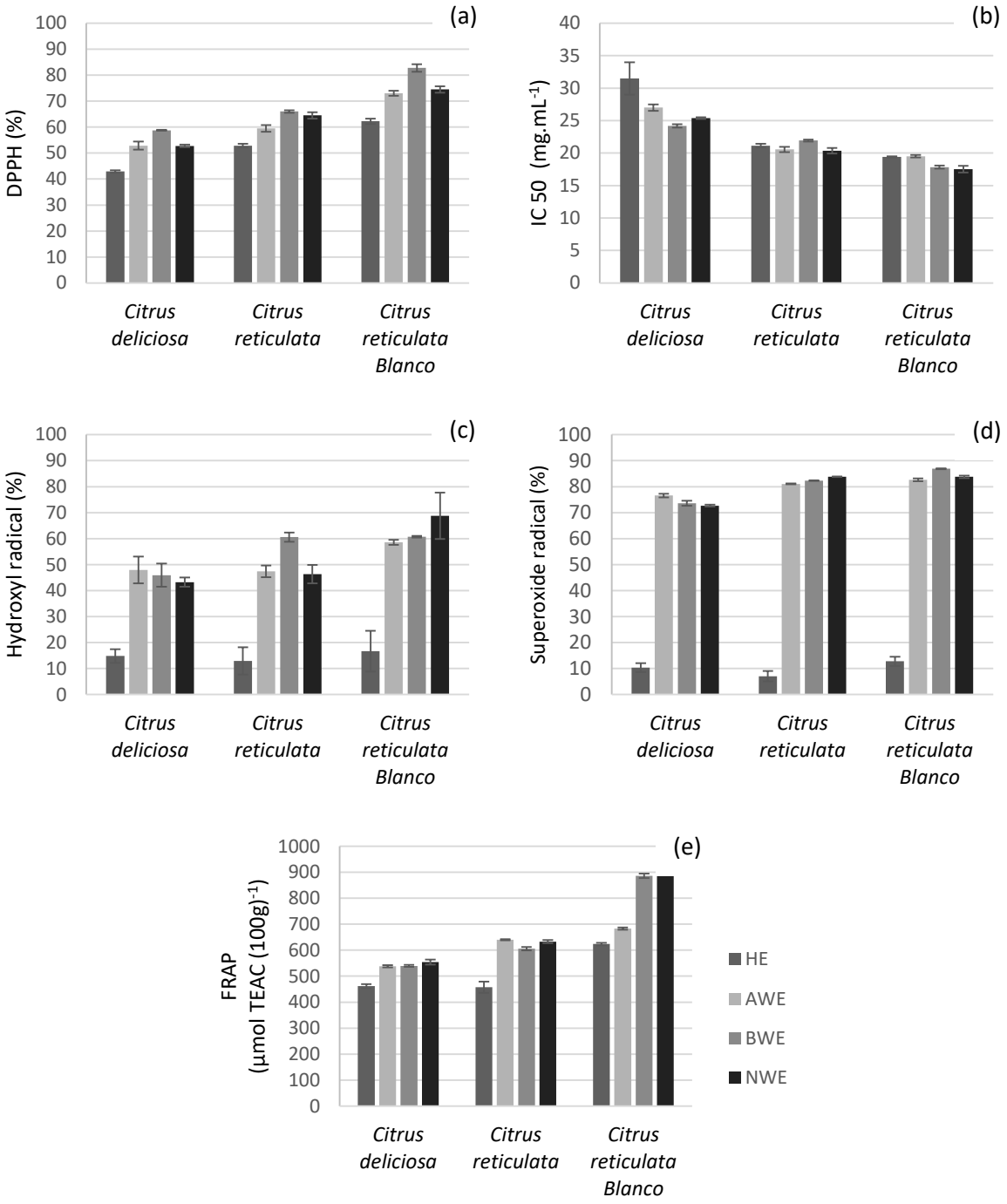
The BEW and NEW provided greater extraction of phenolic compounds and flavonoids than AEW. These results are related to the antioxidant effects of BEW and NEW, whereas AEW has an oxidizing effect [40]. Lee et al. [41] reported that BEW showed activity similar to the superoxide dismutase (SOD) enzyme and catalase. These enzymes fight against active oxygen-reactive species and protect DNA from damage due to free radicals. The antioxidant capacity of these types of waters and their respective pHs will favor the extraction of these compounds and their maintenance when in contact with to bergamot peel.

Hao et al. [42] observed fresher products by using BEW and NEW for in cilantro. This phenomenon may have also occurred when exposing the bergamot peel to these types of waters, thus favoring the exit of phenolic compounds and flavonoids.

## 3.5 ANTIOXIDANT CAPACITY OF EXTRACTS

Figure 2 shows the antioxidant capacity values of bergamot bark extracts obtained in conventional extraction using 80% ethanol and electrolyzed water (AEW, BEW and NEW).

Figure 2 - Antioxidant activity DPPH (a), $IC_{50}$ DPPH (b), Hydroxyl radical (c), Superoxide radical (d), FRAP (e).




Where*:* HE- Hydroalcoholic Extract; BEW – Basic electrolyzed water; AEW – Acidic electrolyzed water; NEW - Neutral electrolyzed water.

In this analysis antioxidant reacts with the DPPH radical, converting it to its reduced form, indicating its ability of the antioxidant to sequester the free radical ($H^+$).

DDPH values ranged from 42.90 % (HE) to 82.74 % (BEW) (Figure 2a). The variety that presented the best antioxidant activity in this method was *Citrus reticulata* Blanco, and BEW showed 20 % efficiency in relation to hydroalcoholic extract.

Antioxidant capacity can be to be considered strong when the percentage of sequestration of the DPPH radical reaches 70 %, moderate between 50 and 70 %, and low when it is below 50 % [43]. Taking this into consideration, the hydroalcoholic extract for the *Citrus deliciosa* variety showed low antioxidant capacity, while the extracts obtained with acidic, basic, and neutral electrolyzed water showed strong antioxidant capacity in relation to the *Citrus reticulata* Blanco variety. The other extracts presented intermediary levels of activity.

The $IC_{50}$ values (the concentration capable of reducing the DPPH by 50 %) are shown in Figure 2b. According to Arbos et al. [44], the lower the $IC_{50}$ value the greater the antioxidant capacity, and values above 25 mg $(mL)^{-1}$ are considered to represent low levels of antioxidant potential. In the present study, only the hydroalcoholic extracts (HE) from the *Citrus deliciosa* variety and the extract obtained with acidic electrolyzed water presented low antioxidant potential. All the extracts obtained with basic and neutral electrolyzed water presented values above 25 mg $(mL)^{-1}$ for the three varieties.

The hydroxyl radical ($^{\bullet}OH$) is a highly reactive oxygen species due to its ability to attack most biological substrates, DNA, fatty acids, and proteins. Consequently, it is important to evaluate the protective capacity of extracts in relation to this radical [45]. The protective capacity of the extract obtained from *Citrus reticulata* Blanco with neutral electrolyzed water was 68 %, while the figure for the hydroalcoholic extract was 43 %. The lowest antioxidant activity (14 %) by this method was in the *Citrus deliciosa* variety using the hydroalcoholic solvent (Figure 2c).

The results for the percentage inhibition of the superoxide radical varied from 7.07 % to 86.88 %. The highest percentage (86 %) was found in the *Citrus reticulata* Blanco variety using BEW for solvent. It has been widely reported that there is a close correlation between antioxidant capacity and iron reducing power [46]. In the present study (Figure 2e), values of 461.37 to 914.50 μmol TEAC $(100\ g)^{-1}$ were found. The best result was found in *Citrus reticulata* Blanco using BEW as solvent, showing a difference of 39 % more than hydroalcoholic extract.

Analyzing the tests of antioxidant capacity in the present study, as was the case for the determination of bioactive compounds, the extracts obtained from functional electrolyzed water showed better antioxidant capacity in all the methods when compared to the hydroethanolic (conventional) extract. The basic and neutral waters presented better retention of bioactive

compounds and, consequently, greater antioxidant capacity. Regarding the varieties of bergamot, the *Citrus reticulata* Blanco extracts showed the highest antioxidant capacity of all the analyzed methods.

The results for the hydroethanolic extracts varied in terms of the hydroxyl and superoxide methodologies compared to DPPH and FRAP. This may have been related to the type of solvent that was used. According to Pérez-Jiménez et al. [47], divergences between analysis methodologies can be based on the chemical principles on which these methods are constructed, as well as variations in the antioxidant complex of a food matrix, which can provide different responses to each method.

Considering there is no previous parameters for the antioxidant capacity of the extracts obtained with functional electrolyzed water, the principles of this solvent in the plant matrix should be further explored. Research related to using electrolyzed water suggests the positive effects of this solvent on oxygen reactive species, which may be related to the better results of the antioxidant capacity of these extracts.

Yahagi et al. [48] suggest that free radicals formed during the production of electrolyzed functional water combat reactive oxygen species, which are responsible for the degradation of compounds and lipid oxidation in food. Similar studies were carried out by Lee et al. [41], who demonstrated that alkaline electrolyzed water reduces the amount of reactive oxygen species. This could explain why the BEW provided better results compared to the AEW in this study.

## 4 CONCLUSION

The bergamot peel flours comply with specific legislation for consumption according to physicochemical and microbiological analyzes.

Bergamot peel powder can be considered to be a source of bioactive compounds and a functional ingredient. The acidic, basic, and neutral electrolyzed waters presented higher extraction capacity of bioactive compounds than 80 % ethanol. Observing the Citrus reticulata variety, AEB extracted 25 % more total phenolics and 50% flavonoids, while AEA was 14 % higher in antioxidant activity (FRAP).

The *Citrus reticulata* and *Citrus reticulata* Blanco varieties showed higher values of bioactive compounds in all the analyses compared to *Citrus deliciosa*. The *Citrus reticulata* variety presented a higher content of phenolic compounds and flavonoids, while *Citrus reticulata* Blanco showed higher antioxidant capacity in all of the analyzed methods.

Further studies should be carried out to explain the phenomenon that have occurred for the better recovery of phytochemicals, since this is the first subleshed study about electrolyzed water as an extractive solution for bioactive compounsds.

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### 4.2.1 Texto de conexão entre artigo 2 e 3.

Com os resultados obtidos no artigo 2, verificou-se que as farinhas de casca de bergamota têm características de farinha de frutas e são apropriadas para consumo conforme resultados obtidos na composição centesimal e microbiológica.

Foram avaliadas as variedades *Citrus deliciosa*, *Citrus reticulata* (tangerina) e *Citrus reticulata* Blanco (ponkan). Dentre estas, a tangerina apresentou maiores teores de fenólicos e flavonoides totais. Com isso, mesmo que a variedade ponkan tenha apresentando maior capacidade antioxidante, pelos métodos DPPH, FRAP e radical hidroxila e superóxido, este resultado foi atribuído a outros compostos analisados, como vitamina C e carotenoides. A tangerina foi escolhida para seguir as análises, pois o objetivo deste trabalho era a extração de fenólicos e flavonoides totais.

Para avaliar os três tipos de água eletrolisada (AEA, AEB e AELA) como solvente, primeiramente realizou-se a extração convencional, com agitação e temperatura controlada, comparando com etanol 80 %. Conforme os resultados, os três tipos de AE apresentaram maior capacidade de extração de compostos bioativos do que o etanol 80 %, apresentando um rendimento 43 % superior. A AEB apresentou melhores resultados de fenólicos (3681,31 mg GAE (100 g) $^{-1}$), flavonoides (571,65 mgEQ (100 g$^{-1}$)) e DPPH (82,75 %), já AELA radical superóxido (86,88 %), hidroxil (68,83 %) e FRAP (914,50 µmol TEAC (100 g) $^{-1}$).

## 4.3 ARTIGO 3: Extraction of bioactive compounds from skin of *Citrus reticulata* using ultrasound and electrolyzed water

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# Ultrasound extraction of bioactive compounds from peel of *Citrus reticulata* using electrolyzed water

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## ABSTRACT

The aim of this study was to evaluate the use of acid electrolyzed water, basic electrolyzed water, and slightly acidic electrolyzed water (AEW, BEW, and SAEW, respectively) as solvents in ultrasound (US) to extract bioactive compounds from *Citrus reticulata*. The influence of the intensity (17–85 W/cm$^2$) and pulse cycles (0.5–1.0 dimensionless) was investigated in relation to the extraction of total phenolics (TP), total flavonoids (TF), and antioxidant activity (FRAP). The extract obtained with US+SAEW presented the highest values for TP (4,324.32 mg GAE (100g) $^{-1}$ of tangerine peel) and FRAP (663.69 µmol TEAC (100g) $^{-1}$ of tangerine peel). The highest TF content was found for US+AEW (691.76 mg EQ (100g) $^{-1}$ of tangerine peel). Response surface methodology showed that higher US intensities improved the extraction of phenolic compounds. Regarding the flavonoid compounds, the highest extractions were obtained at the central points (intensity (51 W/cm$^2$) and cycle (0.75). The results showed that US and electrolyzed water successfully extracted bioactive compounds from tangerine peel; the processing time was also reduced by around 87.5%. These results were higher than those in the literature regarding conventional extraction techniques.

