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Extração enzimática em cascas de uva: processo sustentável para obtenção de corante antociânico

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EXTRAÇÃO ENZIMÁTICA EM CASCAS DE UVA: PROCESSO SUSTENTÁVEL PARA OBTENÇÃO DE CORANTE ANTOCIÂNICO

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“Não há emoção.
Há apenas a
quietude, Não há
pensamentos. Há
apenas o silêncio.
Não há ignorância.
Há apenas a
atenção. Não há
divisão.
Há apenas a
percepção.
Não há ego,
Há apenas a Força.”

(Código Jedi)

RESUMO

A cada ano a indústria de vinhos descarta uma alta quantidade de resíduos líquidos e sólidos, constituído em grande parte por bagaço de uva. Este resíduo pode ser considerado um subproduto da indústria e apresenta quantidades significativas de antocianinas, as quais podem apresentar diversas aplicações. Apesar da extração de antocianinas ser um passo importante na recuperação de pigmentos em matrizes vegetais não existe um método de extração padrão na literatura. Dessa forma, existem estudos tanto em relação ao uso de métodos tradicionais quanto emergentes, sendo estes últimos normalmente mais favoráveis ao Meio Ambiente. Neste estudo foi usado o método de extração enzimática, técnica emergente, que se baseia em alterações da parede celular de matrizes alimentares para exposição dos materiais intracelulares. Assim, o objetivo geral do trabalho foi realizar a extração enzimática de antocianinas presentes em casca de uva para posterior aplicação como corante alimentício em quefir e bebida carbonatada. Foram encontrados diferentes temperaturas e porcentagens de preparado enzimático ótimos dependendo do cultivar analisado. Após aperfeiçoamento foi selecionado o uso de casca de Cabernet Sauvignon, a uma temperatura de 40 ºC e 0,25 % de Pectinex Ultra Color®. O corante natural produzido foi aplicado em bebida carbonatada e quefir, ambos sob análises de tempo de meia-vida de antocianinas e parâmetros físico-químicos durante 16 dias de armazenagem. Dentre os resultados obtidos foi destacado que o quefir com adição do corante manteve características semelhantes ao encontrado na literatura para quefir natural. Em análises em bebida carbonatada, houve maior estabilidade de antocianinas nas amostras armazenadas sem presença de luz. A aplicação do extrato antociânico foi favorável em ambas matrizes alimentares, sendo recomendado estudos futuros visando o aumento do tempo de meia vida da estabilidade das antocianinas.

Palavras-chave: Antocianinas; pigmentos; planejamento experimental; enzimas; bebida carbonatada; quefir.

ABSTRACT

Every year, wine industry discards a high amount of liquid and solid waste, consisting largely of grape pomace. This residue can be considered a by-product of the industry and presents significant quantity of anthocyanins, which can provide several applications. Despite the extraction of anthocyanins be an important step on the recovery of pigments in vegetables, there is not a standard extraction method in the literature. In this way, there are studies regarding the use of traditional and emerging methods, which the latter usually being more environmentally friendly. In this study, controlled enzymatic extraction method was used, an emerging technique that is based on alterations of the cell wall of alimentary matrices for exposure of the intracellular materials. Thus, the aim of this study was to perform the enzymatic extraction of anthocyanins present in grape skins for subsequent application as a food dye in kefir and carbonated beverage. Eight different samples of grape pomace were used to improvement the extraction process. Preliminary responses of the study defined that low extraction times are required for the enzymatic extraction process, where the maximum ones being found when the process was conducted for thirty minutes using Pectinex Ultra Color®. In addition, different temperatures and percentages of enzyme preparation were found depending on the variety analyzed. After improvement, the use of Carbenet Sauvignon skin was selected, at a temperature of 40 ° C and 0.25% of enzymatic preparation. The natural dye produced was applied in carbonated beverage and kefir, both under analysis of the half-life of anthocyanins and physical- chemical parameters during 16 days of storage. Among the results obtained, it was pointed out that kefir with dye addition maintained similar characteristics to that found in the literature for natural kefir. In analyzes in carbonated beverage, there was greater stability of anthocyanins when the samples were stored without light. The application of the anthocyanin extract was favorable in both food matrices, and future studies are recommended aiming to increase the half-life of the stability of anthocyanins

Keywords: Anthocyanins; pigments; experimental planning; enzymes; carbonated water; kefir.

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CAPÍTULO 1 - INTRODUÇÃO

De acordo com a Organização Internacional da Uva e do Vinho (OIV, 2016) foi obtido uma produção mundial de vinhos em aproximadamente 274 milhões de hectolitros anuais, sendo a plantação de uvas uma das mais importantes atividades agroindustriais mundiais. Visto esta intensa produção, a cada ano a indústria descarta uma alta quantidade de resíduos líquidos e sólidos, constituído em grande parte por bagaço de uva (engaço, cascas e sementes). Este composto corresponde a 25% do peso bruto da uva e possui um alto teor de polifénois (GUERRAND, 2001; TORRES et al., 2002).

Dentro os compostos presentes são destacadas as antocianinas da casca, devido a possibilidade de aplicação com sucesso em diversos estudos. Estes estudos abordam principalmente as características de coloração ligadas a valores de acidez e pH do meio (ABE et al., 2007; BOULTON, 2001; FRANCIS, 2000; GARCÍA-LOMILLO; GONZÁLEZ-SANJOSÉ, 2017; JARA-PALACIOS et al., 2015; KAMMERER et al., 2014; LIANG et al., 2011; MAIER et al., 2009; SATO et al., 2001; TOALDO et al.,2013). Estas produzem pigmentos solúveis em água que podem adquirir tons de vermelho, roxo e azul (DAMODARAN SRINIVASAN, 2008).

Contudo, para obtenção das antocianinas a extração representa uma etapa importante na recuperação desses pigmentos a partir de matrizes vegetais, sendo que não existe um método de extração padrão na literatura **(**FONTANA; ANTONIOLLI; BOTTINI, 2013). Desse modo existem estudos tanto em relação ao uso de métodos tradicionais quanto emergentes/não convencionais (BAIANO, 2014). Geralmente as técnicas não convencionais são favoráveis ao meio ambiente e representam uma alternativa em relação às extrações à base de solventes (WANG; WELLER, 2006).

A extração enzimática baseia-se na quebra ou enfraquecimento da parede celular e exposição dos materiais intracelulares, para propiciar a degradação parcial dos polissacarídeos e liberação dos compostos bioativos (BHANJA et al., 2008; LI et al., 2006; ROBLEDO et al., 2008). Tal método vai ao encontro com a tendência da sustentabilidade atual, devido ao gradual aumento na

conscientização dos consumidores quanto aos produtos e serviços adquiridos e suas ações em relação ao Meio Ambiente (DA SILVA, 2015; PANDEY; SOCCOL; MITCHELL, 2000; ROSENTHAL et al., 2001; WANG; DONG; TONG, 2013; WU et al., 2014).

Dessa forma é essencial para melhoria do uso desse método de extração a procura de parâmetros economicamente viáveis. De um modo geral, as pesquisas se focam no uso de porcentagens mínimas de preparado enzimático, baixas temperaturas de extração e tempos curtos de extração (MAIER et al., 2008; PANDEY; SOCCOL; MITCHELL, 2000; WANG; DONG; TONG, 2013; WU et al., 2014).

Tais corantes naturais atóxicos podem ser utilizados para adição em cereais, produtos lácteos e cárneos, entre outros (GARCÍA-LOMILLO; GONZÁLEZ-SANJOSÉ, 2017). Vale destacar que para esses tipos de aplicações é importante o estudo de diversos fatores que podem afetar a estabilidade das antocianinas ao longo do armazenamento como por exemplo, temperatura e presença de luz (DE ROSSO; MERCADANTE, 2007; DELGADO-VARGAS; JIMENEZ; PAREDES-LOPEZ, 2000; KONCZAK; ZHANG, 2004; MARKAKIS, 2012).

A aplicação de corantes provenientes de extrações com uso de solventes orgânicos ou uso do bagaço puro seco já é comum em artigos de alto impacto. Porém, pouco se encontra na literatura sobre a aplicação com uso da extração enzimática em casca de uva, o que evidencia necessidade de maiores pesquisas em relação a este método para produção de corante natural.

1. Objetivos

1.1. Objetivo geral

O objetivo geral desse trabalho foi realizar a extração enzimática de antocianinas presentes em casca de uva para posterior aplicação como corante alimentício em quefir e bebida carbonatada.

1.2. Objetivos Específicos

Dentro esse contexto os objetivos específicos foram:

1. Avaliar a ação de diferentes tempos e porcentagens de preparados enzimáticos para obtenção de um extrato aquoso rico em antocianinas;
2. Qualificar e quantificar antocianinas presentes em diferentes variedades de cascas de uvas;
3. Caracterizar aspectos físico-químicos das cascas de uvas do estudo;
4. Analisar extratos selecionados sob aspectos de porcentuais de recuperação de antocianinas individuais e totais e características físico-químicas;
5. Selecionar melhor processo de extração enzimática compostos antociânicos e potencializar resposta de casca que apresentar melhor custo benefício;
6. Aplicar extrato antociânico aquoso em quefir e bebida carbonatada como corante alimentício natural;
7. Determinar qualidades físico-químicas dos alimentos adicionados com corante natural ao longo do tempo de armazenagem;
8. Determinar estabilidade de antocianinas individuais e totais nos alimentos adicionados com corante natural ao longo do tempo de armazenagem.

Este trabalho está organizado na forma de capítulos. O **Capítulo 2** apresenta a fundamentação teórica dos assuntos abordados ao longo da presente dissertação. No **Capítulo 3** estão descritos os procedimentos empregados para a realização do trabalho, na forma de Materiais e Métodos. Os três artigos produzidos a partir dos resultados obtidos estão apresentados no **Capítulo 4**, e o **Capítulo 5** compreende uma discussão geral do trabalho realizado, assim como as conclusões obtidas a partir do mesmo.

CAPÍTULO 2 - REVISÃO BIBLIOGRÁFICA

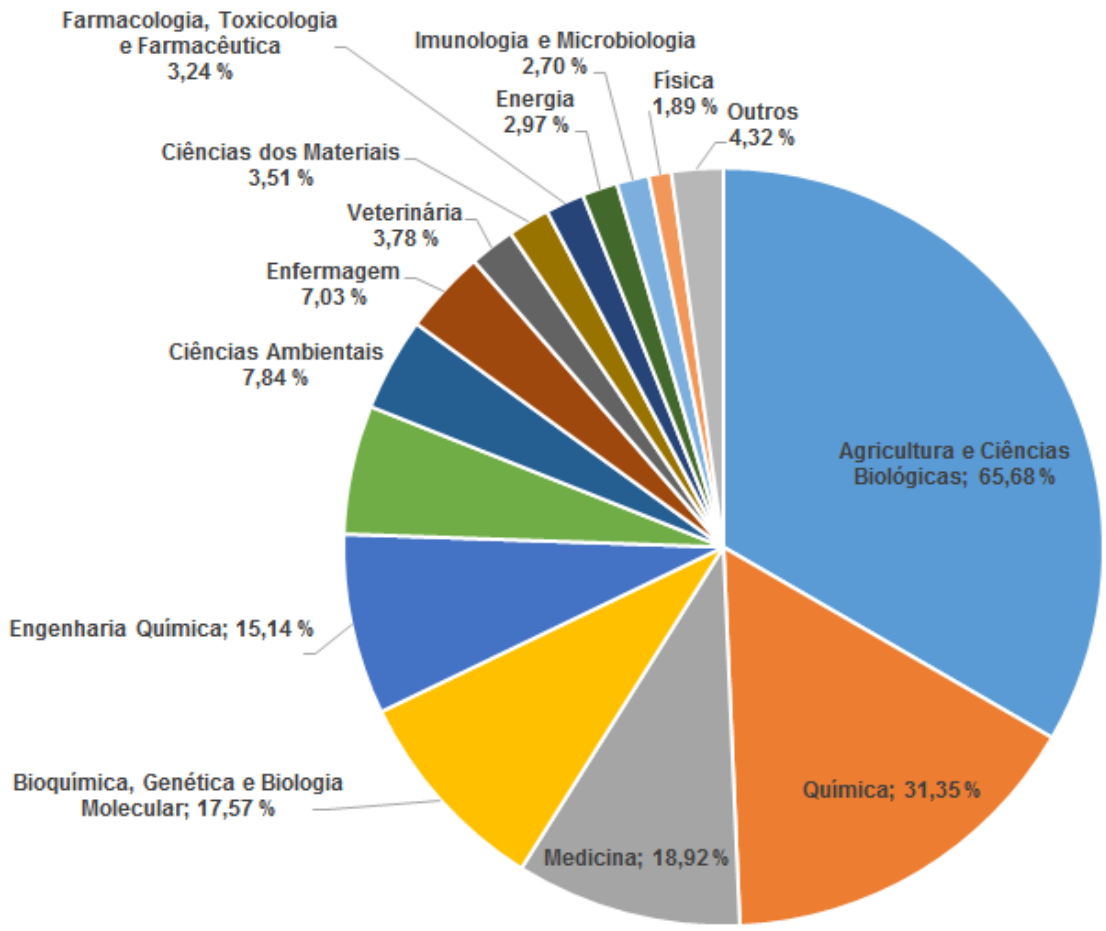
2.1. Resíduos da indústria vitivinícola

A produção de vinhos e sucos de uva implica na geração de altas quantidades de resíduos, sendo o bagaço um dos principais (MUSEE; LORENZEN; ALDRICH, 2007). Este é constituído por sementes, cascas e engaço, é comumente adicionado como ingrediente em fertilizantes e rações animais. Porém, existem impasses em relação a estas aplicações, uma vez que o alto teor de compostos fenólicos presentes nestes inibem a germinação das sementes e reduzem a capacidade de digestibilidade dos animais ruminanentes (BAYRAK, 2013; MUSEE; LORENZEN; ALDRICH, 2007; TORRES et al., 2002).

Entretanto, os mesmos compostos bioativos presentes no bagaço que dificultam as atuais utilizações, possuem propriedades de atividade antioxidante, antimicrobiana, antiviral e anti-inflamatória para a saúde humana (BIESALSKI et al., 2009; CABRITA; RICARDO-DA-SILVA; LAUREANO, 2003; FONTANA; ANTONIOLLI; BOTTINI, 2013;).

Nos últimos cinco anos houve um aumento de mais de 200% em estudos envolvendo o bagaço de uva. A recuperação dos compostos presentes nesse resíduo demostrou ser preferencialmente pesquisado pelas áreas de ciências biológicas e agrícolas (65,68%) com estudos aplicados em produtos alimentícios (SCOPUS, 2017) (Figura 1).

Figura 1 - Áreas de pesquisa envolvendo bagaço de uva



Fonte: Adaptado Scopus (2017)

2.1.1. Características do bagaço de uva

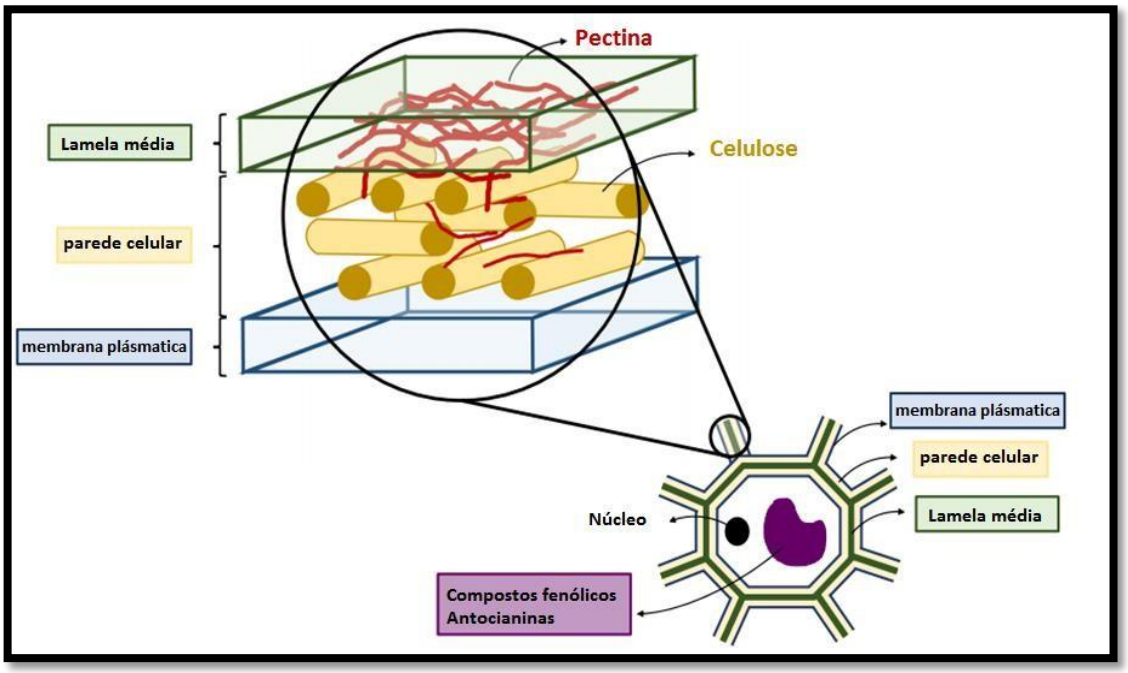
A caracterização do bagaço de uva é essencial para aprimorar o entendimento sobre o potencial de obtenção de extratos como fonte de ingredientes funcionais em alimentos (BUSTAMANTE et al., 2008). Este resíduo é constituído de diversas partes da videira, sendo os principais engaços, cascas e sementes. A uva é botanicamente classificada como angiosperma e compartilha as mesmas características das paredes celulares estruturais das demais dicotiledôneas (CARPITA; GIBEAUT, 1993). Nela, os resíduos insolúveis presentes no bagaço possuem um teor de lignina entre 16% a 24% e, em geral, as substâncias pécticas são o principal constituinte presente nas paredes das células, com valores de 37% a 54%. A celulose também se apresenta em altos teores, em

torno de 27% a 37% (GONZÁLEZ-CENTENO et al., 2010).

Em relação a adição de ingredientes funcionais alimentícios, a casca de uva se destaca entre as porções do bagaço devido a coloração arroxeada empregada como corante natural. Além disso, a alta presença de fibras tem sido empregada na adição de farinhas (LAVELLI et al., 2017; MARCHIANI et al., 2016; SELANI et al., 2011).

A parede celular é a principal camada protetora contra agentes externos em cascas, sendo uma barreira para a difusão de componentes, incluindo aromas e fenóis (DOCO et al., 2003). Essa barreira pode ser subdividida em três camadas sobrepostas. A camada mais exterior, a cutícula, é composta de ácidos hidroxilados chamados de cutina, que é coberta por ceras hidrofóbicas. Posteriormente encontra- se a epiderme intermediária, constituída de uma ou duas camadas, e por fim, a hipoderme, a camada interior. Na figura 2, pode ser observado as principais camadas envolvidas na proteção dos compostos fenólicos da uva.

Figura 2 - Camadas de células protetoras de compostos fenólicos presentes na casca de uva



Fonte: Adaptado de (DAL MAGRO et al., 2016).

Além das características em relação aos compostos bioativos e camadas protetoras, existem outros aspectos do bagaço da uva que merecem destaque para estudo dos métodos de extração e quantificação das antocianinas. Entre eles é relevante destacar a umidade, os sólidos solúveis e o teor de cinzas (DENG; PENNER; ZHAO, 2011; KOSHITA et al., 2011; YU; AHMEDNA, 2013).

O parâmetro umidade depende do cultivar e seu estado de maturação, de modo a afetar diretamente a quantidade de alguns compostos bioativos presentes. Em estudo de González-Centeno et al. (2010), foram analisados dez cultivares diferentes da espécie *Vitis vinífera* e a porcentagem de umidade do bagaço de uva variou entre 50 % e 72%. Enquanto que as diferenças em relação ao sólidos solúveis totais são associados diretamente a presença de diversos tipos de antocianinas e consequentemente afetam a coloração característica do cultivar (KOSHITA et al., 2011).

García-Lomillo e González-Sanjosé (2017) citaram em revisão diversos parâmetros e características considerados importantes em bagaços de uva para determinar abordagens em aplicações alimentares. O conteúdo de minerais,relacionada a cinzas, foi destacado entre os parâmetros devido ao seu potencial de agregação com as camadas protetoras presentes e consequente aumento de rigidez das mesmas.

Vale destacar que no presente estudo foram analisados tanto bagaço provenientes de *Vitis vinifera* quanto de *Vitis labrusca*. No Brasil cultivares *Vitis labrusca* são mais produzidas que *Vitis viníferas* originárias da região do Mediterrâneo. As cultivares *V. labrusca* são comumente conhecidas como uvas americanas e de maneira geral possuem maior resistência a pragas e maiores teores totais de antocianinas, sendo grande parte usada para produção de sucos ou consumo *in natura*. Por outro lado, as *V. vinifera* são variedades para produção de vinhos, principalmente na Europa. Portanto, ambas variedades são de interesse do presente estudo, pois geram alta quantidade de resíduos dentro da indústria apesar de terem finalidades e características muitas vezes diferentes (CAMARGO; MAIA, 2005; CREASY; CREASY, 2016; RIBEIRO et al., 2015).

