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Gabriel Moraes Reis

Vitis vinifera L. cv Pinot Noir: BAGAÇO E BORRA DO VINHO COMO POTENCIAL FONTE DE COMPOSTOS BIOATIVOS

Santa Maria, RS
2016

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Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Ciências Farmacêuticas, Área de Concentração em Controle e Avaliação de Insumos e Produtos Farmacêuticos da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Mestre em Ciências Farmacêuticas**.

Orientadora: Prof. Drª. Carine Viana Silva

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Santa Maria, RS
2016

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RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Ciências Farmacêuticas
Universidade Federal de Santa Maria

Vitis vinifera L. cv Pinot Noir: BAGAÇO E BORRA DO VINHO COMO POTENCIAL FONTE DE COMPOSTOS BIOATIVOS

AUTOR: GABRIEL MORAES REIS
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Data e Local da Defesa: Santa Maria, 01 de julho de 2016.

Indústrias alimentares e agrícolas geram quantidades substanciais de resíduos que poderiam ser fontes naturais de antioxidantes ricos em compostos fenólicos. O objetivo deste estudo foi identificar e quantificar os compostos fenólicos e atividades antiradicalar de dois subprodutos (bagaço e borras) de *Vitis vinifera* L. cv Pinot noir. Encontramos uma distribuição diferente das classes de fenólicos (flavonóides, flavonóis, ácidos fenólicos e estilbenos) e atividade radicalar singular contra os radicais livres (hidroxil, superóxido e radicais peroxil). A principal classe de compostos fenólicos em bagaço foi flavanóis e na borra do vinho foram flavonóis, como a catequina (117,9 ± 2,5 mg g^{-1}) e quercetina (42,4 ± 1,2 ug g^{-1}) sendo os compostos individuais mais abundantes, respectivamente. Encontramos também um elevado potencial em atividade sequestradora contra os radicais superóxido no bagaço (80%) e peroxil (67%) na borra. Estes resultados mostram a possibilidade de utilização de Pinot noir subprodutos como aditivos promissores ou como uma fonte para o desenvolvimento de novos produtos em diferentes segmentos da indústria alimentícia e cosmética.

Palavras chave: resíduos de vinificação. Quercetina, Catequina, atividade antiradicalar

ABSTRACT

Master Course Dissertation
Graduation Program in Pharmaceutical Science
Universidade Federal de Santa Maria

***Vitis vinifera* L. cv Pinot Noir: POMACE AND WINE LEES AS POTENTIAL SOURCE OF BIOACTIVE COMPOUNDS**

AUTHOR: GABRIEL MORAES REIS
ADVISER: CARINE VIANA SILVA
Defense Place and Date: Santa Maria, July 01st, 2016.

Food and agricultural industries generate substantial quantities of phenolic-rich by-products that could be valuable natural sources of antioxidants. The aim of this study was to identify and quantify the phenolic compounds and radical scavenging activities of two by-products (pomace and lees) from *Vitis vinifera* L. cv Pinot noir. We found a different distribution of phenolic classes (flavanols, flavonols, phenolic acids and stilbenes) and singular scavenging activity against free radicals (hydroxyl, superoxide and peroxyl radicals). The major class of phenolics in pomace was flavanols and in lees was flavonols, with catechin (117.9±2.5 μg g^{-1}) and quercetin (42.4±1.2 μg g^{-1}) being the most abundant individual compounds. We also found high potential on scavenging activity against superoxide radicals in pomace (80%) and radical peroxyl (67%) on lees. These results show the possibility of using Pinot noir by-products as promising additives or as a source for the development of new products in different segments of the food and cosmetic industries.

Keywords: winery by-products, quercetin, catechin, radical scavenging activity

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LISTA DE ABREVIATURAS E SIGLAS

GAE	Equivalentes de ácido gálico / acid galic equivalent
GP	Bagaço de uva / grape pomace
GS	Engaço / grape stalk
LC-MS/MS	Cromatografia de alta eficiência acoplada a espectrômetro de massa / Liquid chromatography coupled mass spectometry
WL	Borra de vinho / Wine lees

SUMÁRIO

1 INTRODUÇÃO

A crescente demanda por alimentos seguros vem sendo fortemente acompanhada pela busca por processos limpos de produção, acarretando custos cada vez maiores para a indústria de alimentos no tratamento dos resíduos líquidos e sólidos que são gerados. Esse é o caso da indústria vinícola, que responde por um volume substancial de resíduos orgânicos sólidos. O bagaço de uva como subproduto representa aproximadamente 20% das uvas colhidas (LAUFENBERG; KUNZ; NYSTROEM, 2003).