**Keywords:** Tangerine; phenolics; flavonoids; ultrasound; electrolyzed water; response surface methodology.

## PRACTICAL APPLICATIONS

The combination of unconventional techniques (ultrasound and electrolyzed water as solvent) is characterized as a new methodology for the extraction of bioactive compounds from tangerine peel. This technique is environmental friendly due to the use of fruit residues, as well as the absence of organic solvents, and lower levels of energy use. The extract provided a higher content of total phenolics and total flavonoids than conventional methods. The extract can be incorporated into foods to provide nutritional quality and antioxidant properties. This technique is easily incorporated at the industrial level and is low cost after the purchase of the relevant equipment.

## 1 INTRODUCTION

Citrus fruits are one of the most important horticultural crops, with worldwide agricultural production over 100 million metric tons per year. These crops of Asian origin, are nowadays distributed throughout the world, mainly in the tropical, subtropical and temperate regions, as in Brazil, Portugal, Spain, Italy, Greece, Morocco, Turkey and Egypt. The genus Citrus encompasses 17 species of citrus fruits such as *Citrus reticulata* Blanco (mandarin orange, tangerine), *Citrus sinensis* L. (sweet orange), *Citrus aurantium* L. (bitter orange), *Citrus lemon* L. (lemon), *Citrus paradise* M. (grapefruit) (Moulehi et al., 2012, Ferreira, Silva, Nunes, 2018).

*Citrus reticulata*, also known as tangerine, belongs to the *Rutaceae* family; it is an important citrus plant and is an excellent source of bioactive compounds. Its domestic and industrial usage results in the production by-products, such as tangerine peel. Researchers have reported that tangerine peel presents high nutritional value due to the presence of phenolic compounds and flavonoids (Singanusong et al., 2015; Moulehi et al., 2012). Pharmacological studies of *Citrus reticulata* peel have demonstrated several biological activities, such as anti-inflammatory, antioxidant, antitumor, antimicrobial and antiatherosclerosis properties (Mandal and Mandal, 2016; Duan et al., 2017).

Therefore, *Citrus reticulata* peel is a potentially great interest to the food industry due to its bioactive compounds with antioxidant properties and also could be used in dietary supplements (Filip et al., 2017), replacing synthetic additives, such as butylhydroxyanisole (BHA) and butylhydroxytoluene (BHT), which have toxic and carcinogenic potential (Khan et al., 2010).

Organic solvents and agitation have been used in the disruption of plant cells to extract bioactive compounds. This conventional method requires a long extraction time and the use of large amounts of solvent, which can lead to the degradation of compounds (Heleno et al., 2016). Therefore, the ultrasound has been proposed because it reduces the processing time and/or reduces the use of solvents, as well as promoting greater penetration of the solvent in the cellular material and increasing the extraction yield, besides, this process is considered environmentally friendly (Jaime et al., 2010; Tiwari, 2015).

US probe systems have high cavitation intensity at low operating volumes. The acoustic energy is directly introduced into the liquid, and the power dissipated into the reaction mixture can be typically changed, although the irradiation frequency remains constant. The probe system provides intensity that is approximately 100 times higher than an US bath (Soquetta et al., 2017). The temperature and pressure, as well as the frequency and time of sonication, are factors that regulate the action of US (Toma, Vinatoru, Paniwnyk, & Mason, 2001).

Electrolyzed water is produced by passing a diluted salt solution through an electrolytic cell, into which the anode and the cathode are separated by a membrane. By subjecting the electrodes to direct current voltages, negatively charged ions such as chloride and hydroxide move to the anode and form oxygen gas, chlorine gas, hypochlorite ions, hypochlorous acid, and hydrochloric acid. The positively charged ions, such as hydrogen and sodium ions, move to the cathode and form hydrogen gas and sodium hydroxide (Huang et al., 2008). Two kinds of water are formed simultaneously: acid electrolyzed water (AEW), which has a pH between 2.0 and 6.0 and an oxidation-reduction potential (ORP) between 700 and 1200 mV; and basic electrolyzed water (BEW), which has a pH between 8.5 and 12.2 and an ORP between -800 and -600 (Guentzel et al., 2008).

There are some studies regarding the combined use of ultrasound and electrolyzed water in the literature. Ding et al. (2015) verified that the ultrasound increased the bactericidal activity of SAEW in cherry tomatoes without compromising the quality of the product in study of the natural microflora of the fruits. Kim et al. (2006) investigated the combination of ultrasound and acid electrolyzed water (AEW) to inactivate *Escherichia coli* 0157: H7 inoculated on alfalfa and broccoli seeds. Afari et al., (2016) also found the reduction of *Escherichia coli* 0157: H7 and *Salmonella Typhimurium* DT 104 in Roman lettuce, American lettuce and red tomatoes using neutral electrolyzed water and ultrasound. Luo and Oh, (2016) showed the inactivation kinetics of *Listeria monocytogenes* and *Salmonella enterica*sorovar *Typhimurium* in minimally processed peppers treated with SAEW and ultrasound. This combination was efficient in reducing microorganisms with minimal changes in quality of the product. Forghani, Eskandari

and Oh, (2015) applied SAEW with ultrasound for decontamination of kashk, a dairy product produced in Iran. The authors observed reduction of *S. aureus*, *E. coli* and *A. fumigatus*.

However, no other study has been reported using US and electrolyzed water to extract bioactive compounds from C. reticulata. Consequently, the main objective of this study was to evaluate a combination of electrolyzed water and US to extract bioactive compounds from *Citrus reticulate* peel.

## 2 MATERIALS AND METHODS

### 2.1 PREPARATION OF THE SAMPLES

Tangerines (*Citrus reticulata*) were collected in July 2016 in the district of Tupanciretã, Rio Grande do Sul (Brazil). After peeling, the peel were dried in air circulating oven (Marconi, model MA035/5) at 35 ± 5 °C for 72 h and comminuted in an analytical mill to 4 °C (Quimis, model Q 298ª21, Brazil). The samples were standardized using 38 mesh (0.5 mm) sieves and were vacuum packed (24 x 40 cm, 12 micron) and stored at 4 °C until analysis.

### 2.2 FUNCTIONAL ELECTROLYZED WATER

The AEW and BEW electrolyzed waters were produced by electrolysis at 18 °C (Envirolyte® Estonia) from a brine of 0.01 % sodium chloride (Dinâmica® Brazil) with filtered and deionized waters (Permution® Brazil). The SAEW was obtained by mixing AEW and BEW at the same ratio 1:1 (v/v). The physicochemical characteristics (pH, redox potential and free chlorine) were determined (SMEWW, 1999). The results are shown in Table 1.

Table 1-Physical chemical characteristics of AEW, BEW and SAEW.

| | AEW | BEW | SAEW |
|---|---|---|---|
| pH | 3.24 | 9.73 | 6.2 |
| ORP (mV) | 913.5 | -267.67 | 297 |
| CCL (mg/L) | 1 | - | - |

Where: AEW – Acidic electrolyzed water; BEW – Basic electrolyzed water; SAEW - Slightly acidic electrolyzed water.

## 2.3 CONVENTIONAL EXTRACTION (CE)

The experimental conditions for the conventional extraction (CE) were determined according to Soquetta et al. (2016) and M'hiri, Ioannou, Mihoubi Boudhrioua, and Ghoul (2015) and also by preliminary assays. CE was performed in an orbital agitator (shaker) (New Brunswick TM, model Innova 44/44R, USA) for 2 hr at 200 rpm (25 °C), using AEW, BEW, and SAEW as a solvent at a ratio of 1:50 (w/v). These variables were defined after preliminary tests. The extracts were centrifuged at 4,000 rpm (Eppendorf, model ALB 60, Germany) for 10 min (4 °C) and then filtered.

## 2.4 US-ASSISTED EXTRACTION (UAE) WITH ELECTROLYZED WATER

The experimental apparatus for UAE was composed of a jacketed reactor (250 mL) connected to a thermostatic water bath (temperature accuracy of ±1.0 °C) for temperature control and a high-intensity US processor of 400 W and frequency of 24 kHz (Hielscher, model UP 400S, Germany). A titanium probe (Model H22, Tip 22), presenting a maximum US intensity of 85 W/cm2, was used. For the extractions, the ultrasonic probe was placed at the center of the jacketed reactor containing 1:40 (sample: solvent ratio) in electrolyzed water (AEW, BEW, and SAEW). The temperature was adjusted to 25 ± 2 °C by circulating water. All the extractions were carried out for 15 min (defined according to preliminary assays, data not shown) at a constant US power and pulse cycle (related to the time that the US was on) according to the experimental design. The choice of the processing time also took into consideration other studies, such as Bahmani et al. (2018), which found that the extraction yield increased up to 25 min. and then there was a decrease. The extracts were centrifuged at 4,000 rpm (Eppendorf, model ALB 60, Germany) for 10 min at 4°C for the analyses of total phenolics (TP), flavonoids, and antioxidant power.

## 2.5 CONTROL

The control (C) was placed in contact (15 min at 25 °C and ratio of 1:40 (w/v)) with the AEW, BEW, and SAEW. The extracts were centrifuged at 4,000 rpm (Eppendorf, model ALB 60, Germany) for 10 min (4°C) and then filtered. The C was performed to observe the interaction of electrolyzed water (AEW, BEW, and SAEW) with the sample, without agitation (CE) or US (UAE).

## 2.6 TOTAL PHENOLIC

The TP compounds were determined according to the method described by Singleton, Orthofer, and Lamuela-Raventos (1999). An aliquot of diluted sample (0.2 mL) was mixed with 1 ml of 2 N Folin– Ciocalteu reagent and 0.8 mL of 7.5 % sodium carbonate ($Na_2CO_3$) solution was added. After incubation at 25°C for 2 h the absorbance was measured at 765 nm using a UV-vis spectrophotometer (UV-2600, Shimadzu, Japan). The values were expressed as mg of gallic acid in 100 g of tangerine peel flour. All the analyses were performed in triplicate.

## 2.7 TOTAL FLAVONOIDS (TF)

The flavonoids were evaluated according to the methodology proposed by Zhishen, Mengcheng, and Jianming (1999) with some modifications. A 250 μL of aliquot of diluted (1:25) sample was homogenized with 1,250 μL of distilled water and 75 μL of 5 % sodium nitrite ($NaNO_2$) (w/v). After 5 min, 150 μL of 10 % (w/v) aluminum trichloride ($AlCl_3$) was added and after 1 min, 500 μL of 1 M sodium hydroxide (NaOH) was added. The absorbance was measured at 510 nm using a UV-visible spectrophotometer (UV-2600, Shimadzu, Japan) and the blank was made with distilled water. The values were expressed as mg EQ $(100g)^{-1}$ of tangerine peel flour.

## 2.8 FERRIC REDUCING ANTIOXIDANT POWER

The ferric reducing antioxidant power (FRAP) was evaluated according to the methodology proposed by Benzie and Strain (1999). The FRAP reagente (Fe (III)-TPTZ (tripyridyltriazine) solution) was obtained from a combination of 25 ml of 0.3 M acetate buffer, 2.5 ml of a 10 mM TPTZ solution (3.12 g of TPTZ in 1 L of 40 mMHCl), and 2.5 mL of a 20 mM aqueous solution of ferric chloride. In a test tube, 200 μL of the sample, which had been previously diluted in distilled water (1:25), and 1.8 mL of the FRAP reagent were added and kept in a water bath (37 °C/30 min). The FRAP reagent was used as a blank. The absorbance was measured at 593 nm using a UV-visible spectrophotometer (UV-2600, Shimadzu, Japan). The results were expressed as μmolTEAC $(100g)^{-1}$ of tangerine peel flour.

## 2.9 STATISTICAL ANALYSIS

A central composite rotatable design (CCRD), with eight assays and three repetitions of the central point, was performed. The effects of US intensity (17 - 85 W/cm$^2$) and pulse cycle (0.5 - 1.0) on the TPs, flavonoids, and antioxidante power were evaluated (Tables 1 - 3). The experimental data were analyzed using Statistica 7.0 software (StatSoft Inc., Tulsa, OK, USA) with a 95 % significance level.

## 3. RESULTS AND DISCUSSION

Ultrasonic power is very important for improving the extraction yield of target compounds from plant material (Wang et al., 2013). A CCRD was performed to evaluate the extraction in the ultrasonic equipment using AEW, BEW, and SAEW as the solvent.

A model was performed for the phenolic and flavonoid compounds. The independent and dependent variables were fitted using a second-order model equation and this model was evaluated in terms of goodness of fit. Analysis of variance (ANOVA) was used to evaluate the adequacy of the adjusted model. The $R^2$ value provided a measure of how much the model could explain the variability in the observed response.

The combination of SAEW and US promoted a higher extraction of TP compounds in most assays compared to the other extraction methods (Table 2). According to Bahmani, Aboonajmi, Arabhosseini, and Mirsaeedghazi (2018a) new green techniques, such as US, lead to reduced extraction times, lower consumption of solvent, higher efficiency, and better quality of extracted substances.