2.2. Compostos bioativos presentes na casca de uva

Os compostos bioativos provêm de metabólitos secundários de origem vegetal e são caracterizados por uma grande variedade em sua estrutura química (BIESALSKI et al., 2009; CABRITA; RICARDO-DA-SILVA; LAUREANO, 2003; FONTANA; ANTONIOLLI; BOTTINI, 2013; JIMENEZ-GARCIA; TORRES-PACHECO; , ANDRES CRUZ-HERNANDEZ, 2013). Dentre os grupos presentes na casca de uva estão os fenóis, e os principais representantes dessa classe são compostos simples e de baixo peso molecular, com apenas um anel aromático (CROZIER; JAGANATH; CLIFFORD, 2009). Estes componentes são subdivididos em flavonoides e não-flavonoides, sendo que na uva são encontrados principalmente os flavonoides (antocianinas e flavonóis), os estilbenos (como o resveratrol), os ácidos fenólicos (derivados dos ácidos cinâmicos e benzoicos) e uma larga variedade de taninos (FONTANA; ANTONIOLLI; BOTTINI, 2013).

As cascas da maioria das cultivares de uva são uma fonte promissora de compostos fenólicos de baixo custo. Entretanto, a recuperação de polifenóis apresentam diferentes rendimentos pelas diversas tecnologias empregadas. Portanto, considera-se obrigatório o estabelecimento de processos que sejam rentáveis na obtenção de polifenóis (KAMMERER et al., 2004).

Além disso, as quantidades dos compostos fenólicos na casca de uva podem variar conforme fatores intrínsecos e extrínsecos de acordo com tipo de cultivar, *terroir*, grau de maturação, espécie, clima, estado sanitário das plantas, solo e as práticas de cultivo (HANLIN et al., 2011; JORDÃO; CORREIRA; GONÇALVES, 2015; MORENO-PÉREZ et al., 2013; RAPISARDA et al., 1999; TOMÁS-BARBERÁN; ESPÍN, 2001).

As antocianinas presentes na casca de uva merecem destaque devido a sua aplicação com sucesso em diversos estudos, tendo características de coloração ligadas a valores de acidez e pH do meio (ABE et al., 2007; BOULTON, 2001; FRANCIS, 2000; GARCÍA-LOMILLO; GONZÁLEZ-SANJOSÉ, 2017; JARA-PALACIOS et al., 2015; KAMMERER et al., 2014; LIANG et al., 2011; MAIER et al., 2009; SATO et al., 2001; TOALDO et al., 2013).

2.2.1 Antocianinas presentes na casca de uva

As quantidades e a distribuição de diferentes antocianinas na uva dependem prioritariamente do cultivar e de forma geral, as variedades *Vitis labrusca* possuem maiores valores de antocianinas totais quando comparadas com variedades *Vitis vinifera* (NIXDORF; HERMOSÍN-GUTIÉRREZ, 2010; RIBEIRO et al., 2015). De forma
geral as antocianinas mais encontradas em uva são malvidina, cianidina, delfinidina, peonidina e petunidina, todas glicosiladas (LORRAIN et al., 2013).

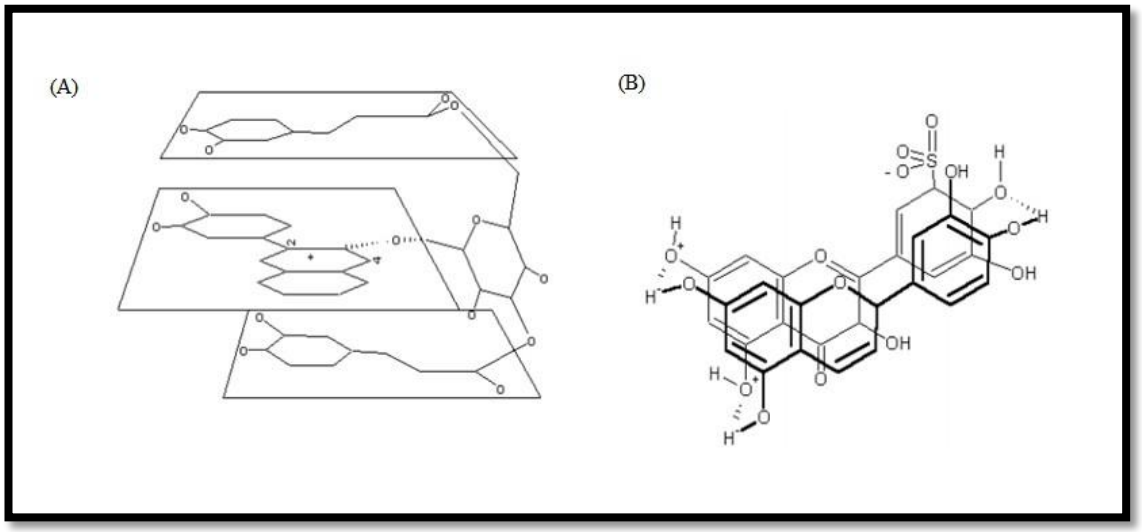
A composição entre os cultivares, como por exemplo entre Ives e Isabel (ambas cultivares de *Vitis labrusca*), prevalecem por fatores genéticos e dificilmente diferem de ano para ano. Dessa forma, diferentes extrações do mesmo cultivar tendem a possuir perfis homogêneos de antocianinas (MORENO-PÉREZ et al., 2013).

Apesar de parte das antocianinas serem extraídas durante a vinificação e produção de suco de uva, extratos de cascas de bagaço apresentam características adequadas e quantidades apreciáveis para desenvolvimento de produtos nutracêuticos (KY; TEISSEDRE, 2015; POMAR; NOVO; MASA, 2005). Um exemplo é o uso de extratos antociânicos de casca de uvas em conjunto com soluções de soro de leite e caseína. Esta interação de componentes impede de forma significativa a perda de cor e degradação das antocianinas (HE et al., 2016).

Ainda é comum a análise da eficácia das extrações com uso apenas dos valores de antocianinas totais encontrada na matéria bruta (FONTANA; ANTONIOLLI; BOTTINI, 2013). Porém, isso pode causar algumas perdas de dados importantes, relacionados principalmente a possibilidade de copigmentações durante o processamento. Esse processo de condensação das antocianinas (tanto co- pigmentações intermoleculares e intramoleculares), geram complexos que são mais estáveis durante o armazenamento e processamento (DAMODARAN SRINIVASAN, 2008) como apresentada na figura 3. Além disso, a presença ou ausência de certas antocianinas individuais, associadas a formas glicosiladas ou agliconas, tem forte influência nas propriedades de coloração (BAUTISTA-ORTÍN et al., 2016; CLIFF; KING; SCHLOSSER, 2007). Dessa forma, nesse estudo foi focado não apenas os

valores totais de antocianinas, mas também características de eficiência de recuperação individuais de cada uma.

Figura 3- Modelos hipotéticos de copigmentação intramolecular (A) e intermolecular (B)



Fonte: Adaptado de Brouillard (1983) e Iacobucci & Sweeny, (1983)

As antocianinas das cascas de uva produzem pigmentos solúveis em água que podem adquirir tons de vermelho, roxo e azul, sendo de interesse das indústrias de corantes alimentícios para obtenção de produtos atraentes, naturais e saudáveis (DAMODARAN SRINIVASAN, 2008; ZHANG; BUTELLI; MARTIN, 2014).

Estudos, tanto com antocianinas purificadas ou extratos ricos em antocianinas em sistemas experimentais *in vitro*, confirmaram o potencial destes compostos como corantes naturais. No entanto, é importante controlar fatores limitantes para sua degradação, como instabilidade a exposição de variações ambientais, incluindo a temperatura, intensidade de luz e oxigênio (DELGADO-VARGAS; JIMENEZ; PAREDES-LOPEZ, 2000; KONCZAK; ZHANG, 2004; MARKAKIS, 2012).

Além das propriedades de coloração desses componentes da casca de uva, estudos têm sugerido que as antocianinas possuem propriedades anti-inflamatórias, anti-carcinogênicas, prevenção de doenças cardiovasculares,

controle de peso e de diabetes, o que incentiva seu estudo em produtos alimentícios (KONCZAK; ZHANG, 2004; MARKAKIS, 2012).

2.3. Métodos para produção de extratos antociânicos

Apesar da extração ser um passo importante na recuperação das antocianinas em matrizes vegetais, não existe um método de extração padrão na literatura (FONTANA; ANTONIOLLI; BOTTINI, 2013). Com isso, a *European Comission* (2012) incentiva a procura de método padrão ambientalmente sustentável dentro da indústria de alimentos, com a necessidade de explorar possíveis bioprodutos antes de descartá-los como resíduos. Atualmente existem estudos tanto no uso de métodos tradicionais como emergentes/não convencionais tratados a seguir (BAIANO, 2014).

2.3.1. Métodos com uso de solventes orgânicos

As extrações que utilizam solventes orgânicos são as técnicas mais difundidas em escala industrial, e entre estas se destaca o uso de Soxhlet. Esse método atualmente é considerado convencional e principal referência para avaliar o desempenho de outros métodos convencionais, principalmente as extrações sólido-líquido (BARBA et al., 2016; WANG; WELLER, 2006). Essas técnicas são as mais difundidas atualmente e tem uso de diferentes solventes orgânicos. Na extração de compostos bioativos em casca de uva os meios mais comuns são acetona ou etanol. Esta permite que os componentes solúveis sejam removidos, de forma individual ou misturadas entre si. A escolha dos solventes deve ser feita com cuidado, a fim de eliminar ou minimizar as interferências da matriz (BARBA et al., 2016; LUTHRIA, 2008; PROESTOS; KOMAITIS, 2008).

Como exemplos de pesquisas que estudam o uso desses métodos para produção de corantes com uso de aproveitamento do bagaço de uva podemos citar pesquisa de (BAAKA et al., 2015) que visa o uso de hidróxido de sódio e diferentes temperaturas com finalidade de aplicar em corante em indústrias têxtil. Dentro da área de alimentos destaca-se pesquisa de (LAVELLI et al., 2017), na qual o bagaço de uva passou por uma extração com uso de etanol e altas

temperaturas para produção de um corante com finalidade de aplicação em purê de maça.

Por fim, de forma geral, o restante dos métodos tradicionais se baseiam no processo de quebra da parede celular pelo alto aquecimento, o que consome alta energia de processamento e podem degradar compostos termolábeis, como as antocianinas (BARBA et al., 2016). Dessa forma, as atuais pesquisas de técnicas emergentes procuram substituir estes métodos por utilização de solventes mais baratos e sem uso de produtos tóxicos como forma de combinação de técnicas de extração mais brandas no aspecto ambiental.

2.3.2. Métodos emergentes

Em contrapartida das extrações convencionais citadas, estão sendo investigadas tecnologias inovadoras nos métodos de extração de compostos bioativos. Abaixo os principais métodos emergentes encontrados na literatura (quadro 1).

Quadro 1 - Principais tecnologias emergentes na extração de compostos bioativos

Método emergente	Modo de ação
Extração com uso de campo elétrico pulsado	Tratamento aplicado entre dois eletrodos a temperatura branda por um segundo, causando eletroporação
Extração com uso de descargas elétricas de alta tensão	Tratamento com uso de eletrodo de agulha de alta tensão para causar ruptura celular
Extração com uso de aquecimento ôhmico pulsado	Tratamento com aumento de temperatura por movimentos ôhmicos. Usado após extração com uso de campo elétrico pulsado

(continuação)

(conclusão)

Método emergente	Modo de ação
Extração assistida por ultrassom	Tratamento que causa transferência de calor e massa por meio das paredes celulares das plantas pela ação de ondas mecânicas de baixa frequência às quais resultam na cavitação
Extração assistida por micro-ondas	Tratamento com uso de energia eletromagnética, na faixa de frequência de 300 MHz-300 GHz
Extração por fluído supercrítico	Tratamento em que o fluído supercrítico permite uma rápida transferência de massa na fase supercrítica sendo mais comum é uso de CO_2 como fluído
Extração por alta pressão	Tratamento utiliza solventes convencionais a temperaturas elevadas (100-180 ° C) e pressões elevadas (1500-2000 psi)
Extração acelerada por solvente (água quente pressurizada)	Tratamento com uso de água em condições acima de 100°C e 0,1 MPa para extração de compostos
Extração enzimática	Tratamento com uso de enzimas para causar enfraquecimento das paredes celulares das plantas

Fonte: Adaptado de BARBA et al. (2015); FONTANA; ANTONIOLLI; BOTTINI (2013); WANG; WELLER (2006).

As técnicas não convencionais buscam ser mais favoráveis ao meio ambiente e representam possíveis alternativas com enfoque em não utilizar solventes orgânicos tóxicos. Porém alguns desses métodos precisam de equipamentos sofisticados, exigem alto gasto de energia, e não estão plenamente desenvolvidos e otimizados para uso em escala industrial (WANG; WELLER,

2006).

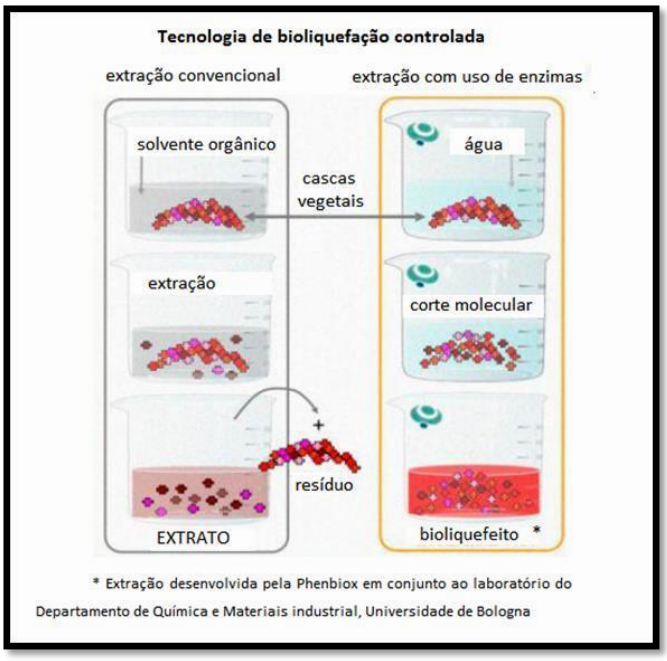
Dentre as extrações citadas tomou-se como vantagem para pesquisa estudar a extração enzimática, visto que a utilização de preparados enzimáticos para extração de compostos bioativos já é comum dentro da indústria vitivinícola, o que facilitaria sua adaptação para escala industrial.

2.3.2.1. Extração aquosa com uso de enzimas

A extração enzimática é baseada na lise ou enfraquecimento da parede celular que provoca a exposição dos compostos bioativos com uso de enzimas degradantes como as celulases, hemicelulases e pectinases, entre outras (BHANJA et al., 2008; LI et al., 2006; PINELO; ARNOUS; MEYER, 2006; ROBLEDO et al., 2008; ROSENTHAL et al., 2001).

A extração pode ocorrer em estado sólido ou em meio aquoso e não prevê a utilização de solventes químicos, sendo comum o uso de água como meio da reação. Esse mecanismo preserva propriedades bioativas e poupa gastos posteriores de energia e tempo para descarte dos resíduos gerados, o que é demostrado na figura 3 (WANG; DONG; TONG, 2013).

Figura 4 - Método de extração enzimática em comparação com extração com uso de solvente orgânico



Fonte: Adaptado de ZANICHELLI; SETTI, (2015).

Rodríguez-Morgado et al., (2014) basearam-se no uso desse método para produção de um extrato solúvel em água a partir de bagaço de uva. Além de evitar o uso de reagentes tóxicos, foi obtido um alto teor de conteúdos polifenólicos (12%) no extrato, dos quais cerca de 70% são flavonoides e 30% são ácidos fenólicos. A partir dos resultados os autores propõem o uso desse extrato antociânico do bagaço de uva como um componente natural cuja implementação na alimentação pode proporcionar benefícios à saúde.

Alguns impasses já foram citados no processo de extração enzimática e podem torná-la antieconômica. Entre esses fatores estão o perfil hidrolítico, o tempo necessário para a extração, a concentração da enzima, o pré-tratamento da semente, o pH do meio, o tempo de incubação, a temperatura e o tipo de vegetal do estudo (DENG; PYLE; NIRANJAN, 1992; MARTÍNEZ-MAQUEDA et al., 2013).

Tendo em vista os impasses e a busca de maior eficácia e menor custo nos métodos de extração, Fernández, Vega, & Aspé (2015) analisaram o uso desse tipo de extração em cascas de uva do cultivar *País*. No estudo foram utilizadas três enzimas (pectinase, celulase e tanase) e suas misturas sequencias como avaliação do aumento na liberação de compostos bioativos. O teste das misturas sequenciais, além do uso individual da enzima, foi baseado no fato da parede vegetal do bagaço conter celulose e pectina, que devem ser degradadas antes da ação da tanase. Porém no caso desse estudo, não houve diferenças significativas entre o uso de mistura ou sua forma individual, tornando mais viável economicamente o uso apenas de uma enzima a 1% em relação ao substrato. Dessa forma é essencial para melhoria do uso desse método de extração a procura de parâmetros com custo/benefício, sendo o uso de porcentagens mínimas de preparado enzimático, baixas temperaturas de extração para evitar degradação das antocianinas presentes na casca e tempos curtos de extração com objetivo de evitar gastos demasiados de energia. (MAIER et al., 2008; PANDEY; SOCCOL; MITCHELL, 2000; WANG; DONG; TONG, 2013; WU et al., 2014).

2.4. Aplicação de corantes de resíduos vitivinícolas em quefir e bebida carbonatada

A extração incompleta das antocianinas presentes na casca de uva durante a prensagem e produção do mosto gera um expansivo recurso de compostos fenólicos que podem ser usados na área de cosméticos, farmacêutica e indústria de alimentos (FONTANA; ANTONIOLLI; BOTTINI, 2013b). Devido ao interesse dos consumidores por alimentos cada vez mais saudáveis e o aumento da consciência ambiental, existem diversas pesquisas que visam a produção de corantes naturais no mercado alimentício (CORTEZ et al., 2017; GOULA; THYMIATIS; KADERIDES, 2017). Esses corantes e seus compostos fenólicos podem ser utilizados para adição em cereais, produtos lácteos e cárneos, entre outros (GARCÍA-LOMILLO; GONZÁLEZ-SANJOSÉ, 2017).

Dentre estas matrizes alimentares, os produtos a base de leite. O quefir, analisado nesse estudo, é um produto fermentado que possui um sabor ligeiramente ácido (favorável a estabilidade de antocianinas), consistência uniforme e contém uma pequena quantidade de álcool etílico em sua composição, sendo comum na ingestão da população adulta (MAGALHÃES et al., 2011).

Recentemente He et al. (2016) comprovaram que a adição de soluções de proteínas de soro de leite e a caseína em extratos de casca de uva impedem significativamente a perda de cor e a degradação das antocianinas presentes no mesmo. Dessa forma, a adição do corante em estudo em junção a um produto lácteo pode vir aumentar a estabilidade das antocianinas e consequentemente prolongar a coloração característica no produto final.

Além dos produtos lácteos, as bebidas carbonatadas também podem ser adicionados de corantes naturais. O interessante em relação a esse tipo de bebida é que a maior parte destes produtos encontrados no mercado contêm corantes artificiais e isso gera uma crítica pela população, devido sua ligação com doenças como diabetes, déficit de atenção e hiperatividade da população mais jovem (ASHURST, 2016; DE ROSSO; MERCADANTE, 2007; MCCANN et al., 2007).

Lavelli et al.(2017) por meio de pesquisa acerca a aplicação sustentável de

casca de uva em bebidas citam que, apesar do aumento do interesse pela população e o potencial de aplicação dessa prática, há necessidade de maiores pesquisas para que sejam superadas barreiras econômicas e regulamentares.

Dessa forma, vale destacar que para esses tipos de aplicações é importante o estudo de diversos fatores que podem afetar a estabilidade das antocianinas ao longo do tempo de armazenagem, como por exemplo, temperatura e presença de luz (DE ROSSO; MERCADANTE, 2007; DELGADO-VARGAS; JIMENEZ; PAREDES-LOPEZ, 2000; KONCZAK; ZHANG, 2004a; MARKAKIS, 2012a).