Table 2- Total phenolic compounds mg GAE (100g) $^{-1}$ flour of *Citrus reticulata* extracted with functional electrolyzed water using ultrasound (real and code variables (in parentheses)).

| Assay | Ultrasound intensity P (W.cm$^{-2}$) | Cycle (-) | Total phenolics mg GAE (100g) $^{-1}$ tangerine skin flour | | |
|---|---|---|---|---|---|
| | | | AEW | BEW | SAEW |
| 1 | 26.89 (-1) | 0.57 (-1) | 3689.18 | 3657.65 | 3518.01 |
| 2 | 75.11 (1) | 0.57 (-1) | 3905.40 | 4081.08 | 4252.25 |
| 3 | 26.89 (-1) | 0.93 (1) | 3641.89 | 3887.38 | 3844.59 |
| 4 | 75.11 (1) | 0.93 (1) | 3878.37 | 4094.59 | 4324.32 |
| 5 | 17 (-1.41) | 0.75 (0) | 3648.64 | 3945.94 | 3936.93 |
| 6 | 85 (1.41) | 0.75 (0) | 3743.24 | 4054.05 | 4283.78 |
| 7 | 51 (0) | 0.5 (-1.41) | 3591.21 | 3869.36 | 4045.04 |
| 8 | 51 (0) | 1.0 (1.41) | 3587.83 | 3918.91 | 4085.58 |
| 9 | 51 (0) | 0.75 (0) | 3707.20 | 3902.02 | 4121.62 |
| 10 | 51 (0) | 0.75 (0) | 3709.45 | 3952.70 | 4189.18 |
| 11 | 51 (0) | 0.75 (0) | 3702.70 | 3983.10 | 4148.64 |
| Control* | - | | 3067.56 | 3155.40 | 3047.29 |
| Conventional with electrolyzed water | Shaker 200 rpm | | 3489.86 | 3681.31 | 3630.63 |

*Extraction without ultrasound. ** Conventional extraction. AEW – Acid electrolyzed water; BEW – Basic electrolyzed water; SAEW – Slightly acidic electrolyzed water.

The highest level of phenolic compounds (4,324.32 mg GAE.100$^{-1}$ g of flour) was achieved in assay 4 (75.11 W/cm$^2$ and 0.93 cycle). The fact that the highest value was not reached at 85 W/cm2 can be explained by Bahmani et al. (2018a), who reported that the use of very high power leads to an increase in the number of bubbles in the solvent. This can consequently decrease the efficiency of ultrasonic energy. This value was higher than that found for TP compounds (960.8–3,162.3 mg GAE.100$^{-1}$ g) reported by Lagha-Benamrouche and Madani (2013) in relation to the CE of *C. sinensis L.* and *C. aurantium L.* with methanol–water. Chen, Yuan, and Liu (2010) used 70% ethanol and US extraction to obtain a TP content of 1,560– 1,900 mg GAE.100$^{-1}$ g dry weight for *C. reticulata* Blanco cv. Ougan peel extract.

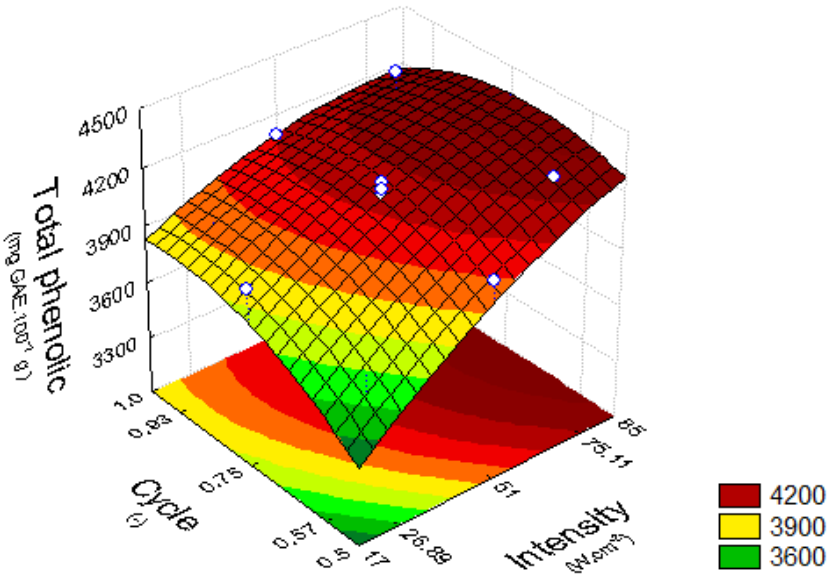
Equation (1) describes the extraction of TP compounds predicted by the model as a function of the coded variables, intensity, and cycle. The reparameterized model only contains the statistically significant terms.

$$\text{Total phenolic} = 4153.55+213.51x_1-47.54x_1^2+57.14\ x_2-70.20\ x_2^2-63.63x_1.x_2 \quad (1)$$

where $x_1$ is the linear intensity, $x_2^1$ is the quadratic intensity, $x_2$ is the linear cycle, $x_2^2$ is the quadratic cycle, and $x_1.x_2$ is the interaction of intensity and cycle.

According to Equation (1), the intensity and cycle variables in linear and quadratic form were significant for the TP compounds. According to the ANOVA analysis (Table 5), the calculated F was higher than the critical F value. The response surface methodology (Figure 1) showed that the extraction yield increased with increasing intensity, independently of the cycle values, which was due to the increase in US energy that improved mass transfer. Bahmani et al. (2018a) increased efficiency by increasing power using US. Ultrasonic intensity has been suggested as a method to affect collapse pressures. At high intensity, the collapse of cavitation bubbles will be more severe as a result of higher ultrasonic intensity. Bigger bubbles would be formed and higher shear forces would be stimulated.

Figure: 1-Response surface for intensity and ultrasound cycle on extraction of phenolic compounds using SAEW as solvent.




Were - SAEW: Slightly Acidic Electrolyzed Water.

The use of US and SAEW has been reported as alternative technologies in the meat industry (Flores et al., 2017) due to their minimum corrosive potential, the fact that they are less harmful to the environment, and the fact that they require hardly any safety precautions (Zacharia, Kamitani, Tiisekwa, Morita, & Iwasaki, 2010).

The results of the present study (Table 2) show that the US intensity had a positive influence on the extraction of phenolic compounds. Dias et al. (2016) also verified that US intensity was significant in the extraction of phenolic compounds from dedo de moça pepper. This result has been related to the energy provided by US, which promotes the better release of the phenolic compounds from the plant matrix, increasing their amounts in the extract. However, high levels of US intensity in the extraction of compounds can accelerate the degradation of phenolic compounds, promoting the formation of free radicals and increasing oxidative reactions. In our study, except for the use of SAEW, there was no evidence of the degradation of phenolic compounds in the US intensity employed in the assays (Table 2), which can be explained by the use of electrolyzed water, especially BEW. According to Hanaoka et al. (2004), BEW has a strong reduction potential that is responsible for the reduction of free radicals, which is due to its low ORP, and it can be used as an antioxidant.

M'hiri et al. (2015) used US to extract phenolic compounds from orange peel; an increase in the intensity range between 100 and 200 W/cm$^2$ improved the number of phenolic compounds that were extracted. The intensification of phenolic extraction was attributed to the propagation of the pressure waves promoted by the US, the solvent used, and also due to cavitation, which promotes the destruction of the cell wall, thereby increasing the release of the cellular content. In the present study, the US intensity range between 51 and 85 W/ cm$^2$ resulted in the highest level of extraction of phenolic compounds (Table 2).

Table 3 shows the results regarding flavonoid compounds. The AEW showed the highest amount of flavonoid compounds in all the assays, except assay 3. Assay 9 extracted the highest amount of flavonoids (691.76 mg EQ.100/g flour).

Table 3- Total flavonoids mg EQ (100g) $^{-1}$ flour of *Citrus reticulata* extracted with functional electrolyzed water using ultrasound (real and code variables (in parentheses)).

| Assay | Ultrasound intensity P (W.cm$^{-2}$) | Cycle (-) | Total flavonoids mg EQ (100g) $^{-1}$ tangerine skin flour | | |
|---|---|---|---|---|---|
| | | | AEW | BEW | SAEW |
| 1 | 26.89 (-1) | 0.57 (-1) | 627.06 | 562.35 | 589.80 |
| 2 | 75.11 (1) | 0.57 (-1) | 650.59 | 621.17 | 591.76 |
| 3 | 26.89 (-1) | 0.93 (1) | 585.88 | 593.73 | 554.51 |
| 4 | 75.11 (1) | 0.93 (1) | 615.29 | 585.88 | 585.88 |
| 5 | 17 (-1.41) | 0.75 (0) | 601.57 | 589.80 | 585.88 |
| 6 | 85 (1.41) | 0.75 (0) | 674.12 | 574.12 | 597.64 |
| 7 | 51 (0) | 0.5 (-1.41) | 613.33 | 574.12 | 562.35 |
| 8 | 51 (0) | 1.0 (1.41) | 636.86 | 491.76 | 589.80 |
| 9 | 51 (0) | 0.75 (0) | 691.76 | 527.05 | 546.67 |
| 10 | 51 (0) | 0.75 (0) | 685.88 | 515.29 | 554.51 |
| 11 | 51 (0) | 0.75 (0) | 660.39 | 515.29 | 530.98 |
| Control* | - | | 499.61 | 393.73 | 393.73 |
| Conventional with electrolyzed water | Shaker 200 rpm | | 483.33 | 571.57 | 517.65 |

*Extraction without ultrasound. ** Conventional extraction. AEW – Acid electrolyzed water; BEW – Basic electrolyzed water; SAEW – Slightly acidic electrolyzed water.

The SAEW presented the best results for the extraction of phenolic compounds, while the AEW for flavonoid. This demonstrated that the flavonoids were more easily extracted in acid pH (pH 3.24), while the phenolics were more easily extracted in neutral pH (pH 6.20).

Equation (2) presents the coded model for the extraction of TF using AEW.

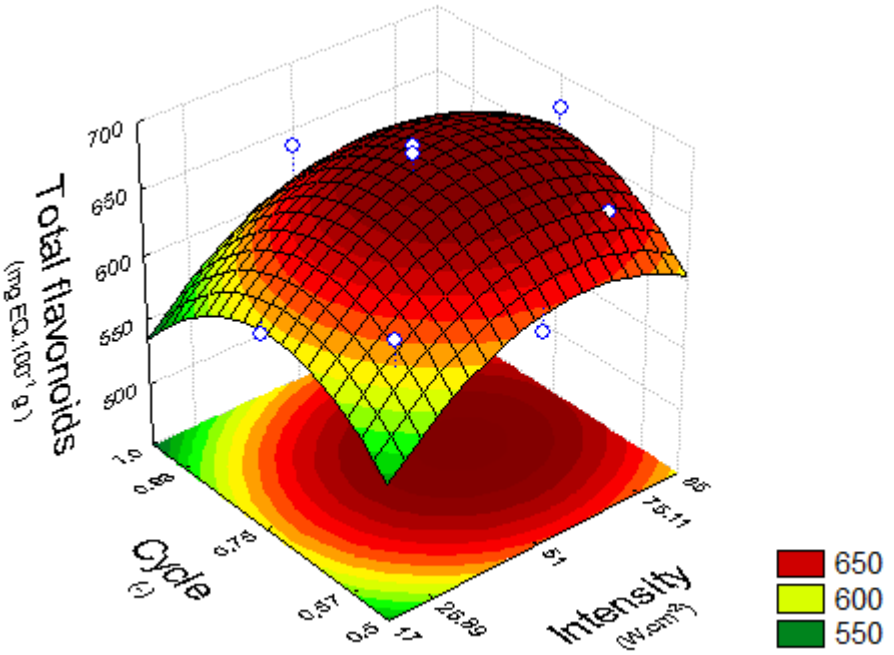
$$\text{Total flavonoids} = 679.38+19.46x_1-23.78x_1^2-30.19x_2^2 \quad (2)$$

where $x_1$ is the linear intensity, $x_2$ 1 is the quadratic intensity, and $x_2^2$ is the quadratic cycle.

According to Equation (2), the intensity in linear and quadratic forms and the cycle in the quadratic form were significant for the extraction of flavonoids using US and AEW as the

solvent. As can be seen in Figure 2, response surface methodology demonstrated that intermediate values for the cycle (0.5–1.0 dimensionless) and intensity (17–85 W/cm$^2$) extracted higher levels of flavonoid compounds by US and AEW.

Figure 2- Response surface for intensity and ultrasound cycle on the extraction of flavonoid compounds using AEW as solvent.




Were – AEW: Acid Electrolyzed Water.

The association of two technologies (US plus solvent) promotes better results compared with CE (Jaime et al., 2010; Khan et al., 2010; Ma et al., 2008). UAE can be used for thermo-sensitive compounds because it promotes the production of bubbles in the extractor liquid below its boiling point, resulting in strong dynamic stress, which facilitates the dilation and hydration of the material, enlarges the cell wall pores, and optimizes the diffusion and mass transfer processes (Chemat, Huma, & Khan, 2011).

Ramful, Bahorun, Bourdon, Tarnus, and Aruoma (2010) classified high-level extraction as more than 550 mg GAE.100$^{-1}$ g and 360 mg EQ.100$^{-1}$ g of fruit for TPs and TF, respectively. All the assays in the present study promoted high-level extraction for flavonoids since the values ranged from 491.76 (BEW) to 691.76 mg EQ.100$^{-1}$ g flour (AEW) (Table 3). The same was observed in the extraction of phenolic compounds (Table 2) since the values ranged from 3,518.01 (SAEW) to 4,324.32 mg GAE.100−1 g tangerine peel (SAEW).