2.4.1. Aplicação em matrizes alimentares de pigmentos antociânicos a partir de extração enzimática

A aplicação de corante provenientes de extrações com uso de solventes orgânicos ou uso do bagaço inteiro seco já é comum em artigos de alto impacto. Porém, pouco se encontra na literatura sobre a aplicação com uso da extração enzimática em casca de uva com finalidade na produção de corantes alimentícios. Muito disso pode ser atribuído ao fator de que essa tecnologia emergente ainda passa por otimização de processo e atualmente o maior enfoque é em estudos de otimização antes de serem avaliados em matrizes alimentares. Como citado no atual livro *Food Waste Recovery: Processing Technologies and Industrial Techniques* (OTLES et al., 2015):

> "O uso de tecnologias ambientalmente amigáveis levou os pesquisadores e a indústria de alimentos a desenvolver novos processos alternativos que podem extrair compostos valiosos de diferentes fontes e resíduos de alimentos de origem diferente [...]. [Essa] comercialização de compostos com alto valor agregado de recuperação de resíduos alimentares trata de várias questões, tais como pesquisa em laboratório, problemas de escalonamento, proteção de propriedades intelectuais e desenvolvimento de aplicações destinadas ao mercado"

A pesquisa destacada para aplicação de corante semelhante proveniente da metodologia emergente com uso de enzimas em casca de uva é de Maier et al. (2009). Os pesquisadores avaliaram a estabilidade de cor e de compostos fenólicos após processamento e armazenamento de géis de pectina e gelatina suplementado com extrato antociânico de bagaço de uva, que apresentou resultados favoráveis. O tratamento térmico e a luz tiveram efeito significativo na

perda de compostos fenólicos durante o armazenamento. Porém, em contraste com o conteúdo de compostos fenólicos individuais e totais, a atividade antioxidante e a cor da amostra mantiveram- se virtualmente inalteradas ou com pouca perda ao longo desse período. Assim, os géis ainda exibiram cores brilhantes e capacidade antioxidante forte mesmo depois de armazenamento de 24 semanas à temperatura ambiente, demonstrando que os compostos fenólicos do bagaço de uva que utilizam como forma de extração a extração enzimática podem ser aplicados com sucesso para enriquecer alimentos processados.

CAPÍTULO 3 - MATERIAIS E MÉTODOS

Os experimentos foram realizados no Laboratório de Enologia e Bebidas, Laboratório de Compostos Bioativos, Laboratório de Enzimologia e Laboratório de Equipamentos Especiais, do Instituto de Ciência e Tecnologia de Alimentos (ICTA) da Universidade Federal do Rio Grande do Sul.

3.1. Seleção de preparado enzimáticos e tempo de extração para em cascas de uva

Para seleção prévia do preparado enzimático foi utilizado bagaço proveniente da agroindústria Sbardelotto (RS) (29° 33' 6.628" S, 50° 30' 20.477" O), safra 2016. As cascas foram separadas manualmente do bagaço e imediatamente guardadas em freezer (-18°C).

Adaptado de FERNÁNDEZ; VEGA; ASPÉ, (2015), para cada 5,0 g da amostra de casca foram adicionados 25 mL de tampão acetato (pH 4,0) a 50°C. Os diferentes preparados enzimáticos (Pectinex Smash XXL®, Novozym 33095®, Pectinex Ultra SPL® e Pectinex Ultra Color®) foram adicionados em proporções de 1.5% (v/v), calculados a partir do peso úmido da amostra (umidade de 81,89 ± 0,08 %). O meio foi mantido no escuro em shaker a 150 rpm por 60 minutos. Posteriormente, o meio foi resfriado em banho de gelo, prensado e filtrado para retirada da casca. Após esse processo, o extrato obtido foi armazenado ao abrigo de luz e de calor até o momento das análises.

A escolha de 1,5% de preparado enzimático foi baseado ao máximo aconselhado para uso em relação a custo/benefício da enzima e a temperatura de 50°C foi determinada de acordo com a temperatura aconselhada em manual empresa fornecedora (GRAINGER; TATTERSALL, 2005; NOVOZYMES, 2005).

A resposta utilizada para analisar eficiência de recuperação de antocianinas dos diferentes preparados enzimáticos foi o total de antocianinas monoméricas obtidas pela análise de pH-diferencial. Esta análise foi baseada em protocolo de GIUSTI; WROLSTAD, (2001). Foram realizadas leituras em espectrofotômetro a

550 nm e 700 nm (UV-Vis - 3000 PRO, Amersham, Biosciences) com uso de tampões a pH 1,0 e 4,5. Os parâmetros foram representados na equação de Lambert-Beer (Equação 1), a fim de calcular a concentração da amostra diluída, considerando o fator de diluição (D) e o coeficiente de extinção molar (M):

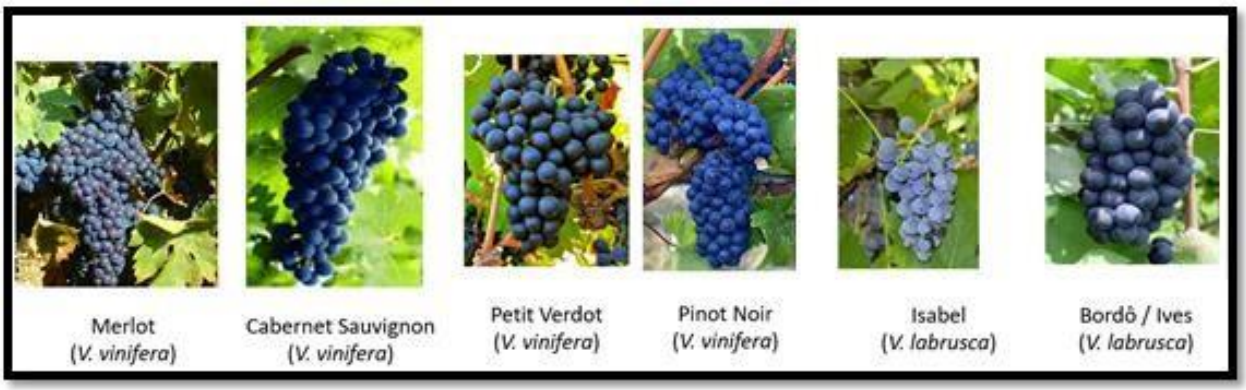
$$C= \{[(A\lambda 520 - A\lambda 700)pH1,0] – [(A\lambda 520 - A\lambda 700)pH4,5]\}*D*F \quad \text{(Equação 1)}$$

A concentração total de antocianinas monoméricas na amostra original foi estimada com base em cianidina-3-glicosídeo. Os resultados foram expressos em g/100g de casca seca. A manipulação das amostras foi realizada sob a luz fraca e temperatura ambiente (25 °C). A partir desta análise se estabeleceu como o preparado enzimático mais favorável para continuidade da pesquisa.

3.2. Caracterização dos bagaços

Os bagaços do estudo foram coletados entre janeiro e março de 2016, no Rio Grande do Sul e Santa Catarina. As cascas foram manualmente separadas e imediatamente mantidas em frezer a -18°C até análise. Imagens de variedades estudadas apresentadas em figura 5.

Figura 5- Variedades *Vitis vinifera* e *Vitis labrusca* estudadas.



Fonte: Autoria própria.

As variedades Merlot, Pinot Noir, Cabernet Sauvignon e Isabel foram cedidas pela vinícola Don Giovanni (Pinto Bandeira, RS) (29° 8' 54.902" S, 51° 26' 23.255" O). O bagaço de Petit Verdot foi doado pela Vinícola Pinheiral

(Major Gercino, SC) (27°25'21.9"S, 49°06'01.6"O). A variedade Ivês veio de três diferentes localidades, sendo que Isabel (1) da Agroindustria Vinhos e Sucos Sbardelotto (Rolante, RS) (29° 33' 6.628" S, 50° 30' 20.477" O), Isabel (2) da Vinícola Buono (Nova Trento, SC) (27°
15' 50.479" S, 48° 56' 58.034" O) e Isabel (3) da Vinícola Pinheiral. Apenas os bagaços de Vinícola Pinheiral são de produção orgânica, sendo o restante de produção convencional.

3.2.1. Caracterização físico-química

As cascas de uva foram caracterizadas pela umidade, acidez total, sólidos solúveis totais e cinzas de acordo com método da *Association of Official Analytical Chemists* (AOAC, 2006). A umidade foi determinada por técnica de gravimetria a 105ºC. A acidez total foi determinada por titulação expressa em porcentagem de ácido tartárico. Os sólidos solúveis totais foram medidos por refratômetro (Model PAL-3, Atago U.S.A. Inc.) e expressos em ºBrix. As cinzas foram determinadas por calcificação em mufla (Model Linn, Elektro therm, Germany) a 550ºC durante 12 horas.

A cor das amostras foi mensurada com uso de colorímetro portátil Konica Minolta Model CR 400, Sensing. Singapore Pte, Ltd. USA) e os parâmetros de cor foram obtidos de acordo com os sistema CIELAB. Os valores de L* (luminosidade) e as coordenadas a* (vermelho-verde) e b*(amarelo-azul) foram determinados após calibração de disco branco (L0*: 94,97;a0*: 0,12; b0*: 1,70).

3.2.2. Caracterização de antocianinas por cromatografia líquida de alta eficiência (CLAE)

A quantificação e qualificação das antocianinas ocorreu por análise em cromatografia de alta eficiência (CLAE) por meio de injeção do extrato exaustivo. Para a obtenção do extrato exaustivo foi preparado a partir da adição a 0,25g da amostra de casca úmida em metanol acidificado (1% HCl). A casca úmida era adicionada a 25mL de metanol, homogeneizada e em seguida filtrada a vácuo. O processo se repetiu até perda total da coloração do filtrado.

Para análises em CLAE, foi adaptada metodologia de Zanatta et al.,(2005)

com uso de coluna de fase reversa C18 Shim-park CLC-ODS (5 µm, 250×4,6mm). A fase móvel possuía gradiente linear de eluição com 5% ácido fosfórico aquoso/metanol 85:15 (v/v) a 20:80 por 25 minutos, com proporção isocrática por 15 minutos. O fluxo da fase móvel foi de 1,0 mL/min, com volume de injeção de 5µL numa temperatura de 29°C. Os cromatogramas foram lidos a 520nm.

A identificação e quantificação dos compostos foi feita pela comparação dos tempos de retenção e áreas dos picos da amostra e de seus respectivos padrões, nas mesmas condições cromatográficas. Para quantificar os compostos, foram comparadas a curvas padrões disponíveis que possuíam concentrações entre 5-100 mg/mL (delfinidina-3-glicosídeo); 5-40 mg/mL (cianidina-3-glicosídeo); 6-70 mg/mL (pelargonidina-3-glicosídeo); 5-50 mg/mL (malvidina-3-glicosídeo); 5-100 mg/mL (cianidina aglicona); 3-40 mg/mL (petunidina-3-glicosídeo) e 3-30 mg/mL para malvidina aglicona.

As faixas de concentração utilizadas para obtenção das curvas padrão e os limites de detecção (LD) e quantificação (LQ) de cada composto foram: delfinidina-3- glicosídeo 58,00 e 193,35 µg/g; cianidina-3-glicosídeo 8,45 e 28,25 µg/g;pelargonidina-3-glicosídeo 2,35 e 7,85 µg/g; malvidina-3-glicosídeo 10,35 e 25,60 µg/g; cianidina aglicona 6,20 e 23,30 µg/g; petunidina-3-glicosídeo 3,30 and 5,50 µg/g; malvidina aglicona 4,70 e 14,15 µg/g.

Os padrões foram adquiridos da Sigma-Aldrich (USA), sendo delfinidina-3-glicosídeo (CAS 528-53-50 ≥95,0 %), cianidina-3-glicosídeo (CAS 7084-24-4 ≥95,0 %), pelargonidina-3-glicosídeo (CAS 18466-51-8 ≥95,0 %), malvidina-3-glicosídeo (CAS 7228-78-6, ≥90,0%), cianidina aglicona (CAS 528-58-5 ≥95,0 %), petunidina-3- glicosídeo (CAS 6988-81-4 ≥95,0 %) e malvidina aglicona (CAS 63-84-5≥95,0 %).

3.3. Extração enzimática

Após a seleção do preparado enzimático citado no item (3.1.) deste capítulo, foram avaliadas as variáveis temperatura (°C) e porcentagem de enzima selecionada de acordo com um planejamento experimental completo 2^2 com três pontos centrais, conforme Tabela 2.

Tabela 1 - Fatores independentes e níveis do planejamento fatorial 2^2 com três pontos centrais

Fatores independentes	Níveis		
	-1	0	+1
Temperatura (°C) (x1)	40	45	50
Porcentagem de preparado enzimático (%E/S) (x2)	0,25	0,75	1,25

Fonte : Autoria própria

Retomando a metodologia adaptada de Fernández; Vega; Aspé, (2015), para cada 5,0 g da amostra de casca úmida das 8 amostras de bagaço de uva foram adicionados 25 mL de tampão acetato (pH 4,0) e adicionado diferentes porcentagens de preparado enzimático de acordo com tratamentos estabelecidos. Após adição, os meios permaneceram sob agitação no escuro em shaker (150 rpm) por 30 minutos. Posteriormente, o tratamento foi resfriado em banho de gelo, prensado e o extrato obtido armazenado ao abrigo de luz e de calor até o momento das análises.

As respostas do planejamento experimental foram analisadas por valores de antocianinas monoméricas totais encontradas pela análise de pH-diferencial com uso de modelos matemáticos, incluindo resposta linear e de interação. Para definir as condições mais favoráveis de extração, para cada uma das oito amostras foi selecionado o parâmetro com maior resposta em relação a extração de antocianinas monoméricas totais presente na casca, e o extrato selecionado foi analisado em CLAE. Os resultados obtidos no cromatograma foram comparados à extração exaustiva para determinar nível de eficiência da extração com uso de enzimas.

3.4. Caracterização físico-química dos extratos obtidos

Após definição de temperatura e porcentagem de preparado enzimático para

cada variedade de bagaço de uva os extratos, além de análise por CLAE, passaram por caracterização de aspectos físico-químicos. Estes foram acidez total, sólidos solúveis totais e cor pelo método da AOAC (2006) já descrito anteriormente. O pH do extrato foi medido a 25°C por potenciômetro (Digimed, Brazil).

3.5. Aperfeiçoamento da extração enzimática com casca selecionada

Para aperfeiçoamento da extração enzimática com casca selecionada pelo planejamento experimental e análises de CLAE, foi fixado o valor de temperatura ótima encontrado e selecionados dois valores de porcentagem de preparado enzimático acima e abaixo do definido como ótimo, tendo dessa forma o total de cinco tratamentos para análise.A resposta dos cincos tratamentos em relação as antocianinas monoméricas encontradas (análise de pH-diferencial) foram plotados para construção de gráfico porcentagem de preparado enzimático (eixo x) por valor total de antocianinas monoméricas no extrato (eixo y). Dessa forma, foi possível determinar os fatores que determinaram o ponto máximo para extração de antocianinas. O ponto máximo de extração foi analisado em CLAE e comparado com ponto selecionado por meio do planejamento experimental para análise de diferenças significativas entre valores individuais e totais de antocianinas encontrados.

3.6. Aplicação do corante natural de bagaço de uva em quefir e bebida carbonatada

O extrato selecionado foi liofilizado para concentração de coloração com perda de umidade de 44% em aplicação para quefir e 42% para bebida carbonatada.

3.6.1. Quefir

O quefir foi preparado utilizando leite homogeneizado pasteurizado (3% de gordura láctea, Santa Clara, Brasil). Os grãos de quefir foram adicionados ao leite numa proporção de 1:10, incubados no meio aeróbico em incubadora tipo B.O.D. (Modelo SP-500, SPLABOR) durante 24 horas a 25ºC ± 2ºC (primeira fermentação). Posteriormente, os grãos de quefir foram removidos por filtração e

bebida de quefir foi mantida a 5ºC ± 2ºC por mais 24 horas (segunda fermentação) (SIMOVA et al., 2002). Após preparo do quefir o corante produzido foi adicionado. A quantidade inicial de antocianinas adicionadas presentes foi 2,61 ± 0,05 mg/mL no quefir.

As amostras foram separadas em copos de polietileno e armazenadas a 7 °C em geladeira no escuro. A análise de características físico-químicas e análise de retenção de antocianinas ocorreu nos dias 0,2,5,7,9,12,14 e 16.

3.6.2. Bebida carbonatada

A água mineral com gás foi adquirida em comercio local. As características da água (Marca Água de Pedra) são pH 7,33 a 25°C, com composição química em mg/mL cálcio (25,69), sódio (24,12), potássio (0,99), fluoreto (0,23), bicarbonato
(129,2), silício (39,82), magnésio (4,55), cloreto (7,32) e vanádio (0,014). A quantidade inicial de antocianinas adicionadas diretamente em água carbonatada na quantidade de 6,69 ± 0,03 mg/mL em bebida carbonatada.

3.6.3. Análises físico-químicas das matrizes alimentares

As matrizes alimentares foram analisadas por leitura de pH, acidez total e sólidos solúveis totais, baseadas na metodologia de AOAC (2006) já descrita anteriormente. A cor foi mensurada com metodologia citada anteriormente.

3.6.4. Extração das antocianinas remanescentes em quefir e bebida carbonatada ao longo dos dias de armazenamento

As amostras de quefir foram diluídas em metanol acidificado (HCl 0,1%) e passaram por seguidas centrifugações (3500×g, 10 min) (Hitachi, Model CR 21GIII). O processo foi repetido até perda total de cor do pellet. Em seguida o líquido obtido foi rotaevaporado (Fisatom, M802, Brasil) à vácuo, aproximadamente a 40°C e sob proteção de luz até obtenção de 10 mL de extrato para injeção em CLAE.

Baseado em metodologia de Garcia-Falcón; Simal-Gándara (2005),

as amostras de bebida carbonatada foram homogeneizadas e desgaseificadas em banho de ultrassom, e alíquotas diretamente injetadas em CLAE. A metodologia utilizada para leitura em CLAE é a mesma que já citada anteriormente (item 3.2.2.).

3.6.5. Cálculo cinético para valores de tempo de meia-vida

A degradação cinética das antocianinas foi analisada com uso da reação de primeira ordem e parâmetros de cálculo cinético para retenção de antocianinas (%) em relação ao tempo de armazenamento. Baseado em De Rosso e Mercadante (2007), a taxa de degradação constante (kt) foi determinada por meio de curvas de meia-vida ($t_{1/2}$) (Equações 2 e 3).

$$[\text{anthocianina}]=[\text{anthocianina}]^{0}\times\exp(-kobs\times t) \quad (2)$$

$$t_{1/2}=\ln^{2}/kt \quad (3)$$

3.7. Análises estatísticas

Todos os dados foram obtidos em triplicata e os resultados expressos em médias ± desvio padrão. Para análises estatísticas foi usado o software Statistica versão 12.0 (StatSoft Inc.. 2011. Tulsa. OK. USA). Os gráficos foram obtidos com uso do software OriginPro 8.2.

CAPÍTULO 4 – ARTIGOS CIENTIFÍCOS

ARTIGO 1

Artigo em formato da revista Food and Bioprocess Technology

Improvement or effect of temperature and enzymatic-assisted extraction conditions for anthocyanins recovery from *V. vinifera* and *V. labrusca* grape pomaces

ABSTRACT

The incomplete anthocyanins extraction during the industrial processes turns pomace into an inexpensive source of phenolic coumpounds. The aim of this study was to analyze the effects of temperature and enzyme preparation percentage on the extraction of grape pomaces from eight grape varieties. The anthocyanins profile in grape skins and their extracts at the improvement conditions of compounds recovery were explored and have their physicochemical characteristics analysed. A factorial 2^2 design was used to select the maximum conditions for extraction, and the variables temperature, enzyme preparation, and their interaction were assessed. The grape skin characteristics such as ash content and origination affected the anthocyanins content and their yield of recovery, where different improvement conditions were found to distinct grape skins varieties. The anthocyanins extraction from Cabernet Sauvignon, the variety which presented the higher percentual of anthocyanins recovery (over 50%), was improvement. The lower tested

temperature (40°C) and percentage of preparation enzymatic (0.25 E/S%) promoted higher anthocyanins extraction for this variety, resulting in a natural food colorant with 2.67g of anthocyanins/100g db skin grape. The extraction improvement allowed the obtention of a non- toxic natural extract rich in anthocyanins, where the characterization of the aqueous extracts infered its application specially into acidic foods.

Keywords: enzymatic extraction; grape pomace, anthocyanin; food colorant

1. Introduction

According to Organization of Vine and Wine (OIV, 2016) estimates, the global wine production is approximately 274 million hectoliters per year. As a result, grape cultivation represents one of the most important agro-industrial activities.. Every year the wine industry rejects large amounts of pressed grape pomace, which corresponds to approximately 25% of the grape (Guerrand, 2001).

The grape anthocyanins profile depends on the variety of grapevine and is frequently associated with genetic factors. Besides those ratios seems to be related to cultivar and independent of the production area, anthocyanins concentration is strongly influenced by viticultural and environmental factors (Ky and Teissedre 2015; Pomar et al. 2005).

The incomplete anthocyanins extraction from skin to wine during the winemaking process turns wine grape pomace into an inexpensive source of phenolic compounds that can be used in the cosmetic, pharmaceutical, and food industries (Fontana et al. 2013). As a result, various extraction methods are studied for the recovery of these compounds.

More than potential food colorants, anthocyanins are endowed of a potent

antioxidant property that possibily explain the diversal health benefits of their dietary consumption. Studies have suggested that anthocyanins possess anticarcinogenic and anti-inflammatory activity, cardiovascular disease prevention, obesity control, and diabetes alleviation properties (He and Giusti 2010; Konczak and Zhang 2004; Markakis 2012).

Nowadays, consumer environmental and health consciousness has placed pressure on the food industry. The possible link between the consumption of artificial food colorants and diversal adverse health affects stimulate the pursuit of natural color additives. As a result of consumer awareness about diet and health, there is a trend towards clean label ingredients. Consequently, the market share for natural colorants is growing, and could possibly surpass synthetic colorants in market value in the future (Cortez et al. 2017; Goula et al. 2017; McCann et al. 2007).

The European Commision (2012) emphasizes that to enhance eco-sustainability on food industry, it is necessary to explore by-products before they become waste. From these considerations, the investigations of recovery technologies to the obtention of natural food colorants from grape pomace is of great importance. Besides the existence of diversal extraction methods, these techniques are still not fully developed and must be improvement to be successfully applied in industrial processes (L. Wang and Weller 2006).

Among the traditional techniques are the solid-liquid extraction by Soxhlet and the use of solvents (Baiano 2014). Techniques that use solvents usually present several disadvantages like solvent toxicity, time-consuming process and the necessity of correct discard of the used solutions (Proestos and Komaitis 2008). Therefore, non- conventional extraction techniques to obtain food grade

phytochemicals from grape pomace and other wastes could be an environment-friendly alternative to the solvent based extractions.

The enzyme-assisted extraction provides the release of bioactive compounds due to the exposure of intracellular materials, which is caused by enzymes that act causing alterations on the cell wall of plants (Bhanja et al. 2008; Robledo et al. 2008). The main advantages in the use of enzymes, compared to the conventional technologies, is the high yield of extraction with preservation of bioactive properties, and the low energy consumption owing to its reduced extraction period and lower amount of solvent required (Alvarez et al. 2015; Barba et al. 2015; Pandey et al. 2000; S. Wang et al. 2013; Wu et al. 2014).

Enzyme-assisted extraction of anthocyanins from wine grape pomace is a promising technique to the obtention of non-toxic natural colorants, and its success and potential depends on the improvement of the technique. Therefore, the aim of this study was to analyse the effects of temperature and enzyme preparation percentage on the enzyme-assisted extraction of grape pomaces from eight grape varieties. The anthocyanins profile in grape skins and their extracts at the maximum conditions of compounds recovery were explored, and have their physicochemical characteristics analysed to predict its applicability into food.

2. Material and methods

2.1. Grape Skins

The grape pomaces were collected from different wineries and juice industries located in two south Brazilian states (Rio Grande do Sul and Santa Catarina) between January and March 2016. Merlot, Pinot Noir, Cabernet Sauvignon and Isabel pomaces were provided by San Giovanni Winery (29° 8' 54.902" S, 51° 26' 23.255" W). Petit Verdot pomace was provided by Pinheiral

Winery (27°25'21.9"S, 49°06'01.6"W). Ives pomace was provided by 3 different locations: Ives (1) by agroindustry Sbardelotto (29° 33' 6.628" S, 50° 30' 20.477" W); Ives (2) by Buono Winery (27° 15' 50.479" S, 48° 56'58.034" W); Ives (3) by Pinheiral Winery (27°25'21.9"S, 49°06'01.6"W).

Grape Ives (3) was cultivated under organic system, while the other grapes came from conventional systems of production. The skins were manually separated from pomace and immediately stored (-18°C).

The analysed grape pomace was obtained after processing for juice production (*Vitis labrusca*) and must production (*Vitis vinifera*). All samples previuosly treated with enzymes during their processing, except for sample Ives (3).

2.2. Physical-chemical analysys of grape skins

Grape skins were characterized regarding the moisture content, total acidity, total soluble solids and ash content following the Association of Official Analytical Chemists (AOAC) (2006). The moisture was determined by a gravimetric technique at 105 ºC. The total acidity was determined by titration and expressed as percentage of acid tartaric. The total soluble solid (TSS) was measured by a refractometer (Model PAL-3, Atago U.S.A. Inc.) at 25 ◦C. The ash was determined in a muffle furnace (Model Linn, Elektro therm, Germany) at 550ºC.

The color measurements of skins were performed using a portable colorimeter (Konica Minolta Model CR 400, Sensing. Singapore Pte, Ltd. USA) and the colorimetric parameters were obtained according to CIELab system. The value L*(luminosity) and the coordinates a* (red–green component), and b* (yellow–blue component) were determined. All the analyses were performed in triplicate.

2.3. Anthocyanins content in grape skins

The determination of anthocyanins total content in grape skins followed the methodology used where anthocyanins from 0.25 g of skin samples were exhaustively extracted with acidified methanol (1 % HCl). The extracts were filtered and quantified by high performance liquid chromatography (methodology described at 2.7.).

2.4. Enzymatic extraction experimental design

2.4.1. Experimental design: Factorial 2^2

A factorial 2^2 was used to select the improve conditions for anthocyanins extraction. The experiment assessed the variables temperature, enzyme preparation, and their interaction on the recovery of monomeric anthocyanins from eight different grape skins.

For the enzymes preparation, the upper (+), centerpoint (0), and lower (−) level values were 1.25, 0.75 and 0.25 %E/S, respectively. The conditions tested for temperature were 50, 45, and 40 °C for the upper (+), centerpoint (0), and lower (−), respectively. At the centerpoint, treatments were made in triplicate in order to estimate the experimental error. The recovery of anthocyanins was examined by response surface methodology.

The effects and interactions of the independent variables temperature and percentage of enzyme preparation on the anthocyanins extraction were analysed by mathematical models. After the generation of response surface graphs, to define the maximum temperature-enzyme dosage conditions for each grape skin cultivar, the intervals were identified and the treatments with higher results responses on

total monomeric anthocyanins were physic-chemically characterized. Also, heir anthocyanin profile were analysed by High Performance Liquid Chromatography (HPLC). The maximum anthocyanins content of the treatments were compared with the content obtained by exhaustive extraction, and were expressed in percentual of recovery.

2.4.2. Enzymatic extraction of grape skins

The enzyme Pectinex Ultra Color® (10000 PECTU/mL) supplied from Novozymes A/S, was used to obtain anthocyanins rich extracts. The enzyme/substrate ratio (%E/S) was calculated on a wet matter basis.

Based on the adapted methodology of Fernández et al. (2015), for each 5 g of grape skin (wet basis) were added 25 mL of acetate buffer (pH 4.0). The enzyme doses defined on the experimental design were incorporated to the buffer solutions, which were then shaken (150 rpm) under protection from light for 30 minutes in different temperatures conditions.

2.4.3. Total monomeric anthocyanins content analysys

The total monomeric anthocyanins content was used as a variable response of the experimental design, and was determined through pH-differential method (Giusti and Wrolstad 2001). Extracts were diluted in 0.025 M potassium chloride buffer (pH 1.0) and 0.4 M sodium acetate buffer (pH 4.5), and were analysed at 520 nm and 700 nm, respectively, in a spectrophotometer (UV-Vis - 3000 PRO. Amersham. Biosciences). The parameters were plotted according the Lambert-Beer equation (equation 1) in order to calculate the concentration of sample considering the dilution factor (D) and the molar extinction coefficient (F):

$$C = \{[(A\lambda_{520} - A\lambda_{700})pH_{10}]\} - \{[(A\lambda_{520} - A\lambda_{700})pH_{45}]\} \times D \times F \quad (1)$$

The total monomeric anthocyanin in the original sample was expressed in g cyanidin-3-glucoside /100g by dry grape skin.

2.5. Improvement of percentage preparation enzymatic in most advantageous extraction method

Among the eight grape varieties analysed, one was chosen to have its extraction process improvement. The grape skin variety that presented the most favorable result of extraction - higher percentual of anthocyanins recovery associated with a high concentration of anthocyanins in the extract – was chosen to perform the improvement of extraction method. The improvement intended to verify the most effective conditions of temperature and enzyme concentration on the anthocyanins recovery from grape skins.

The total monomeric anthocyanins content was used as maximum response and was determined according to the previously described method of pH-differential (Giusti and Wrolstad 2001).

2.6. Physical-chemical analysis of extracts

The extracts exhaustive and anzymatic with higher percentual of anthocyanins recovery from each grape skin cultivar were characterized following the AOAC methods (2006) for total acidity and total soluble solids. The extracts pH were measured at 25 °C using a DM-22 potentiometer (Digimed, Brazil) by AOAC (2006).

2.7. High performance liquid chromatography (HPLC) analysis

The extracts anthocyanin quantification were performed by HPLC using an HPLC chromatograph (Agilent 1100 Series. Santa Clara. CA. USA). The HPLC was equipped with a quaternary pump system solvent, UV–visible detector, and a C18 Shim-Pak CLC-ODS column (5 µm, 250×4.6 mm). The used mobile phase consisted of a linear gradient elution of 5 % aqueous formic acid/methanol 85:15 (v/v) to 20:80 over 25 min, and its isocratic ratio was maintained for 15 min. The injection volume was 5 µL, the mobile phase flow was 1 mL/min, and the column temperature was maintained at 29 °C. The chromatograms were processed at a fixed wavelength of 520 nm. The anthocyanins often found in different grape skin varieties were identified and quantified, and its standards were purchased from Sigma-Aldrich (USA): delphinidin chloride (CAS 528-53-50 ≥95.0 %), cyanidin 3-0-glucoside chloride (CAS 7084-24-4 ≥95.0 %), pelargonidin 3-0-glucoside chloride (CAS 18466-51-8 ≥95.0 %), malvidin- 3-0-glucoside chloride (CAS 7228-78-6. ≥90.0%), cyanidin chloride (CAS 528-58-5 ≥95.0 %), petunidin 3-glucoside (CAS 6988-81-4 ≥95.0 %) and malvidin chloride (CAS 63-84-5≥95.0 %).

The identification and quantification of compounds were performed by comparing of UV-Vis Spectrum and retention times and peak areas of the samples and respective standards under the same chromatographic conditions. For quantification. a standard curve was constructed in the following concentration ranges: delphinidin-3- glucoside, 5–100 mg/mL; cyanidin-3-glucoside, 5–40 mg/mL; pelargonidin 3-0- glucoside chloride, 6-70 mg/mL; malvidin-3-glucoside, 5–50 mg/mL; cyanidin chloride, 16-80 mg/mL; petunidin-3-glucoside, 3–40 mg/mL; malvidin chloride, 3-30 mg/mL. The coefficient of determination (R^2) for the standard curves were established in a minimum of 98%. The limits of detection

(LOD) and quantification (LOQ) were as follows: delphinidin chloride, 58.00 and 193.35 µg/g; cyanidin 3-0-glucoside chloride, 8.45 and 28.25 µg/g; pelargonidin 3-0-glucoside chloride, 2.35 and 7.85 µg/g; malvidin- 3-0-glucoside chloride, 10.35 and 25.60 µg/g; cyanidin chloride, 6.20 and 23.30 µg/g; petunidin 3-glucoside, 3.30 and 5.50 µg/g; malvidin chloride, 4.70 and 14.15 µg/g.

2.8. Statistical analysis

All determinations were carried out in triplicate and the results were expressed as means ± standard deviation (SD). Statistical of pH-differencial analysis was performed with Statistica software version 12.0 (StatSoft Inc.. 2011. Tulsa. OK. USA). The graphic of improvement on the anthocyanin recovery was performed with the OriginPro 8.2.

3. Results and discussion

3.1. Physical-chemical determinations in grape skins

The physical-chemical determination results of the eight grape skins analysed are presented at Table 1. The characterization of grape skin is important to improve extract preparation procedures and essential to evidence wine grape pomace potential as a source of functional food ingredients (Deng et al. 2011; Yu and Ahmedna 2013). According whit study by Koshita et al. (2011), where analyzed the interactive effects of temperature and total soluble solids in cultivar *Vitis labrusca*, concluded tahta slight differences in grape total soluble solids (largely made up of glucose and fructose) can be associated by differences in anthocyanin content and, consequently, on the grape color. This association is directly proportional. In the present study, the total soluble solids varied widely, from 5.00

(Isabel) to 15.00 °Brix (Cabernet Sauvignon).

Table 1 Results for moisture (%), acidity (% acidity in tartaric acid), total soluble solids (TSS) (ºBrix), ash (%) of different skin grapes pomace

	Ives (1)	Ives (2)	Ives (3)	Isabel	Petit Verdot	Pinot Noir	Merlot	Cabernet Sauvignon
Moisture	89.93±0.35^{A}	81.89±0.08^{BC}	79.03±5.05^{BCD}	76.58±0.39^{CDE}	84.09±0.56^{AB}	69.64±2.73^{F}	72.47±1.23^{EF}	73.43±0.06^{DEF}
Acidity	0.11±0.01^{B}	0.11±0.00^{B}	0.11 ± 0.01^{B}	0.08 ± 0.00^{C}	0.13 ± 0.00^{A}	0.09 ± 0.00^{C}	0.12 ± 0.00^{B}	0.11 ± 0.01^{B}
TSS	5.00 ± 0.02^{E}	12.50 ± .01^{B}	9.17 ± 1.44^{C}	5.00 ± 0.10^{E}	10.00 ± 0.01^{C}	7.50 ± 0.01^{D}	7.50 ± 0.01^{D}	15.00 ± 0.01^{A}
Ash	0.26 ± 0.01^{D}	0.50 ± .03^{CD}	1.36 ± 0.30^{A}	1.19 ± 0.04^{AB}	0.78 ± 0.04^{BC}	1.32 ± 0.37^{A}	1.46 ± 0.15^{A}	0.81 ± 0.02^{BC}

Note: Mean values ± standard deviations.
Different letters within a line indicate significant differences among characteristic of skin grape at $P < 0.05$.

The moisture content for grape skin varieties in this study were from 69.64% (Pinot Noir) to 89.93% (Ives 1), while the ashes content varied from 0.26 to 1.46% (Table 2). The acidity values in percentage of acid tartaric were from 0.08 to 0.13%. González-Centeno et al. (2010) analysed the moisture content of ten different grape varieties, and the moisture content percentages of the analysed by-products exhibited values from 50 to 72%. However, in the case of this research it can be deduced that the moisture found at skins are also related to the pressing process during the production of wines and juicesThe study concluded there was high moisture variability depending on the grape variety analysed. Considering the percentuals of ash and acidity, the present study is similar to Stoll et al. (2016),that reported ashes content of 1.18% and acidity of 0.15% in tartaric acid for grape skins provided from a similar region of the ones from our study.

The phenolic compounds present in grape pomace have been successfully applied as natural food colorants in several researches (García-Lomillo and González- SanJosé 2017; Liang et al. 2011; Maier et al. 2009) found a correlation between color CIELab coordinates (L*a*b*) and the ratio of different types of anthocyanins in grape skin. The study characterised 78 grape varieties by color and

anthocyanins content (by HPLC), and concluded that derivates of cyanidin were responsibe for the greater values L* and b*. The results obtained in our research are in accordance with Liang, since Ives (1), (2) and (3), the varieties with higher values of cyanidin-3-glicoside (2.32, 0.45 and 1.33 g/ 100g dry skin grape, respectively) (Table 3), presented higher L* and b* values.

Table 2 Mean values (± standard deviation) of lightness (L*), redness (a*), yellowness (b*) intensity of the by 8 skin grapes pomace.

Varietes	L*	a*	b*
Ives (1)	15.90 ±1.49^{B}	7.21 ± 1.25^{CD}	0.92 ± 0.28^{C}
Ives (2)	23.60 ± 0.95^{A}	13.75 ± 1.28^{A}	- 0.36 ± 0.04^{D}
Ives (3)	16.02 ± 1.47^{B}	11.02 ± 0.04^{BC}	2.29 ± 0.15^{C}
Isabel	15.11 ± 1.39^{B}	11.52 ± 0.40^{B}	3.69 ± 0.25^{A}
Petit Verdot	14.78 ± 0.40^{B}	12.13 ± 0.23^{AB}	2.24 ± 0.06^{B}
Pinot Noir	12.19 ± 0.48^{B}	9.05 ± 0.66^{BCD}	2.31 ± 0.20^{B}
Merlot	11.95 ± 0.66^{B}	6.94 ± 0.21^{D}	2.02 ± 0.11^{B}
Cabernet Sauvignon	15.94 ± 1.48^{B}	13.8 ± 0.44^{A}	0.04 ± 0.00^{D}

Note: Mean values ± standard deviations.

Different letters within a column indicate significant differences at $P < 0.05$.

3.2. Enzymatic extraction experimental design

The statistical data analysis demonstrated an interaction between enzyme concentration and temperature in the whole range of parameters studied in the present study ($p<0.05$) and presented a positive effect on anthocyanins extraction for the eight

grape varieties studied. Observing the independent variables individually, the temperature presented a positive effect ($p<0.05$) for Ives (2), Petit Verdot, Merlot e Pinot Noir, and a negative effect for Ives (3). The variable enzyme concentration only presented a statistically significant effect for Ives (1) and Pinot Noir. The results of the analyses are presented in table 3.

Table 3. Results of anthocyanin (g/100g dry bases) of enzymatic extraction experimental design

Treatments									
temperature	Percentage enzyme	Ives (1)	Ives (2)	Ives (3)	Isabel	Petit Verdot	Pinot Noir	Merlot	Cabernet Sauvignon
-1	-1	12.59	6.61	2.91	0.58	1.98	1.07	1.24	4.03
-1	+1	17.43	6.21	2.94	0.59	2.60	1.01	1.28	2.69
+1	-1	16.11	7.99	2.65	0.61	4.37	0.89	1.46	3.00
+1	+1	21.00	8.45	2.30	0.68	2.39	1.62	1.71	3.10
0	0	17.31	6.56	2.71	0.79	2.76	0.91	1.28	3.06
0	0	14.74	6.92	2.68	0.64	3.17	0.86	1.05	3.31
0	0	16.96	6.47	2.81	0.64	1.91	0.97	1.16	2.93
P (ANOVA)		0.009	0.016	0.004	<0.001	<0.001	<0.001	<0.001	<0.001
R^2		0.90	0.80	0.96	0.19	0.39	0.75	0.49	0.91

However, the effect of temperature and enzyme concentration on the anthocyanins extraction of Isabel, Pinot Noir, Merlot and Petit Verdot varieties could not be explained by mathematical models ($R^2 < 0,80$). This probably occurred because Isabel and Pinot Noir presented low anthocyanin concentration and consequently lower responses for extraction (dos Santos Lima et al. 2014). Other parameter that must be considered is the grape skins ash content, which influences skin hardness and possibly reduces access of enzymes to the substrate and

difficult anthocyanin extraction (Chardonnet and Donéche 2015). Merlot, one of the grape skin varieties to which anthocyanin extraction was not statistically significant at the range of temperature and enzyme tested ($p > 0.05$), had the highest values for ash (1.46%). Petit Verdot is a variety generally used in blends to improve wine color and flavor, and this practice partially refers to the easy extractability of Petit Verdot compounds from skin (Dimitrovska et al. 2011). This characteristic could explain the lower response by enzymatic extraction to Petit Verdot, considering that the grape skin have already passed through the winemaking process of extraction.

The data analysis of grape skins varieties Cabernet Sauvignon, Ives (1), (2) and (3) were fit to a mathematical model ($R^2 \geq 0{,}80$) and the graphical representation of the measured points and the response surfaces are given in Fig. 1.