The antioxidant activity measured by the FRAP method was highest using SAEW (663.69 μmol TEAC.100$^{-1}$ g) with 85 W/cm$^2$ (Table 4). The SAEW presented the best results for phenolics and FRAP; these results are in accordance with Fu et al. (2017), who reported a correlation between antioxidant activity and TP content. The FRAP analysis was not used to generate a response surface, since there was a lack-offit between the experimental results and those predicted by the proposed model. However, all the results obtained by US were higher than the C and conventional treatments. This finding was in accordance with Bahmani et al. (2018a), who observed that the extraction method has a significant effect on the antioxidant activity of essential oils.

Table 4- Ferric reducing antioxidant power (FRAP) of the flour extract of *Citrus reticulata* obtained with functional electrolyzed water using ultrasound (real and code variables (in parentheses)).

| Assay | Ultrasound intensity P (W.cm$^{-2}$) | Cycle (-) | FRAP (μmol TEAC (100g) $^{-1}$ tangerine skin flour) | | |
|---|---|---|---|---|---|
| | | | AEW | BEW | SAEW |
| 1 | 26.89 (-1) | 0.57 (-1) | 575.50 | 591.81 | 593.47 |
| 2 | 75.11 (1) | 0.57 (-1) | 590.81 | 610.11 | 630.74 |
| 3 | 26.89 (-1) | 0.93 (1) | 581.49 | 603.79 | 613.77 |
| 4 | 75.11 (1) | 0.93 (1) | 588.81 | 620.09 | 644.05 |
| 5 | 17 (-1.41) | 0.75 (0) | 583.82 | 602.79 | 614.10 |
| 6 | 85 (1.41) | 0.75 (0) | 607.78 | 618.76 | 663.69 |
| 7 | 51 (0) | 0.5 (-1.41) | 593.81 | 610.78 | 626.75 |
| 8 | 51 (0) | 1.0 (1.41) | 650.71 | 618.76 | 639.40 |
| 9 | 51 (0) | 0.75 (0) | 607.78 | 624.75 | 640.73 |
| 10 | 51 (0) | 0.75 (0) | 608.78 | 622.76 | 617.77 |
| 11 | 51 (0) | 0.75 (0) | 626.75 | 649.71 | 618.77 |
| Control* | - | | 515.60 | 570.84 | 532.91 |
| Conventional with electrolyzed water | Shaker 200 rpm | | 639.68 | 606.68 | 633.02 |

*Extraction without ultrasound. **Conventional extraction. AEW – Acid electrolyzed water; BEW – Basic electrolyzed water; SAEW – Slightly acidic electrolyzed water.

Bahmani, Aboonajmi, Arabhosseini, and Mirsaeedghazi (2018b) evaluated artificial neural network (ANN) modeling as a method to predict the kinetics of the extraction of essential oils from tarragon (Artemisia dracunculus L.) using US pretreatment with Clevenger US power, sonication time, and extraction time. The interactions of the latter were considered as input vectors, while the extraction yield of essential oils was considered to be the model output.

Table 5- Analysis of variance (ANOVA) for total phenolics, flavonoids and FRAP of the flour extract of Citrus reticulata.

| | SS | dF | MS | Fcal | $R^2$ |
|---|---|---|---|---|---|
| Total phenolic - SAEW | | | | | |
| Regression | 437960.4 | 5 | 87592.08 | 4.26 | 0.81 |
| Residual | 102727.4 | 5 | 20545.48 | | |
| Total SS | 540687.8 | 10 | | | |
| Total flavonoids - AEW | | | | | |
| Regression | 9747.21 | 5 | 1949.44 | 3.70 | 0.78 |
| Residual | 2633.27 | 5 | 526.65 | | |
| Total SS | 12380.48 | 10 | | | |
| FRAP - SAEW | | | | | |
| Regression | 2759.78 | 5 | 551.95 | 3.25 | 0.76 |
| Residual | 848.29 | 5 | 169.65 | | |
| Total SS | 3608.07 | 10 | | | |

Ftab:5;5;0.1=3.45

## 3.1 COMBINATION OF US AND ELECTROLYZED WATER

The extraction of flavonoids, phenolics, and antioxidant activity, using US and AEW, BEW and SAEW, achieved better results than CE and the C. These results agree with those of Chemat et al. (2011) and M'hiri et al. (2015), who found that the use of US improved the extraction of bioactive compounds. The collapse of cavitation bubbles near the cell walls induced by US produces cell disruption, thus causing stronger and enhanced penetration of the solvent into the cells, and intensification of the mass transfer. Furthermore, the shock waves may facilitate swelling and hydration, and as a consequence larger pores in the cell wall of the plant and structural modification of plant tissue (Maric et al., 2018).

The extraction of bioactive compounds depends on solvent properties such as viscosity, density, miscibility, and polarity (Cunha, Fernandes, 2018). Thereby, the choice of solvent is fundamental for the efficiency of the process. The extraction yield increases in line with an increase in polarity and a decrease in the viscosity of solvent. Adding water to a solvent usually creates a more polar medium. By increasing the proportion of water, the polarity of the solvent

also increases and the system is able to extract phenolic substances (Kaderides, Papaoikonomou, Serafim, & Goula, 2019). Thus, electrolyzed water seems to be suitable for this purpose.

The results of this study showed that the use of electrolyzed water was viable. Furthermore, the use of organic solvents, which can be toxic to the environment and humans, was avoided. The results were in agreement with Shirahata et al. (1997), who demonstrated that the electrolysis of NaCl solution produced active oxygen scavengers whose function would be similar to biological substances such as catalase and ascorbic acid. Yahagi et al. (2000) observed that electrolyzed water contains reactive species, such as active hydrogen, which prevent the degradation of compounds by eliminating oxygen.

Zhao et al. (2012) reported that BEW has the potential to eliminate oxygen species and, due to its alkaline pH, the use of BEW in the infusion of tea would increase the antioxidant capacity. The present study verified that electrolyzed water was efficient in extracting phenolics and flavonoids. The use of AEW and SAEW promoted greater extraction, demonstrating the importance of these waters combined with US.

Analysing the results of the C and CE, the importance of agitation was observed in the extraction of phenolic compounds (Table 2). The CE with electrolyzed water extracted higher levels of compounds than the C; however, the results for extraction using ethanol were lower than the extraction with electrolyzed water. Corbin et al. (2015) used the CE method and observed that constant stirring during extraction produced effects on the plant matrix, as well as the circulation of the solvent in the system, which increased mass transfer.

Gogate and Kabadi (2009) studied the combination of US and electrolyzed water in the reduction of the microbial population on fruit surfaces. They demonstrated that cavitation improves the penetration of electrolyzed water and promotes the reduction of microorganisms. The interesting results observed in our study may be related to this penetration of the electrolyzed water in the sample, improving the solubilization and extraction of the bioactive compounds from the C. reticulata peel. The sonication of electrolyzed water can generate reactive oxygen species and can cause damage to biological structures, including plant tissues, which may lead to better extraction of bioactive compounds from plant matrices (Shirahata et al., 1997).

The combined use of US and electrolyzed water is important because it can reduce the consumption of chemicals. Furthermore, this process is more effective than traditional methods (C and CE). The use of AEW and BEW should be further investigated, while SAEW is a viable option due to its almost neutral pH (Zhang, Zhou, Chen, & Yang, 2017).

The extraction from *C. reticulata* using electrolyzed water (AEW, BEW, and SAEW) improved the performance when compared to CE, and was even better when combined with US.

The conventional method using an orbital shaker (200 rpm) is time-consuming (2 hours); however, when US and electrolyzed water were used the time was reduced to 15 min, speeding up the process by 85%. The main advantages of using US are the reduced time and less energy required compared with CE (Khan et al., 2010).

## 4 CONCLUSION

The combination of US and different types of electrolyzed water (AEW, BEW, and SAEW) resulted in the extraction of higher quantities of phenolic compounds, flavonoids, and antioxidant capacity compared to traditional methods. Additionally, the processing time was reduced by 87.5%.

The combination of US and SAEW could be used in the extraction of phenolic compounds, whereas the combination of US + AEW presented better results regarding the extraction of flavonoid compounds. According to the results from the experimental design, the highest TP content was observed at intensities higher than 75.11 W/ $cm^2$; the best condition for TF was the central point (51 W/$cm^2$ and 0.75); and the highest extraction for FRAP was at 85.11 W/$cm^2$ and 0.75. The intensity and cycle variables were significant for TP and TF; the higher the intensity, the higher the yield and antioxidant capacity.

This new methodology, combining US and electrolyzed water, is viable for the extraction of bergamot coat bioactive compounds.

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## CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

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### 4.3.1 Texto de conexão entre artigo 3 e 4.

O objetivo do artigo 3 foi comparar a extração convencional com a extração assistida por ultrassom (EAU) usando três tipos de água eletrolisada, ácida (AEA), básica (AEB) e levemente ácida (AELA), como solução extratora. Foi realizado um delineamento composto rotacional para analisar a atuação das variáveis intensidade e pulso ciclo de ultrassom na extração.

Neste artigo, o maior resultado para fenólicos totais foi 4324,32 mgGAE (100 g) $^{-1}$ e capacidade antioxidante (FRAP) 663,69 µmolTEAC (100 g) $^{-1}$ obtidos na EAU+AELA, já para flavonoides totais o maior valor (691,76 mgEQ (100 g) $^{-1}$) foi obtido com EAU+AEA.

A extração ultrassônica foi superior à extração convencional confirmando a hipótese que as tecnologias não convencionais reduzem o uso de energia, são rápidas e eficazes. Entretanto, diferente dos resultados do artigo 2 onde a AEB apresentou melhores resultados como solução extratora de fenólicos (3681,31 mgGAE (100 g) $^{-1}$) e flavonoides (571,57 mgEQ (100 g) $^{-1}$) , neste estudo, a AELA apresentou resultados superiores de fenólicos totais, enquanto a AEA apresentou melhor resultado de flavonoides totais. Este resultado definiu a necessidade de utilização dos três tipos de água eletrolisada no artigo 4.

Com isto, no artigo 4 foram comparadas a extração assistida por ultrassom (EAU), extração com fluido supercrítico (EFS) e a combinação destas (EAU+EFS), utilizando água eletrolisada (AEA, AEB e AELA) como solvente (EAU) e co-solvente (EFS).

## 4.4 ARTIGO 4 - Electrolyzed water for the extraction of bioactive compounds from *Citrus reticulata* peels by ultrasound and supercritical $CO_2$

Artigo submetido na Food Chemistry

# Electrolyzed water for the extraction of bioactive compounds from *Citrus reticulata* peels by ultrasound and supercritical $CO_2$

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## ABSTRACT

Different types of electrolyzed water (acidic, slightly acidic and basic) were evaluated on obtaining bioactive compounds from tangerine (*Citrus reticulata*) peels by ultrasound-assisted extraction (UAE) and supercritical fluid extraction (SFE) with $CO_2$. The single process of SFE or the integrated process of UAE + SFE was more favorable in the extractions than the control process (extraction without UAE and SFE), presenting phenolics and flavonoids up to 41 % and 58 % more concentrated in the extracts, respectively. SFE-$CO_2$-BEW provided the best results for total phenolic compounds (5150.90 mg GAE (100 g) $^{-1}$), flavonoids (941 mg QE (100 g) $^{-1}$) and antioxidant activity (835.28 µmol TEAC (100 g) $^{-1}$). Therefore, a central composite design (CCD) for SFE was further performed to evaluate the influence of temperature, pressure and sample: water ratio on the extract composition and antioxidant activity using basic electrolyzed water. The condition of 40°C, 10 MPa and 1:40 (w/v) ratio extracted phenolics and flavonoids in the order of 5344.59 mg GAE (100 g)$^{-1}$ and 1271.53 mg QE (100 g)$^{-1}$, respectively and the antioxidant capacity was 854.85 µmol TEAC (100 g)$^{-1}$. Overall, the SFE with $CO_2$ using basic electrolyzed water as cosolvent was efficient in the extraction of bioactive compounds from tangerine peels.

**Keywords**: Green technologies. Supercritical fluid extraction. Ultrasound assisted extraction. Tangerine. Phenolic compounds. Flavonoids. Antioxidant activity.

## 1 INTRODUCTION

Tangerine (*Citrus reticulata*) is one of the four most cultivated citrus species in the world. The fresh fruit is appreciated for flavor, aroma and nutritional composition, being widely used in the juice industry [1]. The industrial processing generates a high amount of organic waste such as peels, seeds, pulp, and membranes, constituting an environmental problem [2, 3]. Generally, citrus waste is dried to be use as an animal feed [4]. However, it is rich in bioactive compounds, such as phenolics, which can be used as additive in foods [5].

Phenolic compounds are an important group for scientific and therapeutic interest since they are consistent in order to sequester reactive oxygen and chelating species. They are divided into two groups: flavonoids and phenolic acids. The flavonoids contribute to the yellow background color in citrus [6] and have higher antioxidant activity than vitamins since they are able to retard pro-oxidative effects on proteins, DNA and lipids [7]. Total phenolics have attracted attention due to antioxidant potential and effects on disease prevention, mainly associated with oxidative stress, such as cancer, as well as contributing to the taste and color of citrus fruit [8].

The extraction from cell-matrix is necessary for the analysis and exploration of the constituents [9]. Extraction of bioactive compounds is traditionally carried out by conventional methods using organic solvents, with or without stirring and heating. These methods are being questioned due to the use of toxic solvents and pollutants. In addition, the use of high temperatures causes oxidation and losses of heat-sensitive compounds [10]. Unconventional techniques are being tested to preserve the environment, to reduce or eliminate the use of organic solvents and to reduce energy expenditure. Ultrasound-assisted extraction (UAE) and supercritical fluid extraction (SFE) could be used to minimize the degradation of bioactive compounds, using moderate temperatures and low extraction time [11].