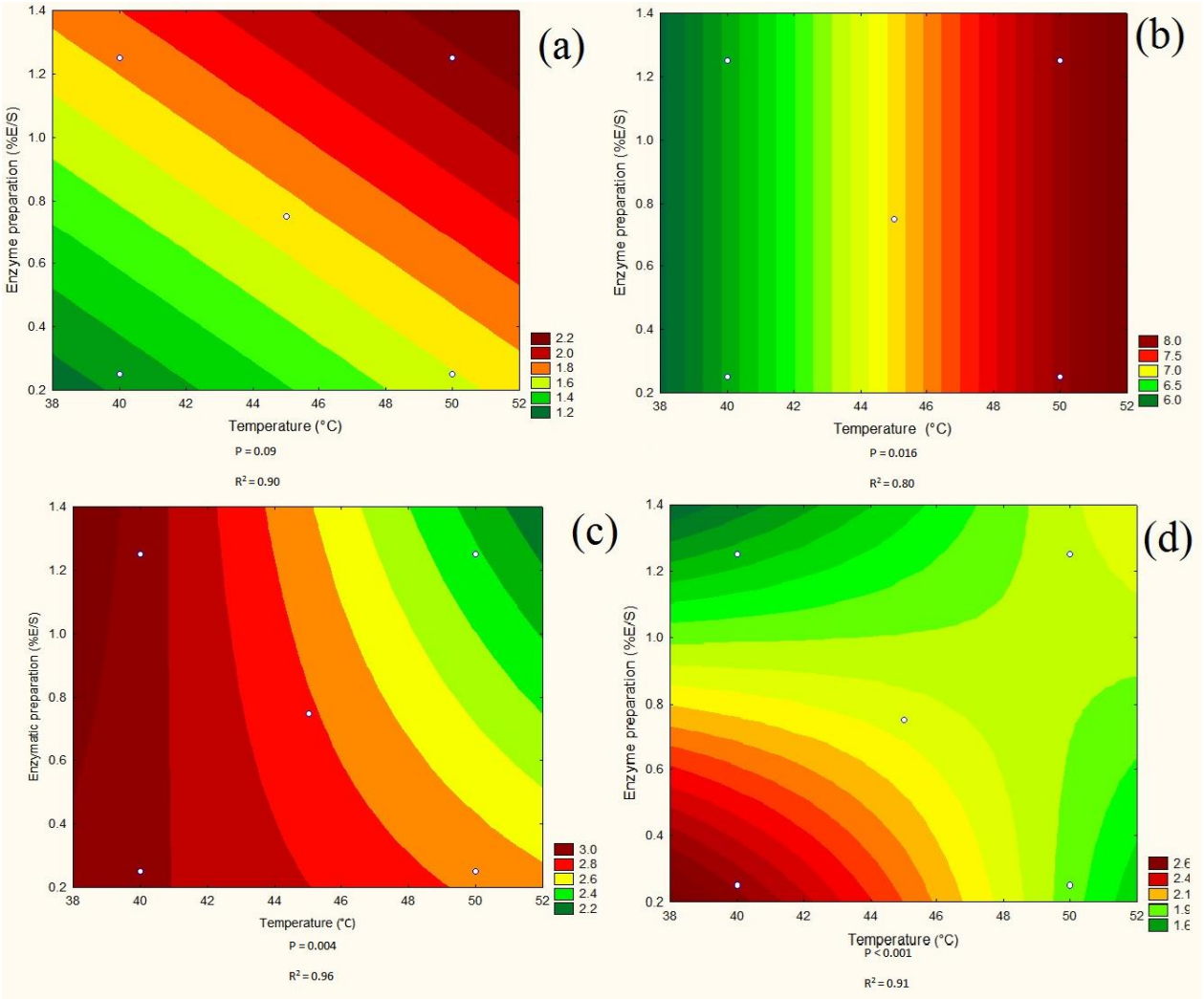


Fig.1 Anthocyanins extraction recovery (%) response surface for each type of winery by-product. Axis x corresponds to the extraction temperature (°C) and axis y corresponds to percentage of enzyme preparation (E/S%). Aqueous extract by grape skin Ives (1) (a), Ives (2) (b), Ives (3) (c) and Cabernet Sauvignon (d).

An increase on anthocyanins extraction was observed to Ives (1) when the highest enzyme concentration and temperature were used. This distinction with variety Ives (2), which also comes from a non-organic system of production, can be related by extrinsic factors such as production area and environmental aspects, which may affect skin rigidity and anthocyanin concentration (Hanlin et al. 2011;

Tomás-Barberán and Espín 2001).

Although Ives (2) and (3) were produced in the same region, these grapes exhibited very different responses to the extraction using enzymes. Ives (3) demonstrated an increased anthocyanins extraction to the highest enzyme concentrations and lower temperatures, while the anthocyanins recovery in Ives (2) was not affected by enzyme concentration. This behavior can be understood by the differences on the viticultural practices between organic and non-organic systems. Ives (2) has passed through an enzymatic extraction during winemaking process, which can lead to greater exposure of bioactive compounds for the production of grape must and consequently decrease the subsequent effect of enzymes on grape pomace (Dal Magro et al. 2016). The same did not occur in must production of organic grape Ives (3).

The anthocyanins recovery in Cabernet Sauvignon grape skin presented very favorable characteristics of extraction, since a higher yield of anthocyanins recovery was obtained when lower enzyme concentrations and temperatures were used. These aspects are essential for enzyme-assisted extraction, considering that processes that require high temperatures and amount of enzymes higher than 1% (relative to the weight of raw material) are considered costly technologies (Martínez-maqueda et al. 2013). The low ash content of Cabernet Sauvignon – which was equal to Petit Verdot (0.78%) – could possibly explain the higher extraction response under lower temperatures.

3.3. Percentual of total and individual anthocyanins recovery measured by HPLC – an analysys of the best exctracts for each grape skin cutivar

The tables 4 and 5 present the concentration of total and individual

anthocyanins contained in the grape skins, which were obtained by exhaustive and enzymatic-asssited aqueous extraction. The compounds were quantified by HPLC. The varieties of *V. labrusca* presented the highest total anthocyanin content (10.08 g/100 db) when compared to the *V. vinifera* varieties (3.58g/100g db). The anthocyanins found in both species only were delphinidin-3-β-glucoside, cyanidin 3-0- glucoside chloride and malvidin-3-0-glucoside chloride. The differences in anthocyanins profile and concentration between the species was evidenced by Ribeiro et al. (2015), that demonstrated that Brazilian grape pomaces from *V. labrusca* and *V. vinifera* differs in various features, including anthocyanins profile. It is worth mentioning that the presence of malvidin 3-glycoside was distinguished, since it was found at considerable amounts in the eight grape skin varieties analysed.

Table 4 Analysis of anthocyanins compounds) (g/100g dry basis) in skin extracts of red grape (*V. labrusca*) pomace, obtained by HPLC

Elution Order	Compound	tr (min)	Ives (1)		Ives (2)		Ives (3)		Isabel		λmax (nm)
			skin grape	extract	skin grape	extract	skin grape	extract	skin grape	extract	
1	Dp-3-β-D-glc	4.5	2.07 ± 0.07	1.26 ± 0.09	0.32 ± 0.02	0.12 ±0.01	n.d.	n.d.	0.21 ± 0.01	0.05 ± 0.00	279(sh), 431,523
2	Cy-3-glc	5,0	2.32 ± 0.24	1.68 ± 0.03	0.45 ± 0.01	0.19 ± 0.00	1.33 ± 0.10	0.11 ± 0.00	n.d.	n.d.	277(sh), 325,374,515
3	Unknown*	5.2	1.78 ± 0.00	0.42 ± 0.07	0.27 ± 0.03	0.05 ± 0.00	0.85 ± 0.03	0.03 ± 0.00	0.10 ± 0.02	0.04 ± 0.00	249, 284, 525
4	pl-3-0-glc-chloride	5.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-
5	pn-3-glc*	5.5	0.93 ± 0.11	0.23 ± 0.02	0.12 ± 0.01	0.02 ± 0.00	0.07 ± 0.00	0.02 ± 0.00	n.d.	n.d.	277(sh), 325,515
6	Mv-3-glc	5.6	1.66 ± 0.13	0.20 ± 0.02	0.39 ± 0.05	0.07 ± 0.01	0.22 ± 0.01	0.04 ± 0.00	0.26 ± 0.01	0.15 ± 0.01	274(sh), 524
7	cy-clhoride	6.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	279(sh), 518
8	Pt-3-glc	6.2	0.44 ± 0.03	0.07 ± 0.01	0.06 ± 0.00	0.03 ± 0.00	0.14 ± 0.00	0.02 ± 0.00	n.d.	n.d.	279(sh),349,527
9	Dp-coum or mv-coum*	6.5	0.33 ± 0.00	0.33 ± 0.00	1.80 ± 0.01	0.60 ± 0.05	3.46 ± 0.23	0.23 ± 0.00	0.11 ± 0.00	0.03 ± 0.00	279,373,531
10	mv-clhoride	6.8	0.55 ± 0.03	0.01 ± 0.00	0.03 ± 0.00	0.01 ± 0.00	0.07 ± 0.00	0.01 ± 0.00	n.d.	n.d.	281,365.531
	Anthocyanins total		10.08 ± 0.61	4.20 ± 0.24	3.44 ± 0.13	1.09 ± 0.07	6.14 ± 0.37	0.46 ± 0.00	0.68 ± 0.04	0.27 ± 0.01	

Note: Mean values ± standard deviations. Abbreviations: nd, no detected; tr, retention time; Dp, delphinidin;Pn, peonidin; Cy, cyanidin; Pl, pelargonidin; Mv, malvidin; Pt, petunidin; glc, glucoside; Coum, 3-0-p-coumarylglucoside. *Quantified in malvidin-3-glicoside. Compounds identified using corresponding standards.

Table 5 Analysis of anthocyanins compounds (g/100g dry basis) in skin extracts of red grape (*V. vinifera*) pomace, obtained by HPLC.

Elution Order	Compound	tr (min)	Petit Verdot		Pinot Noir		Merlot		Cabernet Sauvignon		λmax (nm)
			skin grape	extract	skin grape	extract	skin grape	extract	skin grape	extract	
1	Dp-3-β-D-glc	4.5	0.61 ± 0.04	0.15 ± 0.01	0.49 ± 0.02	0.04 ± 0.00	n.d.	n.d.	0.76 ± 0.05	0.26 ± 0.01	279(sh), 431,523
2	Cy-3-glc	5,0	0.12 ± 0.01	0.02 ± 0.00	0.10 ± 0.00	0.01 ± 0.01	0.04 ± 0.00	n.d.	0.07 ± 0.01	0.02 ± 0.00	277(sh), 325,374,515
3	Unknown*	5.2	0.32 ± 0.02	0.08 ± 0.00	0.42 ± 0.02	0.01 ± 0.00	0.03 ± 0.01	0.02 ± 0.00	0.15 ± 0.01	0.08 ± 0.0	249, 284, 525
4	pl-3-0-glc-chloride	5.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-
5	pn-3-glc*	5.5	0.63 ± 0.01	0.10 ± 0.00	0.86 ± 0.03	0.09 ± 0.00	0.87 ± 0.03	0.22 ± 0.00	0.26 ± 0.01	0.16 ± 0.01	277(sh), 325,515
6	Mv-3-glc	5.6	0.76 ± 0.00	0.14 ± 0.00	1.11 ± 0.00	0.15 ± 0.00	2.01 ± 0.11	0.64 ± 0.01	1.42 ± 0.13	0.60 ± 0.04	274(sh), 524
7	cy-clhoride	6.1	0.01 ± 0.00	n.d.	0.09 ± 0.00	n.d.	n.d.	n.d.	n.d.	n.d.	279(sh), 518
8	Pt-3-glc	6.2	0.01 ± 0.00	n.d.	0.02 ± 0.00	n.d.	n.d.	n.d.	n.d.	n.d.	279(sh),349,527
9	Df-coum or mv-coum*	6.5	0.06 ± 0.00	0.02 ± 0.00	0.49 ± 0.00	0.03 ± 0.00	n.d.	n.d.	0.33 ± 0.02	0.30 + 0.00	279,373,531
10	mv-clhoride	6.8	0.01 ± 0.00	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	281,365.531
	Anthocyanins total		2.53 ± 0.08	0.51 ± 0.01	3.58 ± 0.07	0.33 ± 0.00	2.95 ± 0.15	0.88 ± 0.01	2.99 ± 0.23	1.42 ± 0.06	

Note: Mean values ± standard deviations. Abbreviations: nd, no detected; tr, retention time; Dp, delphinidin;Pn, peonidin; Cy, cyanidin; Pl, pelargonidin; Mv, malvidin; Pt, petunidin; glc, glucoside; Coum, 3-0-p-coumarylglucoside. *Quantified in malvidin-3-glicoside. Compounds identified using corresponding standards

On the present study, the *V. labrusca* grape skin varieties showed higher amounts of malvidin than *V. vinifera*, while the last was the only specie which contained cyanidin chloride. The anthocyanins ratio was also a distinct factor between the species: while malvidin-3-glicoside is the major anthocyanin in *V. vinifera*, for *V. labrusca* only Isabel has this characteristic, and cyaniding-3-glicoside, delphinidin and malvidin 3-0-p-coumarylglucoside are the predominants.

The anthocyanins profile can be influenced by environmental factors and production area, which could be evidenced by the different concentration of anthocyanins in Ives varieties cultivated in distinct states of Brazil. Besides that, the influence of viticultural practices is evidenced by differences in anthocyanins concentration in Ives from organic and non-organic systems originated from adjacent reagions of production. As reported in several researches, the concentration of phenolic compounds in grapes varies addition to according to type of varieties, *terroir*, degree of ripeness, weather, sanitary state of plants, soil and farming practices (Jordão et al. 2015; Moreno-Pérez et al. 2013; Rapisarda et al. 1999).

The higher percentuals of anthocyanin recovery by *V. labrusca* and *V. Vinifera* occurred to Isabel and Cabernet Sauvignon, respectively, both achieving over 50% of recovery (Fig. 2). Despite of the great recovery in Isabel variety, the amount of anthocyanins that is naturally present in its skin is only 0.68 g100g dry basis, which represent the lowest concentration of anthocyanins in this study. As opposed to Cabernet Sauvignon, which present the second higher amount of anthocyanins concentration of the grape skins analysed.

The recovery percentage is related in comparing the amount of anthocyanins obtained by enzymatic extraction in relation to exhaustive extraction. The lowest

percentage of recovery of extraction in *V. labrusca* is Ives (3), and in *V. vinifera* is Pinot Noir, both achieving less than 10% of recovery (Figure 2). These results could be associated with the elevated ash content observed for the variety Ives (2) (1.32%) and Pinot Noir (1.36%) that may increase cell wall rigidity and impair anthocyanins extraction (Pinelo et al. 2006).

In relation to individual anthocyanins (Fig. 2), the higher percentuals of recovery (over than 50%) occurred to glycosylated anthocyanins contained in Ives (1), Isabel and Cabernet Sauvignon. This result may be related to the higher stability of glycosyl compounds, since is a reaction known to improves color and pigment stability. Glycosylated anthocyanins has already been shown as a promising alternative to synthetic colorants in food systems (Jackman et al., 1987). The low stability of malvidin chloride could explain its lower percentage (<20%) of extraction (Fig. 2).

Most of the studies that analysed the efficiency of anthocyanins extraction, independently from the extraction method used, considered the total content of maximum anthocyanins value as the parameter to classify the best extraction conditions (Fontana et al. 2013). This may have caused the loss of important data considering that individual anthocyanins are endowed of particular abilities of co-pigmentation. The presence or absence of certain anthocyanins, associated with their individual characteristics , strongly influences extracts coloring properties (Bautista-Ortín et al. 2016; Cliff et al. 2007).

Many studies regarding the application of anthocyanins - purified or in extract - in experimental systems *in vitro* confirmed the potential of these compounds as natural dyes. Studies mention that quantity and distribution of different anthocyanins affected directly the products final coloration (Delgado-

Vargas et al. 2000; Konczak and Zhang 2004; Ky and Teissedre 2015; Markakis 2012; Pomar et al. 2015).

Malvidin-3-glicoside was one of the majors pigments found in extracts obtained in the present study (approximately 42% of total anthocyanins). This extract characteristic is suitable for food application. The presence of two methylated radicals in malvidin-3-glicoside turn it in one of the most stable anthocyanins, therefore promoting color maintenance when incorporated into food (Tanaka et al. 2008).

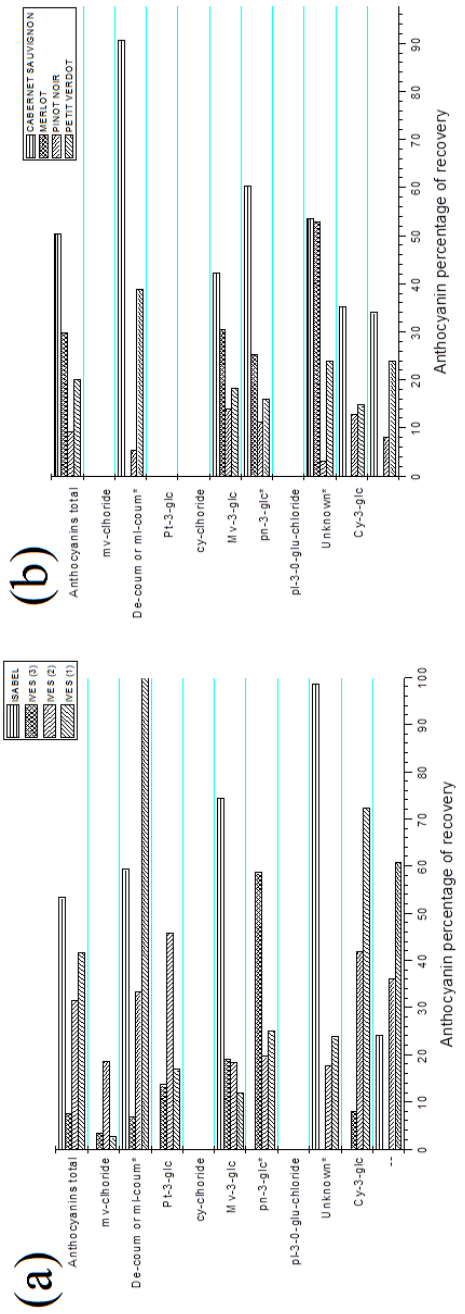


Fig. 2 Individual and total anthocyanins percentage of recovery (enzymatic-assisted extraction/exhaustive extraction) obtained by the best extraction conditions for each type of winery by-product. Axis x corresponds to the individual anthocyanins compounds in skin extracts of red grape (a) (*V. labrusca*) pomace and (b) (*V. vinifera*) pomace. Axis y corresponds to percentage of recovery of enzymatic-assisted extraction.

3.4. Physical-chemical determinations in best enzyme-assisted extraction

Food and Drug Administration (FDA, 2015) reported that additive grape skin extract *enocianina* is defined as a liquid of purplish-red color prepared by aqueous extraction of the fresh deseeded marc remained after grapes have been pressed to produce grape juice or wine. The purplish-red color tendency is related to lower values of the parameters b* and L* and higher values of the parameter a*.

Among the grape varieties analysed, the Ives (3) enzymatic-assisted extracts presented the closest similarity with the *enocianina* color characteristic, followed by Cabernet Sauvignon (Table 6). Furthermore, the extracts color parameters of the present study are similar to a commercial natural red food colorant cited by Castellar et al. (2006), highlighting the fact that the extracts obtained in this study have not gone through any process of concentration. This parameter evidence the interest the use of variety Cabernet Sauvignon as a natural dye in this study.

Table 6 Mean values (± standard deviation) of lightness (L*), redness (a*), yellowness (b*) intensity of the enzymatic-assited extracts rich in anthocyanins.

Varietes	L*	a*	b*
Ives (1)	30.89 ± 0.36^{CDE}	13.85 ± 0.25^{E}	2.77 ± 0.09^{D}
Ives (2)	29.88 ± 0.45^{DE}	8.59 ± 0.69^{D}	1.15 ± 0.01^{E}
Ives (3)	28.70 ± 0.17^{E}	4.17 ± 0.26^{E}	0.40 ± 0.01^{E}
Isabel	46.38 ± 1.93^{A}	15.87 ± 1.13^{C}	9.59 ± 0.33^{A}
Petit Verdot	32.46 ± 1.02^{BC}	15.58 ± 0.90^{C}	4.95 ± 0.49^{C}
Pinot Noir	34.00 ± 0.32^{B}	27.24 ± 0.79^{A}	9.61 ± 0.37^{A}
Merlot	32.14 ± 0.49^{BCD}	18.98 ± 0.74^{B}	6.02 ± 0.43^{B}
Cabernet Sauvignon	31.20 ± 0.70^{CD}	16.37 ± 0.30^{C}	3.91 ± 0.01^{C}

Note: Mean values ± standard deviations.

Different letters within a column indicate significant differences among color groups at $P < 0.05$.

Boulton (2001) reported that the potential of food colorants depends, among other aspects, on factors as pH, concentration of soluble solids, and on the presence of anions in solution (which can relates with solution acidity). It consolidates the importance of this study to characterize the extracts by physical-chemical analysis (Table 7).

Table 7 Results for acidity (% acidity in tartaric acid), total soluble solids (TSS, °Brix), pH of 8 extracts rich in anthocyanins.

Table 7 Results for acidity (% acidity in tartaric acid), total soluble solids (TSS, °Brix), pH of 8 extracts rich in anthocyanins.

	Ives (1)	Ives (2)	Ives (3)	Isabel	Petit Verdot	Pinot Noir	Merlot	Cabernet Sauvignon
Acidity	0.45 ± 0.01^{A}	0.42 ± 0.01^{BC}	0.45 ± 0.00^{A}	0.45 ± 0.00^{A}	0.44 ± 0.01^{AB}	0.41 ± 0.01^{C}	0.40 ± 0.01^{C}	0.44 ± 0.01^{AB}
TSS	2.33 ± 0.11^{D}	2.87 ± 0.15^{B}	2.63 ± 0.06^{C}	2.63 ± 0.06^{C}	2.53 ± 0.06^{C}	2.73 ± 0.06^{CD}	3.13 ± 0.06^{A}	3.37 ± 0.06^{A}
pH	4.94 ± 0.16^{C}	3.66 ± 0.02^{C}	3.57 ± 0.03^{D}	3.85 ± 0.01^{B}	3.80 ± 0.02^{B}	3.72 ± 0.01^{C}	3.85 ± 0.01^{B}	3.95 ± 0.04^{A}

Note: Mean values ± standard deviations.
Different letters within a column indicate significant differences among groups at $P < 0.05$.