UAE and SFE have been used to increase the extraction of bioactive compounds, especially in studies related to the pharmaceutical and food areas [12]. The combination of these techniques was reported by Yang, Lin and Wei [13] and Dias et al. [10], where the authors demonstrated that the combined process can be faster, efficient and selective to obtaining bioactive compounds from raw materials as *Rabdosia rubescens* and *Capsicum baccatum*, respectively.

UAE has been studied as an alternative method to replace conventional thermal processes due to its lower adverse effects on the bioactive compounds [14,15,16]. Ultrasound is a special type of sound wave from 20 kHz to 100 MHz that produces cavitation, which forms

bubbles and causes their collapse after their growth [17]. The mechanism of extraction by ultrasound involves two types of physical phenomena, the diffusion through the cell wall and the waning of the cellular content after the walls break. These phenomena are related to the temperature, pressure, frequency and time of sonication used [18].

SFE is characterized by the change in pressure and temperature for the supercritical fluid condition. Supercritical fluids exhibit desirable transport properties that enhance their extractability. In comparison with liquid solvents used in conventional processes, the supercritical fluids have low viscosity, and this allows them to spread more easily in the solid matrix. They present a low surface tension with rapid penetration into the solid, which consequently increases the extraction efficiency [19].

SFE with $CO_2$ (SFE-$CO_2$) is widely used because it is harmless to human health and environment, and also because the critical temperature is moderate (31.2°C). It avoids oxidation reactions which would occur upon the contact of the extract with the air, thus preserving bioactive compounds. In addition, it is also possible to optimize the extraction parameters for a selective extraction [13]. However, the extraction of phenolic compounds and flavonoids with $CO_2$ requires the addition of an organic cosolvent due to $CO_2$ polarity [20]. Methanol and ethanol are used as cosolvent with excellent extraction yields of compounds using $CO_2$. However, on industrial applications, methanol is not indicated because it is a toxic solvent [21]. Otherwise, ethanol is considered safe. However, for application in food-related areas, it needs to be removed from the extracts [22]. As an alternative, other cosolvents could be used in the extraction, as electrolyzed water.

Functional electrolyzed water is produced by electrolysis of a dilute NaCl solution in an electrolytic cell's direct current that contains an ion exchange membrane separating the anode side and the cathode side, obtaining acidic electrolytic water (AEW) and basic electrolyzed water (BEW) [23,24]. BEW contains hydrogen molecules with high capacity for reduction, which can act in the regulation of the redox potential of cellular function due to the high reducing power [25].

No studies were found in the literature using ultrasound and supercritical $CO_2$ extraction techniques using electrolyzed water as solvent or cosolvent.

Based on this context, the objective of this work was to evaluate the extraction of bioactive compounds and the antioxidant activity of extracts using UAE, SFE-$CO_2$ and UAE + SFE-$CO_2$. For both techniques, different types of electrolyzed water (acidic, slightly acidic, or basic) were used as solvent (for UAE) or as cosolvent (for SFE). An experimental design was carried out to determine the best extraction conditions using $CO_2$.

## 2 MATERIALS AND METHODS

### 2.1 PREPARATION OF SAMPLES

Tangerines were purchased in the city of Tupanciretã (Rio Grande do Sul, Brazil) from a rural farmer. The samples were transported to the laboratory (Santa Maria, Rio Grande do Sul, Brazil) in foam-enveloped cardboard boxes. The tangerines without defects, pests, and rot were selected for the study. Fruit cleaning was performed using a sodium hypochlorite solution (100 mg $L^{-1}$) for 10 min at 5 °C followed by washing with water at the same temperature to remove the excess of chemicals. Thereafter, the tangerines were submitted to manual peeling at temperature below 10 °C. The peels were distributed in aluminum trays and dried at 35 ± 5 °C for 72 h in an air-circulation oven. The dried peels were ground in an analytical mill cooled at 4 °C (QUIMIS, model Q 298A21, Brazil), classified in mesh sieves (0.5 mm) and stored in a vacuum package at -18 °C until further use.

### 2.2 FUNCTIONAL ELECTROLYZED WATER

A brine was prepared with filtered and deionized water (Permution®, Brazil) containing 0.01 wt. % sodium chloride (Dinâmica®, Brazil). The brine was subjected to electrolysis at 18 °C on a bench electrolyzer (Envirolyte®, Estonia). The process generated acid electrolyzed water (AEW) of pH 3.24, Oxidation-Reduction Potential (ORP) of 913.5 mV and $Cl_2$ of 1 $mg.L^{-1}$, and basic electrolyzed water (BEW) of pH 9.73 and ORP of -267.67 mV. Also, slightly acidic electrolyzed water (SAEW) of pH 6.20, ORP of 297 mV and $Cl_2$ of 1 $mg.L^{-1}$ was obtained by mixing AEW and BEW in the 1:1 (v/v) ratio.

The pH was measured in a pHmeter with an electrode (Digimed®, Dme-Cv1, Brazil). The redox potential (mV) was obtained with the aid of a platinum electrode (Digimed®, Dmr-Cp1 Brazil). The concentration of free chlorine in the solutions was determined according to procedures described by Smeww (1999) and the results were expressed in $mg.L^{-1}$ of $Cl_2$.

## 2.3 EXTRACTIONS

### 2.3.1 Control

The control assays were performed by mixing the tangerine peel samples with AEW, BEW and SAEW, at the sample: water ratio of 1:40 (w/v), for 15 min at 25 °C without agitation.

### 2.3.2 Ultrasound-assisted extraction

The experimental apparatus for ultrasound-assisted extraction (UAE) is composed of a jacketed reactor (250 mL) connected to a thermostatic water bath (temperature accuracy of ± 1°C) for temperature control and a high-intensity ultrasound processor of 400 W power and 24 kHz frequency (Hielscher, Model UP 400S, Germany). The ultrasound was equipped with a titanium probe (Model H22, Tip 22, Germany). Ultrasound intensity of 75.11 W $cm^{-2}$ and pulse cycle of 0.57 were the levels used in this study, which were defined in previous research (data not published). For the extractions, the ultrasonic probe was placed at the center of the jacketed reactor containing the sample:water ratio of 1:40 (w/v). The temperature was adjusted to 10 ± 2 °C by circulating water through the jacket and the assays were performed for 15 min Afterward, the samples were centrifuged at 4000 rpm for 5 min and the supernatant was stored in amber flasks at 4 °C until further analyses.

### 2.3.3 Supercritical fluid extraction

Supercritical fluid extraction (SFE) using $CO_2$ was performed in an equipment composed of a band heater to control the temperature inside the extraction vessel, blocking valves, a micrometering valve (HIP 15-11AF2 316SS, Erie, EUA), thermostatic baths (Quimis, Q214M2, Diadema, Brazil), a syringe pump (Teledyne ISCO, 500D, Lincoln-NE, USA) and stainless-steel tube. The stainless-steel vessel has 100 mL of useful volume (internal diameter of 2.5 cm and height of 19.5 cm; support up to 35 MPa).

Samples (10 mL) of 1:40 (w/v) tangerine peel flour: water ratio were loaded in the 100 mL stainless-steel vessel. The extraction was performed according to the methodology described by M'hiri et al. [21] with some modifications. The three types of electrolyzed water (AEW, BEW, and SAEW) were evaluated. The static extractions were performed for 30 min at

80 °C and pressure of 10 MPa. Subsequently, the system was depressurized and the aqueous extracts were stored in amber flasks at 4 °C for the analyses.

### 2.3.4 Ultrasound-assisted extraction coupled with supercritical fluid extraction

The extracts obtained by UAE (described in section 2.3.2), at the extract sample:water ratio of 1:40 (w/v), before centrifugation, were processed by SFE using $CO_2$. The EFS-$CO_2$ was performed according described in section 2.3.3 (using extract instead of milled peels) in order to evaluate the effect of both technologies (UAE and SFE).

## 2.4 CENTRAL COMPOSITE DESIGN

After previously testing the three types of water (section 2.3.3), a Central Composite Design (CCD) was carried out to evaluate the influence of pressure (10 - 20 MPa), temperature (40 - 80 °C) and sample: water ratio (1:20 - 1:40 w/v) in the SFE of bioactive compounds. The assays were performed with BEW because it presented the highest capacity for extraction of phenolic compounds and total flavonoids and provided extracts with high antioxidant activity (measured by Ferric Reducing Antioxidant Power (FRAP)). The assays with basic electrolyzed water were called SFE-$CO_2$-BEW and the control assays with deionized water (DW) were called SFE-$CO_2$-DW.

## 2.5 ANALYSIS OF BIOACTIVE COMPOUNDS

### 2.5.1 Total phenolics

The quantification of total phenolic was performed according to the methodology described by Singleton, Orthofer and Lamuela-Raventos [26]. The extract was diluted (in same solvent) 1:50 (v/v), after this solution (0.2 mL) was mixed with 0.8 mL of 7.5 % sodium carbonate solution ($Na_2CO_3$) and 1 mL of Folin-Ciocalteu 2 N. Samples were incubated at 25 °C for 2 h and the absorbance was measured at 765 nm using a UV-Vis spectrophotometer (UV-2600, Shimadzu, Japan). The results were expressed as mg of gallic acid in 100 g of tangerine peel flour using a calibration curve of gallic acid of concentrations from 5 to 70 mg $L^{-1}$.

### 2.5.2 Total flavonoids

The determination of total flavonoids was performed according to Zhishen; Mengcheng and Jianming [27] with modifications. Each sample of extract was diluted in distilled water 1:10 (v/v). A 250 μL aliquot this solution was homogenized with 75 μL of 5 % (w/v) sodium nitrite ($NaNO_2$) and 1250 μL of distilled water for 5 min. A 150 μL aliquot of 10 % (w/v) aluminum trichloride ($AlCl_3$) was added and, after 1 min, 500 μL of 1 M sodium hydroxide (NaOH) was also added. The absorbance was measured at 510 nm using a UV-Vis spectrophotometer (UV-2600, Shimadzu, Japan). Distilled water was used as a blank. The results were expressed as mg of quercetin in 100 g of tangerine peel flour using a calibration curve of quercetin of concentration from 50 to 80 mg $L^{-1}$.

### 2.5.3 Ferric reducing antioxidant power

The antioxidant activity of the extracts was determined using the Ferric Reducing Antioxidant Power (FRAP) method (iron reduction power) according to the procedures described by Benzie and Strain [28]. A solution of Fe (III) – tripyridyltriazine (TPTZ) (10 mM) was prepared from 3.12 g of TPTZ in 1 L of 40 mM HCl. For obtaining the FRAP reagent, an aliquot of 2.5 mL of this solution was mixed with 2.5 mL of a solution obtained from 20 mL aqueous solution of ferric chloride and 25 mL of 0.3 M acetate buffer.

For the determination of iron-reducing power of the extracts, 200 μL of each sample previously diluted in distilled water (1:25) was added in test tubes and mixed with 1.8 mL of the FRAP reagent. The tubes were kept in a water bath for 30 min at 37 °C and the absorbance of the samples was measured at 593 nm on a UV-Vis spectrophotometer (UV-2600, Shimadzu, Japan). A calibration curve was constructed in the range of 0-25 μM from the Trolox Equivalent Antioxidant Capacity (TEAC) and the results were expressed in μMol TEAC $(100\ g)^{-1}$ of tangerine peel flour.

## 2.6 STATISTICAL ANALYSIS

The results were statistically analyzed by Tukey's test using a significance level of 95 % (p-value < 0.05). The experimental design was evaluated using Statistica® 9.1 software (Statsoft Inc., USA).