Gauche et al. (2010) indicated that lower pH and acid conditions is the most effective medium for anthocyanins stability. The grape varieties and conditions of extraction did not affected greatly the acidity and pH parameters of the extracts. However, the extracts presented differences in soluble solids values, which according to Walker et al., (2001) may be related to distinct maturation indexes of the grapes.

The extracts physical-chemical characteristics suggest their application as functional ingredients for acidic foods such as yogurts and some beverages with functional properties. This is due to a wide range of biofunctional properties that have been reported for these extracts, such as antimutagenic, anticarcinogenic properties, protection against cardiovascular diseases and anti-aging activities (Yu and Ahmedna 2013).

3.5. Improvement of the most advantageous extraction method

Previous studies have revealed the importance of improvement on the recovery in enzymatic extraction before scaling-up (Maier et al. 2008) improved the process of extraction from grape pomace and increased the extraction yield of phenolic compounds, reaching up to 63.6% of anthocyanins recovery.

Cabenet Sauvignon, the grape skin variety which presented the higher percentual of anthocyanins recovery associated with a high concentration of anthocyanins in the extract, was chosen to perform the improvement of the process. The maximum temperature value provided by the experimental analysis (40°C) was fixed prevent degradation of anthocyanins present,and a range of enzymatic preparation was tested (0.000%, 0.125%,0.250%,0.375% and 0.500%

E/S) (Fig. 3).

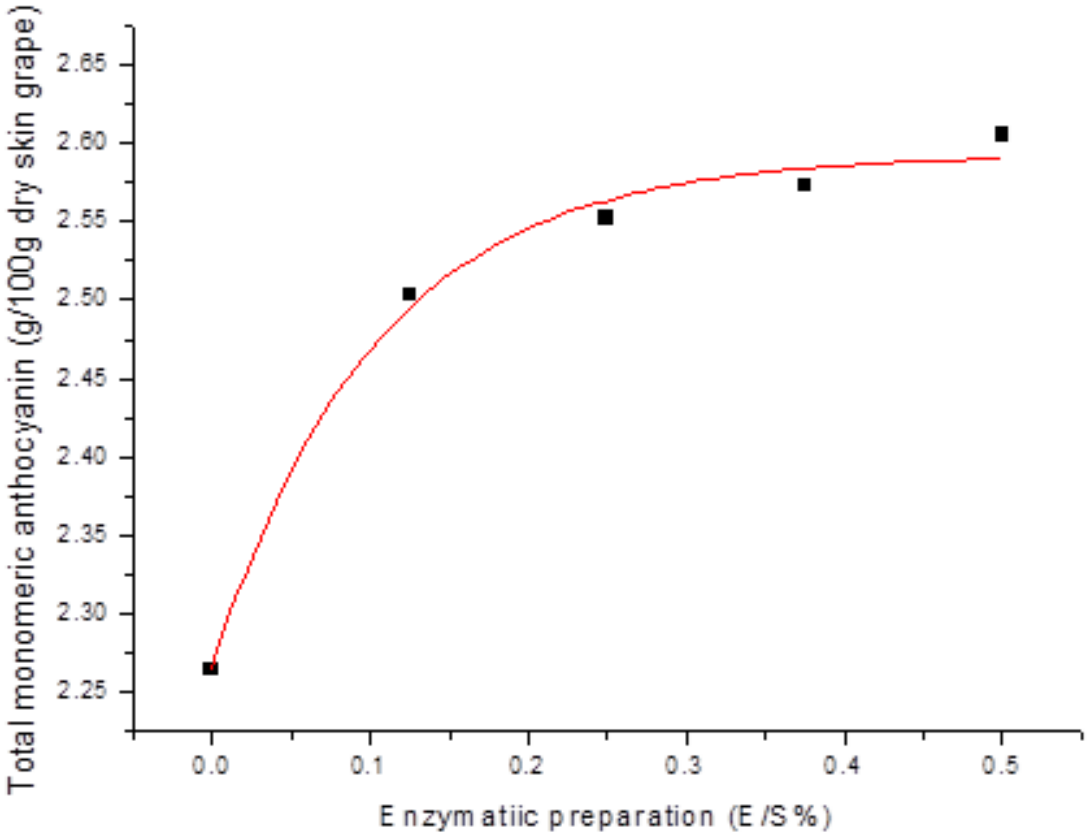


Fig. 3 Improvement on the anthocyanin recovery in Cabernet Sauvignon extract (R^2=0.99)

Based on the improvement results, the extract with the maximum value of total monomeric anthocyanins (0.5 E/S%; 2.60 g/100g skin grape) was analysed by HPLC. The chromatographic analysys of anthocyanins in grape skin extract of Cabernet Sauvignon with 0.5% enzymatic concentration, obtained for individual anthocyanin (g/100 g db) was: dp-3-β-D-glc 0.48 ± 0.01; cy-3-glc 0.05 ± 0.01; unknown compost (t_r= 5.2) 0.16 ± 0.00; pn-3-glc 0.23 ± 0.01; mv-3-glc 1.08 ± 0.10; de-coum or ml-coum 0.68 ± 0.00. Therefore, resulting in 2.67 ± 0.13 (g/100 db) of total anthocyanins. Through this improvement, it was possible to

increase the yield of anthocyanins extraction by 15.07% compared to the extraction performed at 40°C without the use of enzyme Pectinex Ultra Color® (0.000 E/S%; 2.26 g/100g skin grape).

Comparing the results of 0.5% enzyme usage (Table 5) with 0.25%, any significant differences ($p > 0.05$) were achieved, either for individual anthocyanins neither for total anthocyanins content. Therefore, 0.25% E/S is the enzymatic preparation that should be used in terms of efficiency.

4. Conclusions

The study confirms the potential of the enzymatic-assisted extraction of anthocyanins from wine skin grape, which is definitely an attractive and low cost source of phytochemicals. Different conditions of temperature and enzyme concentrations were found to distinct grape skins varieties. The grape skin characteristics such as ash content and origination (region of cultivation, viticultural practices, etc) affected the anthocyanins content and their yield of recovery, where acylated anthocyanins presented the higher recovery yields.

The improvement of anthocyanins extraction allowed the obtention of a non-toxic natural extract rich in anthocyanins. The skin grape selected is Cabernet Sauvignon, whit higher percentual of anthocyanins recovery (over 50%) in 40°C, 0.25% E/S at 30 minutes of extraction. This aqueous extract is endowed with characteristics that enable its application as functional food colorant, specially in acidic foods, without the necessity of concentration or solvent removal – a holdback frequently found at colorants orginated from conventional techiques of extraction.

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ARTIGO 2

Artigo em formato da revista Food Chemistry

Stability assessment of anthocyanins obtained from skin grape applied in kefir and carbonated water as a natural dye

Abstract

In the recent years the food additives industry has been looking for use of bioproduts as a source of food pigments. In this way, the aim of this study was to assess the stability of anthocyanins from residues of grape applied as food coloring in kefir and carbonated water.The degradation of anthocyanins applied in food matrices followed the first - order kinetic behavior during storage, in the light exposure or in the dark. For stability analysis of anthocyanins in kefir, a retention of anthocyanins was 67% during storage and a half-life time ($t_{1/2}$) was approximately 27 days. The highest stability in the carbonated water samples was showed by anthocyanin malvidin-3- glycoside, which obtained about four times longer $t_{1/2}$ when was stored in the dark. The light had negative effects on the color of colored carbonated water. The colored kefir showed physical properties similar to kefir without additives.

Keywords: natural dye; stability; kinetic behavior; grape pomace; enzymatic extraction

1. Introduction

In the recent years, the by-products from the wine industry have been used as a source to recover natural pigments and phenolic compounds that can be used as additives in cereals, dairy and meat products, among other food matrices (García-Lomillo & González-SanJosé, 2017).

The interest of consumers for this type of product has increased and the food coloring industry has been promoting for the use of skin and residues of grape to obtain food pigments, in order to support a sustainable agriculture due to a decrease of costs related with raw materials (Cortez, Luna-Vital, Margulis, & Gonzalez de Mejia, 2017; Goula, Thymiatis, & Kaderides, 2017).

There are several extraction methods for recovery of bioactive compounds from grape pomace. Among the traditional techniques, the solid-liquid procedure with the use of organic solvents is highlighted, however, this method is toxic and requires long extraction times. In addition, to involves subsequent processes of disposal and recovery of solvents that were used (Baiano, 2014; Proestos & Komaitis, 2008).

Among the emerging processes, the enzymatic extraction appears as economically favorable, due to the water is used as a solvent and promotes the degrading action of enzymes. This causes the consequent expulsion of the intracellular materials, thus obtaining a dye rich in bioactive compounds that can confer coloration in foods (Bhanja, Rout, Banerjee, & Bhattacharyya, 2008; Robledo et al., 2008).

Many different food matrices areknown in which can be added natural dyes to improve their sensorial and functional characteristics, among it is possible to emphasize the products based on milk. Un example is the kefir, a refreshing fermented milk beverage that has a slightly acidic flavor, uniform consistency and contains a small amount of ethyl alcohol in its composition (White, Kilara, Hui, & Chandan, 2008; Magalhães, Pereira, Campos, Dragone, & Schwan, 2011).

He, Xu, Zeng, Qin, & Chen, (2016) found that the addition of solutions of whey proteins of milk and casein in extracts of anthocyanins from grape skin, significantly impede the loss of color and degradation of anthocyanins. Thus, an addition of natural dye from grape in dairy foods may prevent degradation and increase their shelf life with a maintenance of color parameters. The authors reported that the proteins of milk are responsible for this behavior due to their protect effect which promotes positively the stability of anthocyanins.

In addition, of dairy products, the natural dyes also can be added to soft drinks. Most of these products that are found on the market contain artificial colorants and this generates a criticism of the population due to its connection with diseases such as diabetes, attention deficit and hyperactivity. Thus, the addition of natural additives in various types of carbonated drinks becomes an excellent alternative (Ashurst, 2016).

When researches are conducted researches for evaluating different systems of stability in food models with the addition of anthocyanins, the pH is one of the main factors. This parameter can affect the molecular structure, thus change its color. Therefore, conditions of high acidity permit a redness shade and a better structurally stability. The increase of these values affects the quinoidal base form, showing approximate color similarities of violet and blue tones (Cavalcanti, Santos, & Meireles, 2011).

Therefore, the natural dyes can be added in both products, because in a previous study with purified anthocyanins and extracts rich in anthocyanins that were applied in experimental systems *in vitro*, the potential referencia of these compounds as natural dyes were confirmed. Nevertheless, is important to control limiting factors that influence its degradation and stability when exposure to environmental variations, including temperature, light intensity and oxygen (de Rosso & Mercadante, 2007; Delgado- Vargas, Jimenez, & Paredes-Lopez, 2000; Konczak & Zhang, 2004; Markakis, 2012).

The aim of this study was to assess the stability of anthocyanins from residues of grape applied as food coloring in kefir and carbonated water. In addition, the physico-chemical properties of kefir and carbonated beverage were analyzed during storage.

2. Materials and methods

2.1. Materials

The residues of grape (Cabernet Sauvignon) was harvested between January and March in the State of Rio Grande do Sul and were donated by the San Giovanni winery (29 ° 8'54 .902 ° S, 51 ° 26 '23.255 "W). The preparation of enzyme Pectinex Ultra Color® (10000 PECTU / mL) (Novozymes, 2005) was used. Carbonated commercial water (Água da Pedra, Brazil) was purchased at a local store with pH 7.33 (at 25 ° C). The chemical composition of the beverage in mg/mL was calcium (25.69), sodium (24.12), potassium (0.99), fluor (0.23), bicarbonate (129.2), silicon magnesium (4.55), chloride and vanadium (0.014). The pasteurized homogenized milk (3% milkfat, Santa Clara, Brazil) was purchased at a local store.

2.2. Preparation of anthocyanin extract

Firstly, the husks of grape were manually separated from the bagasse and immediately stored at -18 °C. The preparation of concentrated natural dye of anthocyanin, from grape skin, was carried out according to the methodology adapted by Fernández, Vega, & Aspé, (2015), in each 5 g of grape skin were added 25 mL of acetate buffer (pH 4.0), previously heated at 40 °C and stirred at 150 rpm for 30 min. Then, the enzymatic preparation was added at the required ratio 0.25% E/S. The enzyme/substrate ratio (%E/S) was calculated on a wet matter basis.

For a concentration of anthocyanins, the extract obtained was lyophilized in

order to obtain 44% and 42% of their initial weight, these were added in kefir and carbonated water, respectively.

2.3. Preparation of food matrices: kefir and carbonated water

The kefir grains were added to the milk in 1:10 ratio, the mixture was incubated in the aerobic medium by using an incubator type B.O.D. (Model SP-500, SPLABOR) for 24 hours at 25 °C ± 2 °C (first fermentation). Subsequently, kefir grains were removed by filtration and kefir beverage was maintained at 5 ± 2 °C for a further 24 hours (second fermentation) (Marchiani et al., 2016). During the fermentation process it was added 400 mL of anthocyanin extract (produced with 79.99 g of skin grape) in 2 L of kefir. Then, it was stirred in the dark at room temperature until reach a color homogenization and was placed in polyethylene cups of 50 mL each one and stored at 7 °C in the dark for periodically evaluation of parameters at 0, 2, 5, 7, 9, 12, 14 and 16 days of storage. The initial amount of anthocyanin into kefir was 2.61± 0.05 mg/mL.

In the carbonated water, it was added 250 mL of anthocyanin extract. In order to produce the extract was performed an enzymatic extraction from 50.08 g of grape skin. The beverage was stirred slowly for homogenization of sample and was placed in translucide glass amber bottles of 20 mL.

The carbonated water samples were stored for quality evaluation at 0, 2, 5, 7, 9, 12, 14 and 16 days. The samples were kept in Incubator type B.O.D. (Model SP-500, SPLABOR) at 45 ºC ± 2 ºC in light exposure or in the dark. The initial amount of anthocyanin into carbonated water was 6.69 ± 0.03 mg/mL. Only kefir was analyzed in

the dark, due to previous studies of the respondents and also to the fact that this type of product is sold in matte packages in the industry.

2.4. Characterization physico-chemical of kefir and carbonated water

Kefir and carbonated water added to anthocyanin extract were chemically characterized for acidity and total soluble solids following the methods of the Association of Official Analytical Chemists (AOAC, 2006). The pH was determined by using a digital pH meter. The acidity was determined by titration and expressed as the percentage of tartaric acid for carbonated water, while for kefir was used the percentage of lactic acid. Although the majority constituent in the anthocyanin extract was tartaric acid, when this is applied to dairy matrices, is measured as the percentage of lactic acid, according to the methodology of AOAC (2006). The total soluble solids were measured using a refractometer (Model PAL-3, Atago U.S.A. Inc.) at 25 °C and expressed in °Brix.

Color measurements were performed using a portable colorimeter (Konica Minolta Model CR 400, Sensing, Singapore Pte, Ltd. USA) and the colorimetric parameters were obtained according to the CIELAB system. The value *L** (luminosity) and the coordinates *a** (red-green variation) and *b** (yellow-blue variation) were determined. These values were used to calculate the total color difference (ΔE*), follows the equation 1. All analyses were performed in triplicate.

$$\Delta E = (\Delta L*^2 + \Delta a*^2 + \Delta b*^2)^2 \qquad (1)$$

2.5. Extraction of remaining anthocyanins in kefir and carbonated water throughout the storage

Kefir samples were diluted in a solution of acidified methanol (0.1% HCl) and centrifugated (3500 x g, 10 min) (Hitachi, Model CR 21GIII) with withdrawal of supernatants for their respective measurement in HPLC. The procedure was repeated until the entire color of the pellet was completely removed. The obtained liquid was evaporated in a rotary evaporator (Fisatom, M802, Brazil) in vacuum at approximately 40 °C in absence of light until 10 mL of extract has stayed in the flask.

Based on Garcıa-Falcón & Simal-Gándara (2005), the homogenization and degassing of the sample of carbonated water were conducted in an ultrasonic bath; the sample aliquots were transferred directly into the vial and subsequently injected into the high-performance liquid chromatography system (HPLC) to quantify the anthocyanin content.

2.6. High-performance liquid chromatography (HPLC)

The anthocyanin quantification was performed by using HPLC system according to procedure adapted by Zanatta, Cuevas, Bobbio, Winterhalter, & Mercadante, (2005). For this analysis, was used an HPLC chromatograph (Agilent 1100 Series, Santa Clara, CA. USA) equipped with a quaternary pump system and a UV-visible detector with a C18 Shim-Pak CLC-ODS column (5 µm. 4.6 mm). The mobile phase consisted of a linear gradient elution of 5% aqueous formic acid/methanol 85:15 (v/v) to 20:80

over 25 min, and its isocratic ratio was maintained for 15 min. The mobile phase flow was 1 mL/min, the injection volume was 5 µL, and the column temperature was maintained at 29 °C. The chromatograms were processed at a fixed wavelength of 520 nm. This analysis identified and quantified the anthocyanins often in different grape skin varieties. Standards were purchased from Sigma-Aldrich (USA): delphinidin chloride (CAS 528-53-50 ≥ 95.0%), peonidin 3-O-glucoside chloride (CAS 6906-39-4 ≥ 95.0%) and malvidin-3-0-glucoside chloride (CAS 7228- 78-6. ≥ 90.0%).

The identification and quantification of compounds were performed by comparing of UV-Vis Spectrum and retention times and peak areas of the samples and respective standards under the same chromatographic conditions. For quantification, a standard curve was constructed in the following concentration ranges for the anthocyanins: delphinidin-3-glucoside 5–100 mg/mL; peonidin 3-O- glucoside 2–40 mg/mL and malvidin-3-glucoside. 5–50 mg/mL. The coefficient of determination (R^2) for the standard curves was established in a minimum of 98%.

The limits of detection (LOD) and quantification (LOQ) were as follows: delphinidin chloride 58.00 and 193.35 µg/g; peonidin 3-O-glucoside chloride 1.47 and 57.83 µg/g; malvidin-3-0-glucoside chloride 10.35 and 25.60 µg/g.

The CHEMSTATION® software was used to collect and process the data. For the injection, all samples were pre-filtered using a modified PTFE membrane for aqueous and organic solvents with a pore diameter of 0.22 µm (Millipore, SP, Brazil) and each injection was carried out in triplicate.

2.7. Kinetic parameters

The kinetics of anthocyanin degradation was analyzed using the first order reaction and to evaluate the kinetic parameters were plotted the anthocyanin retention (%) against time of storage. The degradation rate constant (k_t) was determined from the first derivative of the curves and was used to calculate the half-life time ($t_{1/2}$) following the equations 2 and 3 of de Rosso & Mercadante, (2007), where [anthocyanin] is the concentration of anthocyanins at a given time and $[anthocyanin]^0$ is the concentration of anthocyanins at time zero.

$$[anthocyanin]=[anthocyanin]^0 \times \exp(-kobs \times t) \quad (2)$$

$$t_{1/2}=\ln^2/kt \quad (3)$$

2.8. Statistical analysis

All determinations were carried out in triplicate and the results were expressed as means ± standard deviation (SD). Statistical analysis of pH-differencial was performed with Statistica software version 12.0 (StatSoft Inc.. 2011. Tulsa. OK. USA). The graphic of improvement on the anthocyanin recovery was performed with the OriginPro 8.2 (Origin Lab Co., MA, USA).

3. Results and Discussion

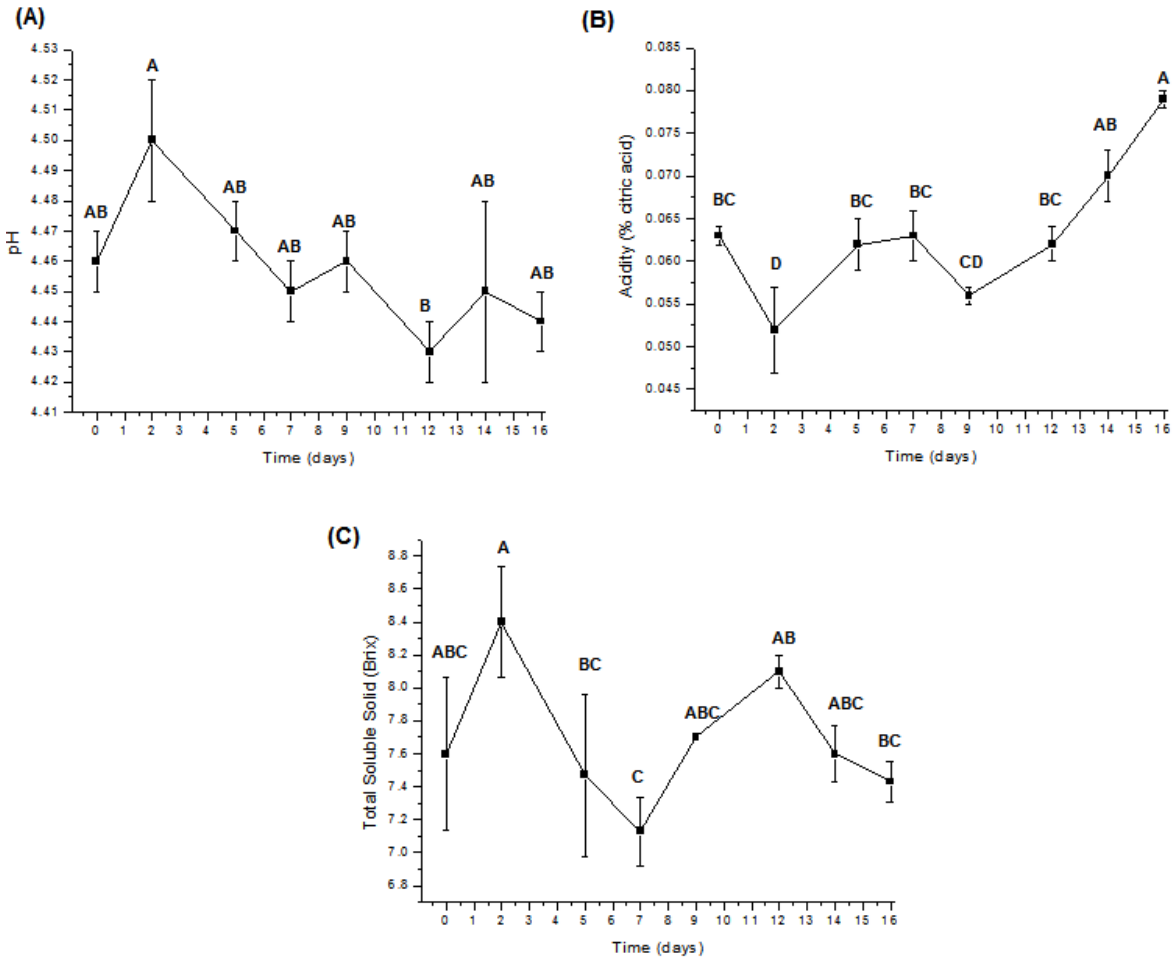
3.1. Physic-chemical characterization of kefir and carbonated water added with anthocyanin extract

Several studies that were conducted on research of anthocyanin natural dyes indicate that is important to analyze the main factors that can affect the stability of anthocyanins throughout the storage process, among these are the low pH values and an acidified medium (Delgado-Vargas et al., 2000; Konczak & Zhang, 2004).

The definition of temperature and experiment time was based on previous tests. In addition, in another study about the application of anthocyanins in an aqueous matrix by de Rosso & Mercadante, (2007) was reported an half-life time between 10 and 30 days during storage at room temperatures (20 °C ± 1 °C). This behavior showed that is possible to use a higher temperature to obtain reliable data of anthocyanin retention in an experiment with accelerated conditions of storage during less time.

The pH levels of kefir did not show significant differences ($p>0.05$) over the 16 days of analysis (Figure 1). The pH value found in the kefir with natural dye addition was similar to values found in kefir without the addition of additives (4.5 to 4.7) in research by Irigoyen et al. (2005). During the 16 days, there was an increase in acidity until reaching 0.08% of tartaric acid on the last day of analysis. Beal, Skokanova, Latrille, Martin, & Corrieu, (1999) explained that the increase in this value is related to high bacterial metabolic activity and its consumption of lactose in the initial days of storage.

In relation to the results of total soluble solids (TSS), the sample presented a decrease on the day 7 ($p > 0.05$). In the study carried out by Tseng & Zhao, (2013), were analyzed the TSS values of yogurt storage that contained the liquid extract of grape pomace over 21 days, and the authors reported similar data with the obtained in our research. However, in this study the measurements de did not show a significant difference in the evaluation of the day 7 of storage in relation to time zero, there was a significant difference only in the day 14 of storage. In addition, total soluble solids in foods mainly include sugars, thus, tests for enriching yogurt with grape pomace suggest a greater preference on the part of consumers in products with high amounts of sugars. This is because the increase in an amount of total soluble solids leads to a decrease in the perception of the bitter taste of kefir (Li, Sun, & Cheng, 2016; Marchiani et al., 2016).



Note:Different letters indicate significant differences among groups at P < 0.05.

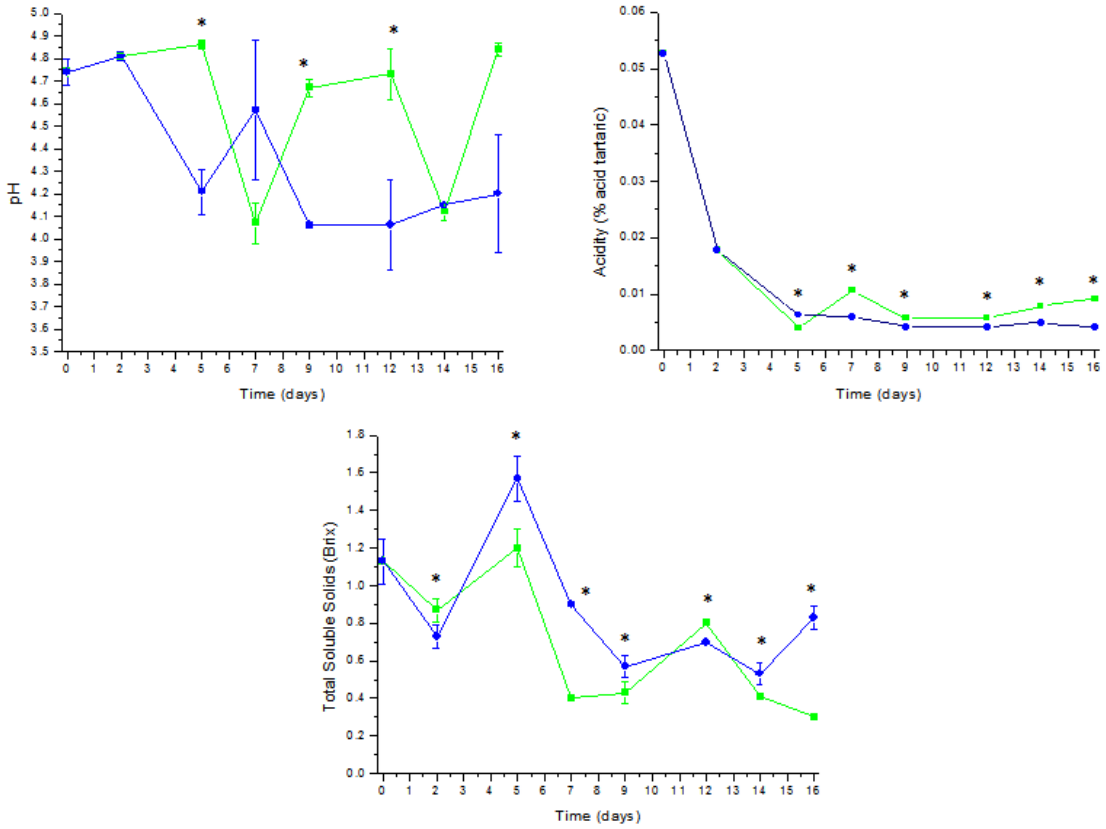
Figure 1. Values of Ph (A), acidity (% citric acid) (B), total soluble solids (°Brix) (C) in kefir colored with grape skin extract

The pH values of the carbonated water samples throughout storage under light exposure or in the dark did not show significant differences on the last day of analysis ($p>0.05$) between the different light conditions; these results were 4.53 and 4.84, respectively (Figure 2). In addition, the samples of both conditions did not present significant differences from the pH value of time zero until the day 5, 9 and 12 ($p>0.05$). The sample that was stored at light exposure had significant decreases in its

values on the day 7 and 14 of analysis ($p \leq 0.05$). For samples stored in absence of light, there was a significant decrease in pH only on the day 9 ($p \leq 0.05$). These oscillations between values probably occurred because tartaric acid is effective in maintaining pH values and acidic conditions (Cavdarova & Makris, 2014).

Throughout the experiment time, the acidity analyses showed significant variations in pH value, these results corresponded to the increase of tartaric acid value on day 7 and 14. In addition, there was a significant increase in values on the day 16. In the samples stored in absence of light, the values of tartaric acid had a significant decline only in the day 2, highlighting that after this first decrease there were no significant differences during the remaining days of experimentation.

Comparing the storage light conditions, the values of tartaric acid were higher for samples stored on light exposure, exact on the day 5 which the sample showed the high value of tartaric acid percentage. In the remainder of the experiment, the carbonated water samples stored in the dark showed higher values than carbonated water stored on light exposure. The values found after 16 days of analysis were 0.009 ± 0.001 % for storage in the light exposure and 0.004 ± 0.001 in the absence of light. Both samples contained 0.053 ± 0.002 % tartaric acid at the zero analysis time



Note: *significantly differ from Carbonates water stored in light and dark ($p<0.05$).

Figure 2. Mean values ± standard deviations for pH, acidity (% acidity in lactic acidity) and total soluble solids (ºBrix) of carbonated water (●) stored in light exposure and (■) on the dark during storage period.

The value of TSS in foods is largely related to the presence of soluble sugars such as glucose and fructose. In this case, the values of soluble solids may depend directly on the presence of certain glycosylated anthocyanins that promotes a consequently increase in the content of coloring (Koshita, Yamane, Yakushiji, Azuma, & Mitani, 2011; Li et al., 2016).

In the samples of carbonated water, there was a significant increase of the values of

TSS in the day 5 for both samples. For the sample stored in light exposure, there was an increase in the TSS values also on the day 12, and for the sample stored in absence of light, the increase occurred again only on the last day of analysis. In spite of this, there was a gradual decrease in the values, at zero time both had a value of 1.13 ° Brix and at the end of the analysis, the results were 0,30 and 0,83 (° Brix) for carbonated water stored in the dark and, in the light exposure, respectively. The values for samples stored in absence of light were remained higher compared to samples stored in the presence of light ($p \leq 0.05$) except on days 2 and 12. At the end of the 16 days of analysis, there was a retention of 70% of soluble solids for the sample stored in light exposure and only 26% for the sample in the absence of light. In this way, the sample stored in the dark probably maintained a higher amount of sugars.

3.2. Color retention on samples of kefir and carbonated water with addition of anthocyanin extract

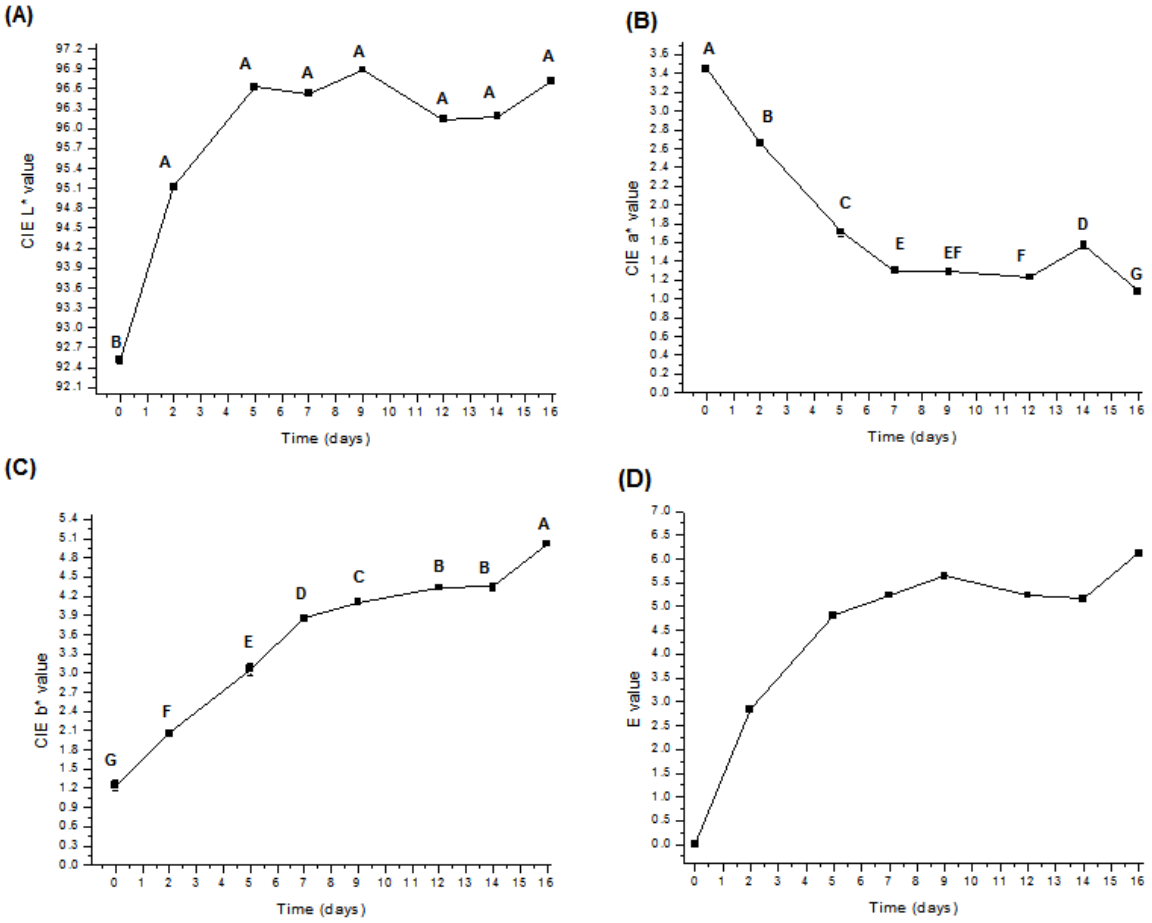
In the recent years increased the interest of the scientific community to looking forward natural alternatives as food additives, in order to benefit the population to access a healthier diet. Among these researches, we can highlight the development of natural pigments as from the most different residues of the food industry (Wrolstad & Culver, 2012). In order to these developed natural dyes can be applied in food, it is important to analyze the color stability of the product after application. In relation to pigments from anthocyanin extracts, this stability during storage is linked to the pH values and structure of the anthocyanins (Dai, Gupte, Gates, & Mumper, 2009). In this way, this work evaluated the color stability in the food matrices as kefir and

carbonated water throughout the storage time.

In addition to the CIELAB parameters, the overall color difference (ΔE) was evaluated over the 16 days of storage. The ΔE value denotes a difference in color between two samples, in the range of 0 to 0.5 signifies an imperceptible difference in color, 0.5 to 1.5 a slight difference, 1.5 to 3.0 a just noticeable difference, 3.0 to 6.0 a remarkable difference, 6.0 to 12.0 an extremely remarkable difference, and above 12.0 a color of a different shade (Kim, Park, & Hwang, 2002), it means that a high ΔE implies a significant color variation.

For kefir samples, the parameter L * had a significant increase in the day 2, after this time it remained stable until the end of the storage. The parameter a * and b * had a significant decrease and increase over the storage, respectively. Regarding the ΔE parameter, the samples obtained a significant difference of perception during all days of storage, except on the second day with noticeable difference (2.84) and last day extremely difference (6.12) of analysis (Figure 3).

This color loss is typical in food matrices added of natural extracts over the days of storage, this behavior was already reported in other studies and is one of the major limitations in relation to the application of this product on a large scale. Therefore, its necessary a further research to prolong the stability of coloration (Kammerer, Schillmöller, Maier, Schieber, & Carle, 2007; Wallace & Giusti, 2008).



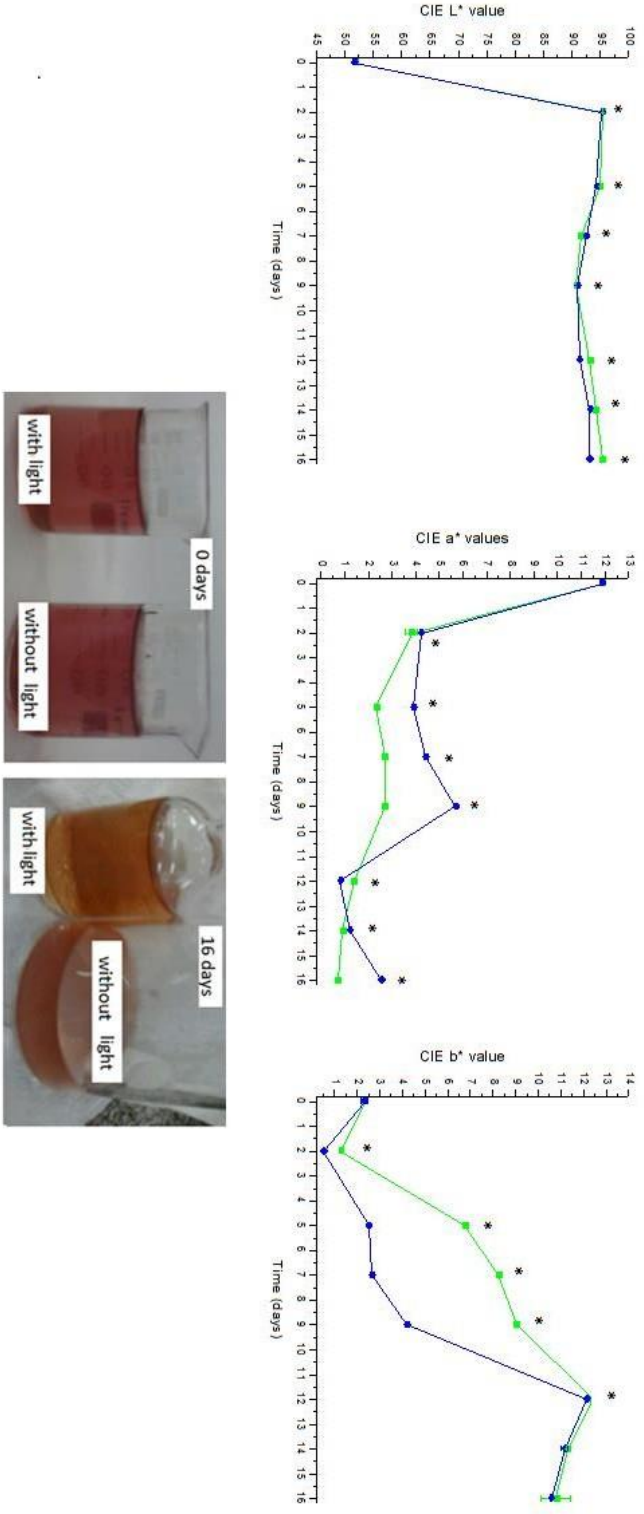
Note: Different letterS indicate significant differences among color groups at P < 0.05.

Figure 3. Values (± standard deviation) of lightness (L*) (A), redness (a*) (B), yellowness (b*) intensity (C) and overall change in color (ΔE) (D) of kefir with anthocyanin extract as coloring during storage period.

In relation to parameter L * in the carbonated water, in general, both samples had a significant increase, with initial values of 51.69 and final values of 95.39 and 92.99 for samples stored in light exposure or in the dark, respectively. In addition, there was a significant difference between these values during all time of analysis. As expected, the values of a * had a decrease and there was an increase of the b * values for both storage conditions. This directly affected the perception of coloration as can be observed in the images zero and 16 days analysis (Figure 7894).

The values of parameter a* at time zero were 11.88 and were changed to 0.70 in samples stored in the dark and 2.52 in light exposure. The values of parameter b * at time zero were from 2.32 to 10.78 and 10.57 respectively in the light exposure and absence of light, and there were no significant differences between these values in the last days analysis (14 and 16 days). All values of total color difference (ΔE) presented values above 12, reaching a different shade of color throughout the storage time, for samples stored in light exposure and in absence of light.

The a * values of carbonated water showed a pronounced changes between days 9 and 12, but still remaining in the same coloration spectrum, i.e. at final day of experimentation did shows statistical differences between samples stored in light exposure or in the dark (Figure 4). This change of color tone of red to green is connected to degradation of anthocyanins present, and that this degradation was also observed in study Rosso & Mercadante (2007).



Note: *significantly differ from Carbonates water stored in light and dark ($p<0.05$).

Figure 2. Mean values ± standard deviations of lightness redness (a*) and yellowness (b*) intensity of carbonated water (●) stored in light exposure and (■) on the dark during storage period and images for 0 – 16 days of storage.

The values of parameter b* of carbonated water stored in light exposure presented double value (6.75) compared to stored in dark (3.91). On the 14 and last day of storage the sample stored in light exposure and dark showed not difference value ($p>0.05$) (Figure 4).

According to Hubbermann, Heins, Stöckmann, & Schwarz, (2006), the stability of the reddish coloration in aqueous samples can be influenced by the presence of sugars, because they decrease the water activity present in the sample and directly affect the stability of the anthocyanins in the solution. This can be observed in the model of storage in the dark, which showed a stability between values found from the days 5 to 7 of storage, which corresponds the increase of the TSS values on the day 5 of sampling. The same behavior did not occur in the sample stored in light exposure, probably because this parameter significantly affects the stability of anthocyanins in solutions.