## 3 RESULTS AND DISCUSSION

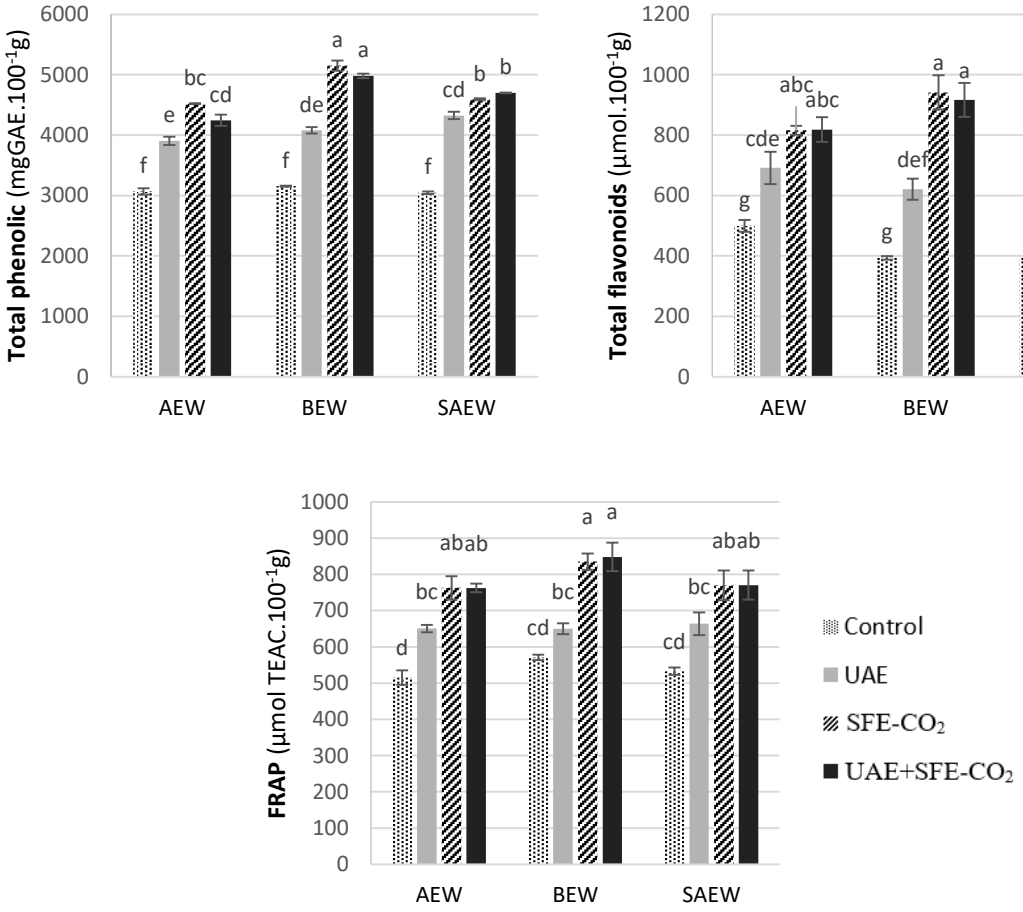
### 3.1 COMPARISON BETWEEN EXTRACTION TECHNIQUES AND TYPES OF ELECTROLYZED WATER

Figure 1 shows the phenolics and flavonoids values and the antioxidant activity (FRAP) of extracts obtained from tangerine peels using different extraction techniques (control, UAE, SFE, and UAE + SFE) with functional electrolyzed water (AEW, BEW, and SAEW) as extraction media.

The control treatment presented the lowest values of phenolics, flavonoids and antioxidant activity in all assays. The highest values were observed for extracts obtained by SFE and UAE + SFE, which there was not a significant difference (p-value < 0.05) between these two methods. Therefore, in such case, only supercritical technology is sufficient, thus saving time, work and energy. Suggesting that tangerine extracts are sufficiently solubilized by $CO_2$, especially with the aid of electrolyzed water. In fact, the positive results of SFE could be explained by the low viscosity solvent, which makes it easily diffuse into the solid matrix, and low surface tension, allowing rapid penetration of the solvent into the solid and thereby increasing extraction efficiency [19].

Some works reported that the UAE combined with SFE-$CO_2$ was a positive strategy for obtaining oridonin extract from *Rhinella rubescens* [13, 29]. The authors presented that ultrasound enhanced cavitation mass transfer and increased the interaction between the solvent and plant tissues. The target components could be dissolved more rapidly in supercritical $CO_2$, increasing the yield in a shorter time. In the same trend, Kawamura et al. [30] extracted leaf components from *Perilla frutescens* using UAE+SFE, and this method integration allowed the extraction of two times more luteolin and apigenin.

Figure 1- Total phenolics (a), total flavonoids (b) and antioxidant activity (FRAP) (c) of tangerine extracts obtained by control, UAE, SFE-$CO_2$, and UAE+SFE-$CO_2$ using three types of electrolyzed water as extraction media.




[abc]Means with equal letters do not differ significantly by the Tukey's test (p-value < 0.05); The bars refer to the standard deviation of triplicate analyses; UAE: Ultrasound assisted extraction; SFE-$CO_2$: Supercritical fluid extraction with $CO_2$; UAE+SFE-$CO_2$: Ultrasound assisted extraction + Supercritical fluid extraction with $CO_2$; AEW – Acidic electrolyzed water; BEW – Basic electrolyzed water; SAEW - Slightly acidic electrolyzed water.

Valadez-Carmona et al. [31] found that the SFE-$CO_2$ can be used as a technique to obtaining an extract enriched in phenolic compounds from cocoa skin. The extract obtained under ideal conditions (60 ° C, 299 bar and 13.7 % ethanol) presented 12.97 mg GAE.$g^{-1}$ extract. Wang et al. [32] used SFE with $CO_2$ to obtain flavonoids from *Pueraria lobate* and the results demonstrated that the extraction time was reduced and improved the recovery of analytes. On the other hand, total phenolics and flavonoids of orange peel extracted using SFE were lower than the conventional extraction using solvent 80 % ethanol as solvent, and proportion sample: solvent 1:10 (w/v) [21]. The authors explained that orange peel is richer in

polar phenolic compounds (glycosylated flavanones) than non-polar flavones (polyoxethoxylated flavones), whereas supercritical $CO_2$ extraction is more suited to non-polar compounds. In our study, the supercritical $CO_2$ with the aid of a polar medium (electrolyzed water) acting as a cosolvent presented interesting results in the extraction of polar compounds such as phenolics (approximately 5000 mg GAE $(100\ g)^{-1}$ of tangerine peels) and flavonoids (approximately 950 µmol QE $(100\ g)^{-1}$ of tangerine peels). Suggesting the potential electrolyzed water, mainly BEW, is efficient as a cosolvent on the SFE of bioactive compounds from tangerine peel.

Comparing the types of electrolyzed water in the control assay, BEW also provided the best results for the extraction of phenolics and for providing extracts with high antioxidant capacity. BEW reduction has a strong reduction potential that is responsible for the reduction of free radicals [33]. Likewise, BEW resulted in a reduction from 1 to 3 log reduction of *Escherichia coli*, *Listeria monocytogenes*, *Campylobacter jejuni*, *Aeromonas hydrophila* and *Vidrio parahaemolyticus* in suspension [34]. The authors suggested that BEW improved the penetration of active chlorine agents and damages cell walls, destabilizing extracellular polymeric compounds around bacterial cell. Overall, the good performance of BEW as a modifier medium was evidenced, because it can damage cell walls, provide better penetration of solvents, and solubilize bioactive compounds for dragging them with the bulk extract.

One of the major requirements of a cosolvent is to be a good solvent in its liquid state for the target analyte [32]. Specifically, its polarity index plays an important role in the extraction of biocompounds [31]. Alcoholic solvents can be used to extract phenolic compounds from natural sources because they produce a high yield of total extract. However, they are not highly selective for phenolic compounds. For instance, mixtures of alcohols and water were found to be efficient in the extraction of phenolic compounds than a mono-component solvent system [22]. In this work, electrolyzed water showed to be efficient in the extraction of bioactive compounds, as it reduces the cost with solvent since the cost with electrolyzed water is mainly related to the initial investment for the purchase of equipment acquisition. Another positive aspect is the elimination of organic solvents, which can be harmful to human health and environment.

## 3.2 CENTRAL COMPOSITE DESIGN

In SFE, proportion, pressure and temperature are some important parameters to be evaluated in the process, mainly when compounds of intermediate polarity such as polyphenols

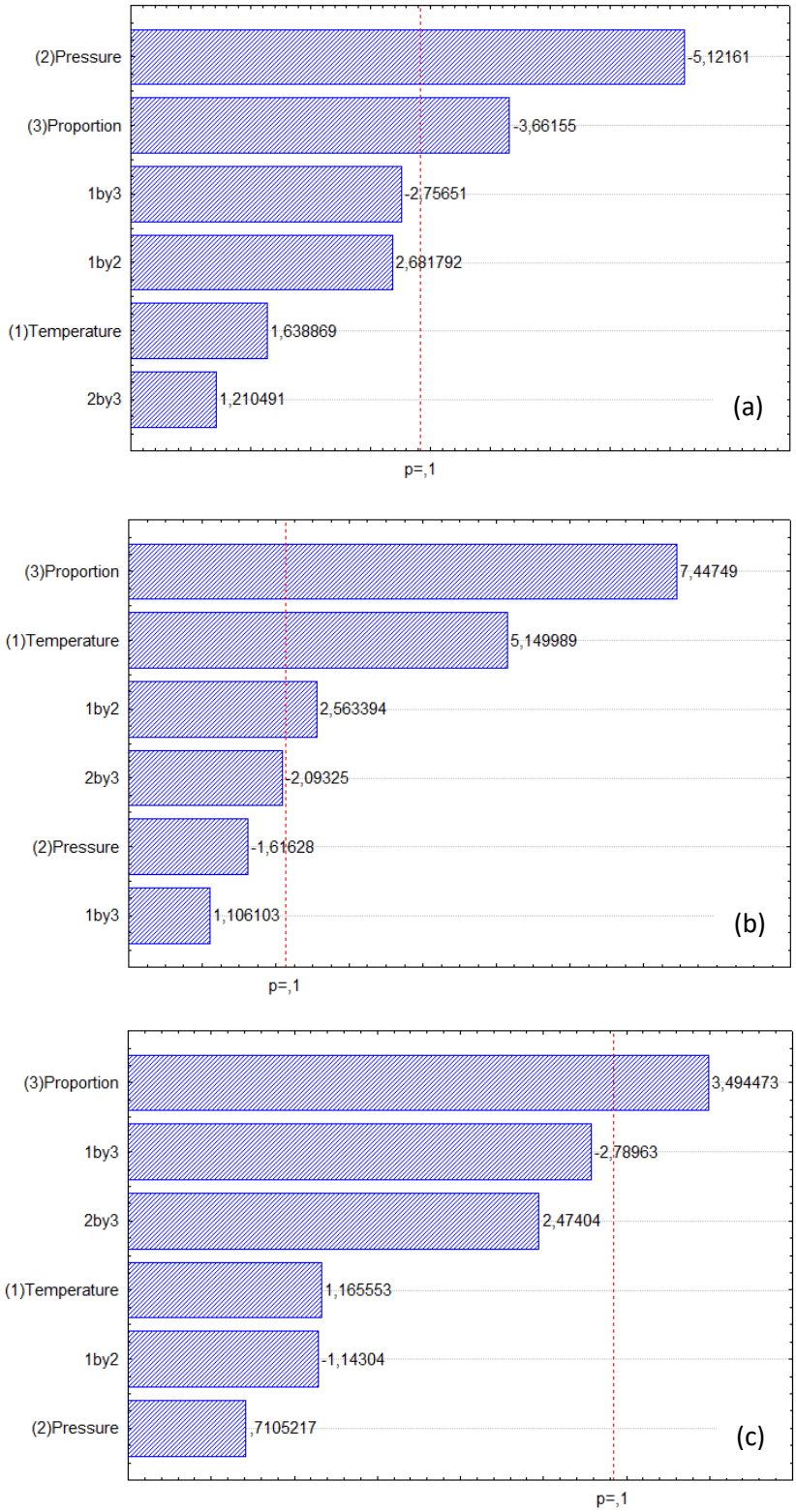
are targeted. In this study, the sample:water ratio was the variable with the highest significance in the experimental design (Figure 2 and Table 1). As higher the solvent:water ratio, higher is the total amount of solids extracted based on mass transfer phenomenon [22].

Table1- Total phenolics (TP), total flavonoids (TF), and antioxidant activity (FRAP) of extracts obtained by SFE.

| Assay | T | P | Sample: cosolvent | SFE-$CO_2$-BEW | | | SFE-$CO_2$-DW | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | TP | TF | FRAP | TP | TF | FRAP |
| **1** | 40 (-1) | 10 (-1) | 1:20 (-1) | 4221.62 | 1243.29 | 588.26 | 4092.34 | 836.07 | 563.48 |
| **2** | 80 (1) | 10 (-1) | 1:20 (-1) | 4324.32 | 1271.53 | 630.56 | 4288.28 | 1089.02 | 688.41 |
| **3** | 40 (-1) | 20 (1) | 1:20 (-1) | 4764.86 | 932.71 | 552.15 | 4259.00 | 687.08 | 557.58 |
| **4** | 80 (1) | 20 (1) | 1:20 (-1) | 4608.11 | 1182.12 | 687.64 | 4040.54 | 926.45 | 613.62 |
| **5** | 40 (-1) | 10 (-1) | 1:40 (1) | 5344.59 | 1150.59 | 783.43 | 4344.59 | 989.80 | 658.94 |
| **6** | 80 (1) | 10 (-1) | 1:40 (1) | 5067.57 | 1056.47 | 854.85 | 4668.91 | 527.05 | 639.64 |
| **7** | 40 (-1) | 20 (1) | 1:40 (1) | 4594.59 | 968.24 | 635.78 | 4925.67 | 1085.88 | 749.65 |
| **8** | 80 (1) | 20 (1) | 1:40 (1) | 4925.68 | 991.76 | 839.41 | 4736.48 | 891.76 | 694.64 |
| **9** | 60 (0) | 15 (0) | 1:30 (0) | 4201.47 | 939.04 | 712.07 | 4149.60 | 785.21 | 696.45 |
| **10** | 60 (0) | 15 (0) | 1:30 (0) | 4967.24 | 988.95 | 741.25 | 4337.29 | 803.74 | 637.98 |
| **11** | 60 (0) | 15 (0) | 1:30 (0) | 4414.41 | 1028.16 | 746.98 | 4060.87 | 827.45 | 643.21 |

SFE-$CO_2$-BEW: Supercritical fluid extraction with $CO_2$ and basic electrolyzed water as co-solvent; SFE-$CO_2$-DW: Supercritical fluid extraction with $CO_2$ and deionized water as co-solvent; T: Temperature (°C); P: Pressure (MPa); Sample: cosolvent ratio (w/v); TP: Total phenolics (mg GAE (100 g)$^{-1}$); TF: Total flavonoids (mg EQ (100 g)$^{-1}$); FRAP: Ferric Reducing Antioxidant Power (µmol TEAC (100 g)$^{-1}$).