3.3. Content of individual and total anthocyanins in kefir and carbonated water added with grape skin extract

Kefir is already considered a healthy food because inhibit pathogenic microflora, like Salmonella (White, Kilara, Hui, & Chandan, 2008). The addition of anthocyanins extracted from grapes as a natural dye may transform it into a functional food with antioxidant properties (Konczak & Zhang, 2004).

The anthocyanins found in kefir colored with anthocyanin extract were in descending order malvidin-3-glycoside, delphinidin-3-β-D-glycoside, peonidin-3-glycoside and an unidentified anthocyanin with a considerable amount.

Throughout storage, the degradation of anthocyanins in kefir had a kinetic behavior that follows the first-order reaction, in agreement with other studies carried out by Gris, Ferreira, Falcão, & Bordignon-Luiz, 2007; Hubbermann et al., 2006 and Maier, Fromm, Schieber, Kammerer, & Carle, 2009. The data of anthocyanin retention against storage time was used to perform the degradation curves and calculate the kinetic constant (kt) through an exponential fit. The curves showed a good correlation factor in the range ($0.93<R^2<0.96$). Then, the k_t value was used to calculate the time necessary to reach 50% of degradation ($t_{1/2}$) (Table 3).

As a result of anthocyanins quantification during the 16 days of storage, the anthocyanin with the highest losses was delfinidin-3-b-glucoside, reaching the half-life from the day 12. However, all other anthocyanins in this study showed the greater stability of 60% over the 16 days of storage, with peonidin-3-glycoside maintaining 89% stability after the day 16. The half-life of peonidine was the longest, approximately over 3 months. Nevertheless, the $t_{1/2}$ value of total anthocyanin content was less because the peonidine was not the anthocyanin present in higher quantity in the extract. However, total anthocyanins maintained a stability of 67% over the time of storage and the $t_{1/2}$ was approximately one month (27 days). Which was similar to the anthocyanin of greater quantity in the extract, i.e. the malvidin-3-glycoside with a $t_{1/2}$ almost of 24 days (Figure 5).The use of anthocyanins from grape skin as a natural dyes may be replace the synthetic dyes and could be evaluated the incorporation of other compounds to preserve and improve the color stability.

In a research conducted by (Karaaslan et al., 2011), the significant loss of anthocyanins during 14 days of storage of yogurt was also reported. In this study, an acidified ethanol extraction was used due to the presence of phenolics in yogurts.

Residues solids from grape were used to produce anthocyanins, testing Cabernet Sauvignon, Chardonnay, Shyrah and Merlot varieties. For this study, the cultivar Cabernet Sauvignon showed the greater presence of general phenolic compounds. Thus, it is recommended to use the fruit and residues solids of this variety in order to extract anthocyanins to be added as a natural dye in yogurts

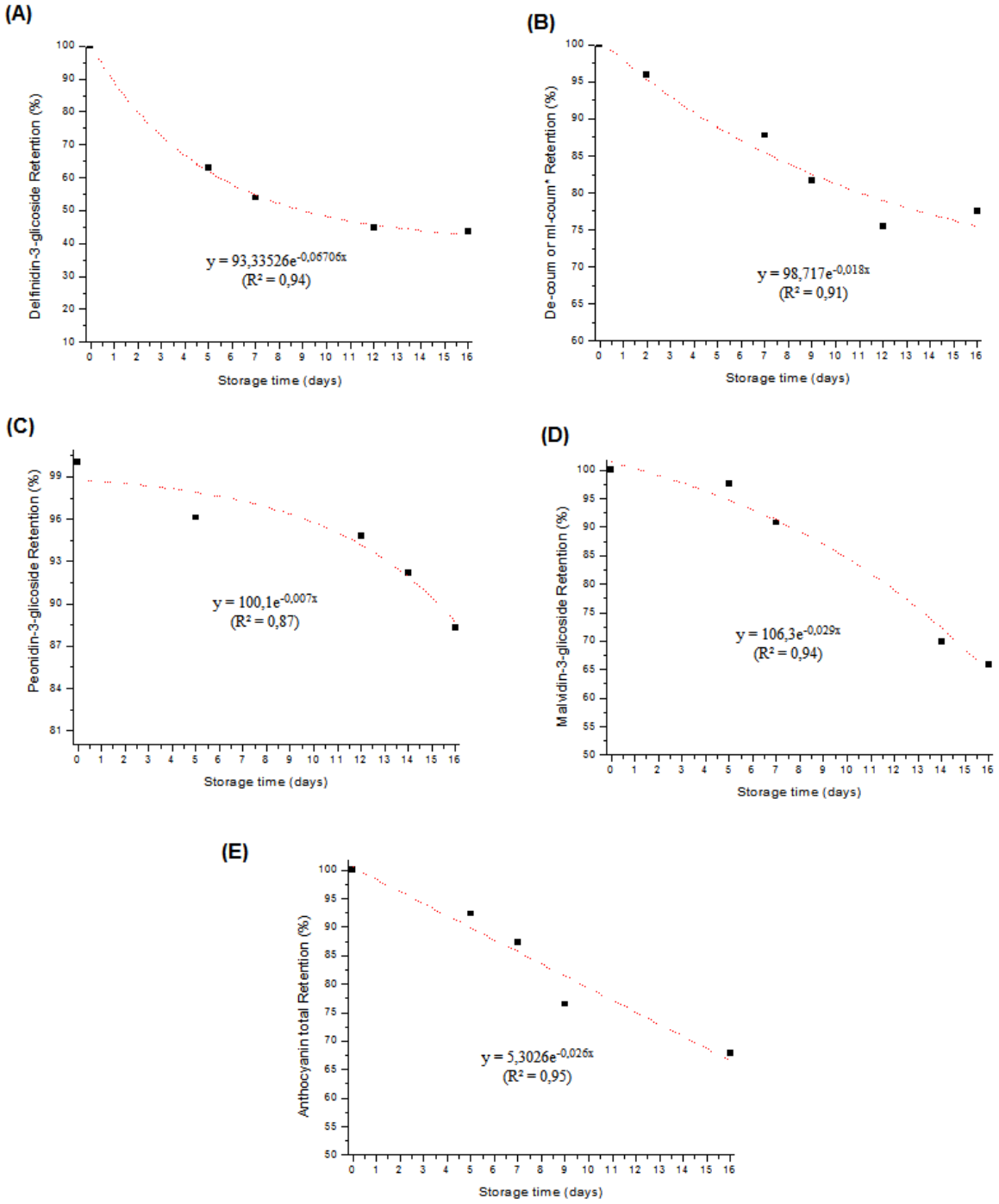


Figure 5. Percentages values of delfinidin-3-glicoside (A), delphinidin-3-0-p-coumarylglucoside or malvidin-3-0-p-coumarylglucoside (B), peonidin-3-glicoside (C), malvidin-3-glicoside (D), and total anthocyanins (E) in kefir with colorant stored in the dark for 0 – 16 days of storage.

The anthocyanins found in the extract and added in the carbonated water were, in

descending order, malvidin-3-glicoside (3.67 ± 0.01 mg/100 mL carbonated water), non-identified anthocyanin (delphinidin-3-0-p-coumarylglucoside or malvidin-3-0-p-coumarylglucoside) (2.19 ± 0.02 mg/100 mL carbonated water) and peonidin-3-glicoside (0.83 ± 0.00 mg/100 mL carbonated water).

In analyses for anthocyanin quantification in carbonated water, the anthocyanins had a retention time of 6.5 minutes and maximum λ in 279, 373 e 531 nm. One of the non-identified anthocyanins that were added in high quantities was responsible for this measurement. Among the different anthocyanins that were found, malvidin-3-glycoside showed the highest stability in the samples of carbonated beverage, which obtained about 3 times more $t_{1/2}$ when was stored in the dark (15.05) than in the light exposure (4.47). For peonidin-3-glycoside it obtained double the $t_{1/2}$ for samples stored in the dark (12.84) than in the light exposure (4.80). The anthocyanin with shorter $t_{1/2}$ was the non-identified anthocyanin with a $t_{1/2}$ of 6 days for samples stored in the dark and 4 days for storage in the light exposure (Figure 6).

In order to achieve a red coloration are commonly used synthetic dyes as the Red 40 (E129) and Bourdeaux S (E123), the high consumption of products with synthetic colorants is related to diseases linked to allergies in susceptible people (De Andrade et al., 2014). Therefore, The Brazilian National Agency for Public Health Surveillance (ANVISA) established legal regulations about the use of synthetic food dyes in non-alcoholic beverages. According to regulation the anthocyanins (E163i) can be added without restriction (*quantum satis*) while the maximum levels accepted of Red 40 and Bourdeaux S are 0.01 g/100 mL and 0.005 g/mL of beverage, respectively (ANVISA, 2007). In our research was possible reach to a desired tonality (red) by using a less quantity of anthocyanins (approximately 7 mg/100 mL).

In dairy drinks there is no restriction legislation about the addition of natural dyes, however, according to Normative Instruction MAPA nº 16 of 08/23/2005 a maximum level of 0.005 g/100 mL of Red 40 or Ponceau 4R can be added in this food matrix. It is noteworthy that these recommendations follow health ideas due to possible harmful effects of the excessive consumption of these artificial dyes, while the natural dye application reported in this study may cause health benefits due to the beneficial properties of the bioactive compounds present in the coloring (MAPA, 2005).

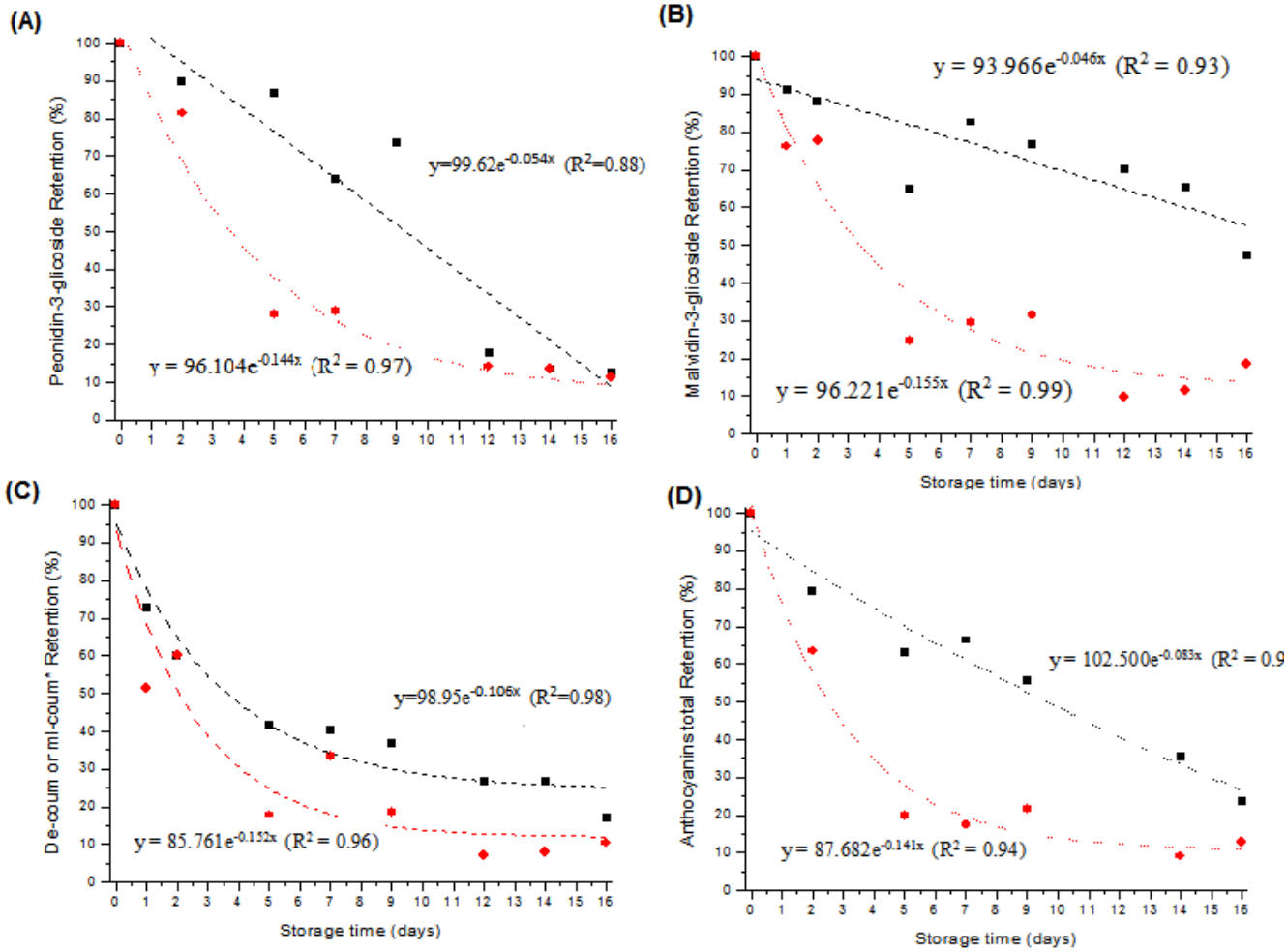


Figure 6. Percentages values of peonidin-3-glicoside (A), malvidin-3-glicoside (B), delphinidin-3-0-p-coumarylglucoside or malvidin-3-0-p-coumarylglucoside (C) and

total anthocyanins (D) in carbonated water with colorant (● - red) stored in the light exposure and (■ - black) stored in the dark for 0 – 16 days of storage.

Comparing with the study conducted by de Rosso & Mercadante, (2007), who studied the stability of anthocyanins in isotonic beverages based on acerola, the authors reported similar results to the peonidian-3-glucoside responses of our study. In this study, the half-life time of the anthocyanins for samples stored in the light exposure and in the dark was from 10 to 11 days. In addition to analyzing the anthocyanin behavior in the aqueous model (beverages), also were analyzed under conditions with buffer (pH = 2.5) which showed an improvement of about 70% in stability during storage time. This result indicates that for future studies it would be interesting to look for an aqueous system with lower pH for assessing the application of these anthocyanin extracts.

4. Conclusion

The stability of the anthocyanins followed a kinetics of the first-order reaction and had different responses depending on the matrix in which it was applied, kefir or carbonated beverage. In relation to color properties, further studies are needed to increase their stability. The kefir with the addition of anthocyanins as a natural dye had physical properties similar to natural kefir without additives according to literature. The light exposure of carbonated beverage during storage significantly affected several parameters, among these; the main was the significant loss of coloration at the end of the 16 days of storage. Thus, it is recommended to store carbonated beverage added of anthocyanins as a natural dye in amber bottles.

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CAPÍTULO 5 – DISCUSSÃO GERAL E CONCLUSÃO

5.1. Discussão geral

O bagaço é constituído de diversas partes da videira e muitas vezes não apresenta um padrão nos porcentuais de cada um dos componentes bioativos no todo, devido as características individuais de cada uma das partes da planta. Para todas as análises citadas no trabalho foi usado especificamente a casca de uva, como forma de evitar grande variedades de resposta em relação a parâmetros de quantificação antocianinas e respostas físico-químicas. Vale ressaltar que a escolha foi devido a necessidade de homogeneização de amostras, sendo que na indústria é recomendável o uso do bagaço por inteiro (incluindo engaço e sementes).

Além disso, a maioria dos estudos se baseia em amostras secas e/ou produzia o bagaço por meio de prensagens laboratoriais, o que pode não favorecer a realidade de bagaços produzidos após processo industrial. Isso devido ao muito do bagaço da produção de vinho ou suco durante prensagem ter a adição de enzimas, o que não ocorria na escala laboratorial. Nesse estudo a casca utilizada foi úmida, o que é pouco encontrada nas metodologias de extração na literatura. A escolha desse diferencial foi para facilitar uma futura aplicabilidade industrial além de evitar perda de antocianinas durante a secagem em estufa ou liofilização, sendo a última uma forma custosa em grande escala.

O processo de extração enzimática pode ser realizado com sucesso desde que estabelecidas condições ideais de cada variável que influencia no resultado final. Um estudo da quantidade de enzima e tempo de ação da mesma representa uma etapa fundamental, bem como a escolha da enzima correta, uma vez que no mercado encontram-se diversas marcas e fabricantes. Assim, a pesquisa com a seleção prévia do tempo de extração e do preparado enzimático que deveria ser utilizado. Para isso foram obtidas diferentes amostras de preparado enzimáticos semelhantes aos presentes na literatura. O tempo de extração em estudos iniciais foi de cerca de três a seis horas, isso devido a recomendação de artigos base. Porém após testes laboratoriais foi observado que a extração máxima de

antocianinas totais se concentrava na primeira hora de extração.

Além disso, foram testados oito preparados e apenas Pectinex Smash XXL®, Novozym 33095®, Pectinex Ultra SPL® e Pectinex Ultra Color® possuíram respostas prévias que recomendaram seu uso na pesquisa. Não tiveram respostas recomendáveis para o estudo Lallzyme Beta®, Pectinex Ultra Clear®, Pectinex BE XXL® e Rohapect 10L®.

Assim, o resultados da pesquisa indicaram como melhor tempo de extração 30 minutos e uso de preparado enzimático Pectinex Ultra Color®. Para esse estudo foi determinado o uso de amostras cultivar Bordô/Ives devido a sua alta quantidade de antocianinas presentes e produção comum no Brasil. A alta quantidade de preparado enzimático (1,25% em relação a matéria úmida) foi determinada como forma de garantir extração total das antocianinas presentes.

Mesmo após definido uma enzima de ação adequada para o processo de extração, verificou-se a necessidade de analisar de forma minuciosa outros parâmetros, como concentrações dos preparados enzimáticos e determinação de temperatura exata dentro da faixa ótima recomendada pelo fabricante. Destacou-se nesse experimento que houve diferentes respostas dos diversos cultivares de bagaço de uva com uso de mesma metodologia de extração. Esse fator foi considerado relevante na pesquisa, pois não foi encontrado na literatura comparação de diferentes espécies de uva em relação ao método de extração enzimática.

As condições estabelecidas pela ação da enzima deve ser avaliada por experimentos independentes e o uso de um planejamento experimental pode ser de grande utilidade. Tal procedimento experimental usado para avaliar essa condições indicou um tempo de extração de 30 minutos na temperatura de 40°C, sendo que tal temperatura não dever ser maior por degradar as antocianinas.

Após análise minuciosa dos aspectos relacionados a melhora da metodologia de extração, é relevante discutir quais alimentos poderiam ser favorecidos com a adição desse corante natural e atóxico. Por meio de dados obtidos das características do extrato e análise dos alimentos presentes na literatura, foi decidido a aplicação em dois alimentos como sugestão de atender

tanto o consumo de uma população mais jovem quanto a adulta.

Dessa forma o quefir foi selecionado devido a propriedades como perfil de sabor ácido que auxiliaria na estabilidade das antocianinas e na coloração, mas não interferiria no sabor do produto. Para atender a população mais jovem, foi aplicado o corante em bebida carbonatada com visão de diminuir o consumo de corantes sintéticos durante infância e adolescência. Para atender um público mais adulto foi decidida adição em quefir. Foram analisadas propriedades físico-químicas dos produtos ao longo de seu armazenamento e manutenção de antocianinas do mesmo. A presença de luz afetou de forma negativa o perfil de cor da bebida carbonatada e houve diferenças no tempo de meia-vida em relação as antocianinas individuais de ambas matrizes alimentares.

5.2. Conclusão

As melhores condições da extração enzimática apresentaram diferenças em pontos de temperatura e porcentagem de preparado enzimático com maior resposta de extração de antocianinas dependendo do cultivar de estudo. Além disso, a eficiência de recuperação analisada em relação a extração exaustiva foi essencial para determinar qual melhor aplicabilidade de escolha de matéria prima. Em relação a esse parâmetro foi destacado resultados de extrato de Ives (3), que apesar de possuir altas quantidades de antocianinas em seu extrato, a eficiência de recuperação encontrada foi abaixo de 10%. Além disto, é importante destacar que entre as características físico-químicas, nos quais os parâmetros de cor foram semelhantes a de corantes artificiais. A subsequente aperfeiçoamento do procedimento escolhido da cultivar Cabernet Sauvignon garantiu a extração máxima das antocianinas com uso de extração enzimática dentro dos pontos selecionados.

A posterior aplicação do extrato antociânico foi favorável em bebida carbonatada e quefir, sendo recomendado estudos futuros visando o aumento do tempo de meia vida da estabilidade das antocianinas adicionadas nestas matrizes alimentares. Em relação aos parâmetros físico-quimicos estudados nestas matrizes alimentares destaca-se que o quefir com adição do corante manteve características semelhantes ao encontrado na literatura para quefir natural. Nas análises da bebida carbonatada foi destacado resultados de coloração, com maior estabilidade de antocianinas para amostras armazenadas sem presença de luz.

Por fim, foi comprovada potencialidade da extração enzimática por meio de aperfeiçoamento de processo e sua aplicação favorável em bebida e produtos lácteos ácidos.

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