Figure 2 - Pareto chart for identifying the influences of variables of SFE-$CO_2$-BEW on the flavonoid (a) and antioxidant activity (b), and of SFE-$CO_2$-DW on the antioxidant activity (c).




SFE-$CO_2$-BEW: Supercritical fluid extraction with $CO_2$ and basic electrolyzed water as co-solvent; SFE-$CO_2$-DW: Supercritical fluid extraction with $CO_2$ and deionized water as co-solvent;

According to the results of SFE-$CO_2$-BEW, the highest phenolic compounds content (5344.59 mg GAE. (100 g) $^{-1}$) was obtained at 40 °C, 10 MPa and sample: water ratio of 1:40 (w/v) (assay 5). The temperature is explained due to the possibility of degradation of these compounds as they are unstable at the extraction temperature above 60 ° C [35]. The highest flavonoids content (1271.53 mg EQ (100 g) $^{-1}$) was obtained at 80 °C, 10 MPa and sample:water ratio of 1:20 (w/v) (assay 2). For FRAP, the best antioxidant activity (854.85 μmol TEAC (100 g) $^{-1}$) was obtained at 80 °C, 10 MPa and sample:water ratio 1:40 (w/v) (assay 6).

For the three responses evaluated in this study (phenolics, flavonoids and FRAP), the best results were observed at pressure of 10 MPa. This behavior is explained by Wang et al. [32], where the fluid density can be very sensitive to temperatures near the critical pressure of the system. An increase in pressure tends to increase the fluid density, which modifies the solute solubility. The use of lower pressure (10 MPa) in SFE-$CO_2$-BEW extraction is positive, considering the need for less energy in the process.

In assays 5 and 6 (Table 1), the phenolic compounds decreased in higher temperatures. The increase of temperature improved the extraction by increasing both the solubility of the solutes and the diffusion coefficient. However, this increase can degrade some phenolic compounds [22, 36]. Significant effects of pressure and temperature in the optimization of SFE of total phenolics of grape skin was also presented in the scientific literature. This behavior is because the combination of pressure and temperature influences the solvation power of $CO_2$. The optimal conditions were identified at 45 °C and 16 MPa [37], and it was similar to the findings presented in this study.

In the results of SFE-$CO_2$-DW (Table 1), the highest content of phenolic compounds (4925.67 mg GAE (100 g) $^{-1}$) was extracted at 40°C, 20 MPa and sample: water ratio of 1:40 (w/v). In such case, a higher pressure (20 MPa) was required to obtain the phenolics while in BEW the best result was obtained with lower pressure (10 MPa). In the evaluation of flavonoids, the best result (1089.02 mg EQ (100 g) $^{-1}$) was obtained at 80 °C, 10 MPa and a sample:water ratio of 1:20 (w/v), which were the same conditions observed for extraction using BEW. The antioxidant activity was higher (749.65 μmol TEAC (100 g) $^{-1}$) in lower temperature (40 °C) and higher pressure (20 MPa) and same sample:water ratio of 1:40 (w/v).

Table 2 shows the analysis of variance (ANOVA) for phenolics, flavonoids and antioxidant activity (FRAP) of tangerine extracts using SFE-$CO_2$-BEW and SFE-$CO_2$-DW. The calculated F was higher than the critical F value for total flavonoids and FRAP both in SFE-$CO_2$-BEW as SFE-$CO_2$-DW.

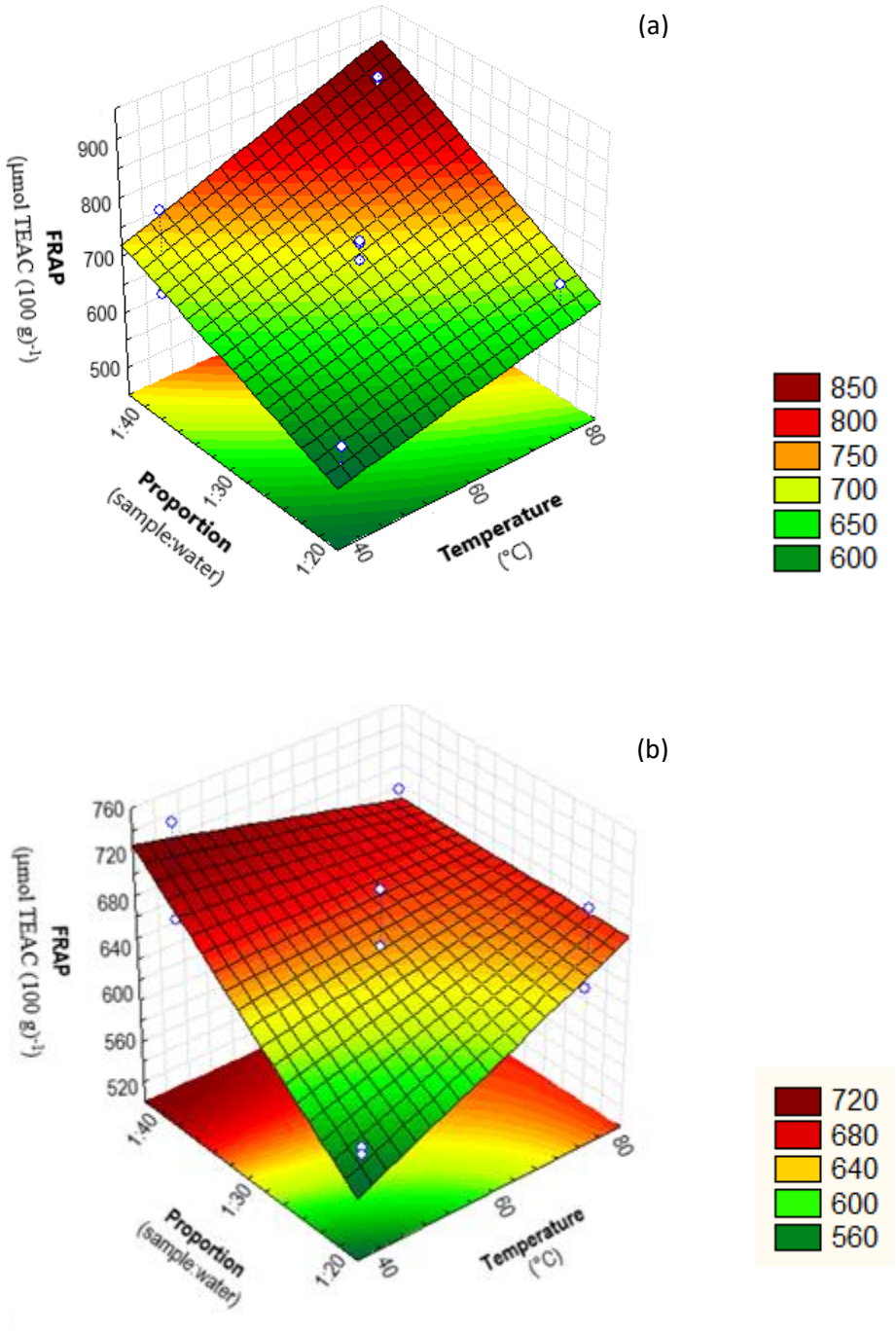
Table 2- Analysis of variance (ANOVA) for phenolics, flavonoids and antioxidant activity of *Citrus reticulata* extracts using SFE-$CO_2$-BEW and SFE-$CO_2$-DW.

| | | | SS | df | MS | $F_{cal}$ | $R^2$ |
|---|---|---|---|---|---|---|---|
| **SEF-$CO_2$-BEW** | Total phenolics | | | | | | |
| | | Regression | 893301 | 6 | 148883.5 | 1.19 | 0,64 |
| | | Residual | 497080 | 4 | 124270.0 | | |
| | | Total SS | 1390381 | 10 | | | |
| | Total flavonoids | | | | | | |
| | | Regression | 116874 | 6 | 116874 | 13.83 | 0.77 |
| | | Residual | 33787.3 | 4 | 8446.82 | | |
| | | Total SS | 150661.1 | 10 | | | |
| | FRAP | | | | | | |
| | | Regression | 93531 | 6 | 15588.5 | 16.13 | 0.96 |
| | | Residual | 3865.9 | 4 | 966.47 | | |
| | | Total SS | 97396.68 | 10 | | | |
| **SEF-$CO_2$-DW** | Total phenolics | | | | | | |
| | | Regression | 716896.7 | 6 | 119482.7 | 2.92 | 0,81 |
| | | Residual | 163500.4 | 4 | 40875.1 | | |
| | | Total SS | 880397.1 | 10 | | | |
| | Total flavonoids | | | | | | |
| | | Regression | 254188 | 6 | 42364.66 | 7.46 | 0.91 |
| | | Residual | 22693.9 | 4 | 5673.47 | | |
| | | Total SS | 276882.2 | 10 | | | |
| | FRAP | | | | | | |
| | | Regression | 30654 | 6 | 5109 | 7.78 | 0.92 |
| | | Residual | 2626.9 | 4 | 656.7 | | |
| | | Total SS | 33280.57 | 10 | | | |

$F_{tab}$:6;4;0.1=4.01; SFE-$CO_2$-BEW: Supercritical fluid extraction with $CO_2$ and basic electrolyzed water as co-solvent; SFE-$CO_2$-DW: Supercritical fluid extraction with $CO_2$ and deionized water as co-solvent; SS – Sum of Sequential Squares; Df – Degrees of freedom; MS – Sequential Middle Squares; Fcal – Test Statistics; $R^2$ – Determination coefficient.

In the same trend, the interaction of temperature and proportion variables on the antioxidant capacity of extracts obtained by SFE-$CO_2$-BEW and SFE-$CO_2$-DW is presented (Figure 3).

Figure 3 - Response surface of antioxidant activity (FRAP) for extractes obtained by SFE-$CO_2$-BEW (a) and SFE-$CO_2$-DW (b).




SFE-$CO_2$-BEW: Supercritical fluid extraction with $CO_2$ and basic electrolyzed water as co-solvent; SFE-$CO_2$-DW: Supercritical fluid extraction with $CO_2$ and deionized water as co-solvent;

The higher the proportion of sample: water and higher values temperatures of extraction (in most of the cases; one exception is for DW with sample: water ratio of 1:40 (w/v); Figure 3b) favor obtaining higher values of antioxidant activity in both extractions. The absolute values (up to 854.85 μmol TEAC $(100\ g)^{-1}$) are higher when using electrolyzed water. The antioxidant capacity is determined not only by concentration, but also by several other factors, such as reactivity to radicals, and the distribution, location and destination of radicals derived from antioxidants. These factors must be evaluated and considered separately (Yeddes et al., 2013). Most of the results of SFE using BEW as extraction medium were higher than the results obtained by SFE with deionized water. Also, lower pressure was needed when using BEW, thus demonstrating the efficiency and viability in using electrolyzed water as a promising cosolvent in high-pressure technology.

## CONCLUSION

Green methodologies such as UAE, SFE and UAE + SFE were evaluated using different types of electrolyzed water as extraction media for obtaining bioactive compounds from tangerine peels. The SFE presented better results of phenolic compounds, flavonoids compounds and antioxidant activity when using basic electrolyzed water. As a way forward and according to the Central Composite Rotatable Design, the condition that provided the best results on an integrated evaluation was temperature for 40 °C, pressure 100 bar and proportion 1:40 which the results were 5344.59 mg GAE $(100\ g)^{-1}$ for total phenolic and temperature for 80 °C, pressure 100 bar and proportion 1:20 for flavonoids (1271.53 mg EQ $(100\ g)^{-1}$).

As a promising result, the use of basic electrolyzed water showed better results of bioactive compounds compared to deionized water. For instance, the phenolics and flavonoids contents could be increased by approximately 10 % and 15 %, respectively. Therefore, SFE-$CO_2$-BEW extraction was feasible and efficient in the extraction of bioactive compounds from tangerine peels, which can encourage further studies in this area using other raw materials.

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## 5 RESULTADOS E DISCUSSÃO GERAL

A variedade de bergamota *Citrus reticulata* apresentou maior teor de fenólicos totais e flavonoides totais, nas extrações convencionais, do que as variedades *Citrus deliciosa* e *Citrus deliciosa* Blanco. Com isso, esta variedade foi escolhida para a aplicação das metodologias não convencionais na extração de compostos bioativos desta matéria-prima.

As tecnologias não convencionais testadas foram a extração assistida por ultrassom (EAU), extração com fluido supercrítico e água eletrolisada ácida (AEA), básica (AEB), levemente ácida (AELA) e neutra (AEN), como solvente ou co-solvente (EFS). Na tabela 6 estão apresentados os resultados mais significativos das técnicas utilizadas na extração de compostos bioativos de *Citrus reticulata.*

Tabela 6 – Fenólicos totais, Flavonoides totais e atividade antioxidante (FRAP) de *Citrus reticulata* usando diferentes metodologias de extração.

| **Ensaio** | **Extração** | **Fenólicos totais** (mg GAE (100 g)$^{-1}$) | **Flavonoides totais** (mg EQ (100 g)$^{-1}$) | **FRAP** (µmol TEAC (100 g)$^{-1}$) |
|---|---|---|---|---|
| 1 | Controle+AEA | 3067,56 | 499,61 | 515,60 |
| 2 | Controle+AEB | 3155,40 | 393,73 | 570,84 |
| 3 | Controle+AELA | 3047,29 | 393,73 | 532,91 |
| 4 | EC+Etanol 80% | 2783,33 | 282,35 | 457,48 |
| 5 | EC+AEA | 3489,86 | 483,33 | 639,68 |
| 6 | EC+AEB | 3681,31 | 571,57 | 606,12 |
| 7 | EC+AEN | 3630,63 | 517,65 | 633,02 |
| 8 | EAU+AEA | 3905,40 | 691,76 | 650,71 |
| 9 | EAU+AEB | 4094,59 | 621,17 | 649,71 |
| 10 | EAU+AELA | 4324,32 | 597,64 | 663,69 |
| 11 | EFS-$CO_2$+ AEA | 4520,27 | 815,95 | 762,35 |
| 12 | EFS-$CO_2$+ AEB | 5344,59 | 1271,53 | 854,85 |
| 13 | EFS-$CO_2$+ AELA | 4592,34 | 757,12 | 770,44 |
| 14 | EAU+EFS-$CO_2$+ AEA | 4245,50 | 818,56 | 762,35 |
| 15 | EAU+EFS-$CO_2$+AEB | 4981,98 | 916,60 | 848,21 |
| 16 | EAU+EFS-$CO_2$+ AELA | 4681,98 | 876,08 | 770,44 |
| 17 | EFS-$CO_2$+ AD | 4925,67 | 1089,02 | 749,65 |

AEA – Água eletrolisada ácida; AEB – Água eletrolisada básica; AELA – Água eletrolisada levemente ácida; AEN – Água eletrolisada neutra; EC – Extração convencional; EAU – Extração assistida por ultrassom; EFS-$CO_2$ – Extração com fluido supercrítico usando $CO_2$; EAU+EFS-$CO_2$ – Extração assistida por ultrassom combinada com extração com fluido supercrítico usando $CO_2$.

Fonte: Autor, (2019).

Observando as extrações convencionais (EC), ensaios 4, 5, 6 e 7, mostrados na Tabela 6, os resultados de compostos fenólicos, flavonoides e atividade antioxidante dos extratos obtidos com água eletrolisada (AEA, AEB e AELA) foram superiores aos encontrados com solvente hidroalcoólico 80 %, sugerindo a ação positiva das três águas eletrolisadas como soluções extrativas de compostos bioativos de casca de *Citrus reticulata*.

Para fenólicos totais, a EC+AEB obteve 25 % a mais deste composto do que a EC+etanol 80 %, este resultado pode ser relacionado ao pH alcalino. Este fato também foi evidenciado por Wei e Yang, (2015) onde o uso de AEB na infusão de chá aumentaria a capacidade antioxidante, sendo esta identificada com potencial para eliminar espécies de oxigênio, explicado pelo seu pH alcalino. Para flavonoides, EC+AEB extraiu 50% a mais do que EC+etanol 80 %. Já a atividade antioxidante (FRAP) a EC+AEA foi superior a EC+etanol 80 % em 13 %.

De acordo com o delineamento composto central rotacional o rendimento da extração assistida por ultrassom usando água eletrolisada como solvente foi maior com o aumento da intensidade, independente dos valores do ciclo, devido ao aumento da energia do ultrassom, resultante do aumento da transferência de massa. Foram definidas as variáveis intensidade (75,11 $W.cm^{-2}$) e pulso de ciclo (0,57) ótimas para a extração de fenólicos totais e flavonoides totais de casca de *Citrus reticulata*.

A EAU+AEA extraiu as maiores quantidades de compostos flavonoides (691.76 mg EQ $(100g)^{-1}$). Enquanto a EAU+AELA apresentou melhores resultados na extração de compostos fenólicos (4324.32 mg GAE $(100g)^{-1}$) e FRAP (663.69 µmol TEAC $(100g)^{-1}$).

A EAU+AEA que apresentou os melhores resultados na extração de flavonoides, tanto no artigo 3 quanto no artigo 4, sugerindo que o uso de ultrassom modifica as propriedades do solvente, além de uma melhor extração, devido a melhor penetração deste na matriz vegetal, em função dos poros criados pelas cavitações das ondas ultrassônicas (BAHMANI et al., 2018). O pH baixo tende a destruir os compostos da parede celular (por exemplo polissacarídeos) aumentando a permeabilidade. Um ORP circundante mais alto pode perturbar a distribuição de íons nas superfícies das células interna e externa, o que leva ao rompimento de envelopes celulares e arraste de componentes intracelulares (DING; LIAO, 2019).

As técnicas verdes de extração, EAU, EFS-$CO_2$ e a combinação destas EAU+EFS-$CO_2$ apresentaram valores superiores ao tratamento controle. Observando-se 40% de aumento para fenólicos, 58 % para flavonoides e 33 % para FRAP na EFS-$CO_2$-AEB em relação ao controle. Este resultado é promissor, tanto para o ambiente como para a saúde humana, devido a eliminação de solventes tóxicos e redução no uso de energia para o processo de extração. Esse

resultado está relacionado ao mecanismo de ação das técnicas que se caracterizam na melhoria da penetração dos solventes na matriz vegetal, tanto pelos poros causados pelas cavitações formadas, pelas ondas ultrassonicas, quanto pela mudança nas propriedades dos solventes quando atingidas a pressão e temperatura supercrítica.

Os maiores valores de fenólicos, flavonoides e atividade antioxidante foram observados para os extratos obtidos por EFS e EAU+EFS-$CO_2$, que não apresentaram diferença significativa (valor de $p < 0,05$) entre esses dois métodos. Portanto, nesse caso, apenas a tecnologia supercrítica seria suficiente, economizando tempo, trabalho e energia. De fato, os resultados positivos da EFS são explicados pela baixa viscosidade do solvente, o que facilita sua difusão na matriz sólida e a baixa tensão superficial, permitindo sua rápida penetração do solvente no sólido e, assim, aumentando a eficiência da extração (WANG, 2008).

Avaliando a resposta dos três tipos de água eletrolisada, na comparação de técnicas de extração, a AEB apresentou os melhores resultados de fenólicos e flavonoides, na extração controle (ensaio 2; 3155,40 mg GAE (100 g) $^{-1}$ e 393,73 mg EQ (100 g) $^{-1}$), EC (ensaio 6; 3681,31 mg GAE (100 g) $^{-1}$ e 571,57 mg EQ (100 g) $^{-1}$), SFE-$CO_2$ (ensaios 12; 5344,59 mg GAE (100 g) $^{-1}$ e 1271,53 mg EQ (100 g) $^{-1}$) e EAU+EFS-$CO_2$ (ensaios 15; 4981,98 mg GAE (100 g) $^{-1}$ e 916,60 mg EQ (100 g) $^{-1}$). O bom desempenho do AEB foi evidenciado e relacionado por danificar as paredes celulares, proporcionando melhor penetração de solventes e solubilizando compostos bioativos pelo arraste com o extrato. As propriedades básicas, incluindo a concentração de ACC (cloro disponível), nas formas $Cl_2$, $^{-}OCl$ e HOCl, pH e ORP influenciam diretamente na sua eficácia (HANAOKA et al., 2004).

De acordo com o planejamento de experimentos aplicado para a EFS-$CO_2$ e AEB como co-solvente foi observado uma variação de 12%, 51% e 12% nos valores de fenólicos totais, flavonoides totais e FRAP, quando modificadas as variáveis proporção de amostra:co-solvente, temperatura e pressão. Quanto maior a razão amostra:co-solvente, maior é a quantidade de sólidos totais extraídos com base no fenômeno de transferência de massa.

Com base nas três respostas avaliadas neste estudo (fenólicos, flavonóides e FRAP), os melhores resultados foram observados na pressão de 10 MPa. O uso de menor pressão (10 MPa) na extração de EFS-$CO_2$-AEB foi positivo, considerando a necessidade de menor energia para o processo.

Analisando as técnicas não convencionais utilizadas neste estudo, observou-se melhores resultados comparados a técnica convencional. Além disso, o método convencional é demorado (2 h; 200 rpm). A combinação de técnicas verdes e água eletrolisada reduziu o tempo de processo. Na EAU e água eletrolisada a redução foi de 87,5%, sabendo que o tempo do processo

foi de 15 min. Na EFS-$CO_2$ e água eletrolisada como co-solvente, a redução foi de 75% visto que o tempo de processo foi de 30 min.

Portanto, a água eletrolisada poderia ser empregada como solução extratora, sendo que já é considerada um aditivo para alimentos nos Estados Unidos e Japão (XUAN et al., 2017). Além disso não tem nenhuma influência negativa na questão sensorial e na qualidade dos alimentos no uso tanto de AEA como de AEB (DING; OH; LIU, 2019).

A medodologia de extração usando $CO_2$ como fluido supercrítico e AEB como co-solvente foi a melhor técnica para a extração de fenólicos totais (5344,59 mg GAE $(100\ g)^{-1}$) e flavonoides totais (1271,53 mg EQ $(100\ g)^{-1}$), bem como foi o extrato de maior atividade antioxidante, pelo método FRAP (854,85 μmol TEAC $(100\ g)^{-1}$), de casca de tangerina (*Citrus reticulata*), mostrando ser uma técnica inovadora e aplicavel na indústria de alimentos.

Entretanto, estudos adicionais devem ser realizados para identificação e quantificação de todos os componentes do extrato de tangeria, através de cromatografia líquida de alta eficiência, bem como encapsular estes extratos em produtos alimentícios, visando proteção à oxidação lipídica, principalmente em produtos carneos, sabendo da alta capacidade antioxidante que possuem.

Com este estudo, torna-se interessante pesquisas utilizando água eletrolisada ácida, básica, neutra e levemente ácida como solvente ou co-solvente na extração de compostos bioativos de matrizes vegetais.

# CONCLUSÃO GERAL

Os resultados deste estudo mostraram a eficácia de aplicar metodologias não convencionais, como a extração assistida por ultrassom, extração com fluido supercrítico e água eletrolisada como solvente ou co-solvente, para extrair compostos bioativos de casca de bergamota.

As farinhas obtidas de casca de bergamota, das variedades *Citrus deliciosa*, *Citrus reticulata* e *Citrus reticulata* Blanco apresentaram características de farinhas de frutas a partir da caracterização físico-química e segurança microbiológica.

A variedade de bergamota *Citrus reticulata* (tangerina) foi a que apresentou maior teor de fenólicos totais variando de 2127,9 mg GAE $(100\,g)^{-1}$ a 5150 mg GAE $(100\,g)^{-1}$ e flavonoides totais de 248,03 mg EQ $(100\,g)^{-1}$ a 941,44 mg EQ $(100\,g)^{-1}$.

A água eletrolisada básica apresentou melhores características na extração convencional de fenólicos e flavonoides de *Citrus reticulata*. Em contrapartida, na extração assistida por ultrassom, a água eletrolisada levemente ácida apresentou maiores valores de fenólicos totais e capacidade antioxidante (FRAP), enquanto a água eletrolisada ácida apresenta melhores resultados de flavonoides totais.

Na extração com fluido supercrítico, a água eletrolisada básica apresentou maiores teores de fenólicos, flavonoides e capacidade antioxidante (FRAP).

A associação das técnicas verdes, ultrassom, fluido supercrítico, e água eletrolisada, promoveu melhores resultados em comparação com as extrações controle e convencional. O ultrassom, bem como as propriedades supercríticas, unidos com água eletrolisada podem gerar espécies reativas de oxigênio e/ou causar danos às estruturas vegetais, levando a uma melhor extração de compostos bioativos de matrizes vegetais devido a penetração do solvente na amostra, melhorando a solubilização e extração dos compostos bioativos da casca de *Citrus reticulata*.

A extração com fluido supercrítico apresentou melhores resultados do que a extração assistida por ultrassom. A combinação, ultrassom+fluido supercrítico, não apresentou diferença significativa da extração com fluido supercrítico, sendo esta opção descartada pelo maior gasto de energia e tempo de processo.

A técnica de extração utilizando $CO_2$ como fluido supercrítico e água eletrolisada básica como co-solvente apresentou melhores resultados do que a técnica utilizando água deionizada, comprovando o progresso das técnicas quando combinadas com água eletrolisada.

No delineamento composto central aplicado na metodologia EFS+$CO_2$+AEB as variáveis que influenciaram significativamente ($p< 0,05$) foram a pressão e razão amostra:co-solvente, para a extração de flavonoides e a razão e temperatura na capacidade antioxidante (FRAP).

Com isso, apresentamos uma técnica inovadora e sustentável na extração de compostos bioativos de casca de bergamota (EFS+$CO_2$+AEB), com redução de 75 % no tempo de processo comparando com a extração convencional. O uso de resíduos de frutas, aliado a eliminação de solventes e redução de energia durante o processo de extração é “amigável” para o meio ambiente e saúde humana. A utilização destes extratos como atitivo alimentar poderia colaborar na redução da utilização de aditivos sintéticos, os quais muitas vezes são relacionados a efeitos mutagênicos e carcinogênicos.

# TRABALHOS FUTUROS

Como trabalho futuro sugere-se a caracterização do extrato obtido através de cromatografia líquida e gasosa, e posterior aplicação em produtos alimentícios com intuito de comprovar a atividade antioxidante do extrato. A nanoencapsulação também pode ser aplicada para obtenção de extratos mais resistentes e potentes.

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