A recuperação de compostos a partir dos desperdícios da indústria de vinho poderia representar um avanço significativo na manutenção do equilíbrio do meio ambiente, visto que nas vinícolas as grandes quantidades de resíduos gerados representam problemas de armazenagem, de transformação, e de eliminação, em termos ecológicos e econômicos. Esta situação explica o interesse crescente em explorar os subprodutos da vinificação (XUEMING, 2010).

O bagaço da uva consiste das cascas, do talo e das sementes dessa fruta, que sobram após o processo industrial para a produção de vinhos e sucos (MONRAD et al., 2010). No Brasil, a maior parte do bagaço de uva gerado na produção de vinhos, próximo de 59,4 milhões de quilos, considerando 18 kg de bagaço/100 litros de vinho, é tratada como resíduo com baixo valor, sendo utilizado, por exemplo, para a ração animal (ROKENBACH et al., 2011). Sementes e cascas de uvas são onde a maior parte dos compostos fenólicos se acumula. Por essa razão, o extrato obtido do resíduo da uva tem se tornado uma fonte para a obtenção de ingredientes funcionais, tais como antioxidantes naturais e suplementos alimentares (BAGCHI et al., 2000; SHRIKHANDE, 2000; XU et al., 2010).

Após a produção de sucos e vinhos, o bagaço de uva tipicamente retém compostos fenólicos em quantidade de 20 – 30 % nas cascas e 60 – 70 % nas sementes. Entre estes, os flavonoides reconhecidos por apresentar diversas atividades biológicas tais como antioxidante, antimicrobiana, capacidade de sequestrar espécies reativas de oxigênio e eletrólitos, capacidade de inibir a nitrosação, capacidade quelante de metais e pode modular a atividade de algumas enzimas celulares (MONRAD et al., 2014).

As propriedades benéficas de extratos de resíduos de vinícolas estão recebendo atenção não só da comunidade científica, mas também de indústrias setoriais, envolvidos na valorização dos recursos de compostos bioativos. Neste contexto faz-se necessária a caracterização qualitativa e quantitativa dos compostos (poli) fenólicos dos resíduos de vinificação para que possa viabilizar a utilização. A presente dissertação está organizada na forma de artigo científico. Inicialmente é apresentada a introdução, o objetivo geral, os objetivos específicos e a revisão da literatura. Na sequência é apresentado o artigo científico referente aos resultados experimentais desse trabalho, seguido da conclusão da pesquisa realizada.

1.2 OBJETIVOS

1.2.1 Objetivo Geral

Avaliar a composição de bioativos dos resíduos de vinificação de uvas finas, bem como sua atividade antiradicalar *in vitro*.

1.2.2 Objetivos específicos

- Determinar os teores totais dos compostos fenólicos dos resíduos de vinificação.

- Investigar a atividade antioxidante dos resíduos de vinificação obtidos por métodos *in vitro* baseados na geração dos radicais superóxido, hidroxila e peroxila.

- Caracterizar e quantificar dos compostos fenólicos presentes por cromatografia líquida com detecção por espectrometria de massas (LC-MS/MS).

2 REVISÃO BIBLIOGRÁFICA

2.1 PRINCIPAIS SUBPRODUTOS DA INDÚSTRIA VINÍFERA

A implementação da gestão de resíduos na indústria do vinho é um desafio, tornando necessário o desenvolvimento de procedimentos inovadores e eficazes. Neste sentido, a crescente demanda de produtos finais e a urgência de evitar problemas ambientais advindos desta atividade, houve um aumento nas exigências legais para garantir a eficiência dos processos e para apoiar as melhorias dos procedimentos de recuperação e reciclagem (SERRA et al., 2008).

Os tipos de resíduos produzidos dependem dos procedimentos específicos de vinificação, que também afetam as propriedades físico-químicas do material residual, cujas características determinam a sua utilização posterior e rota de valorização em que poderia ser integrado. Os principais resíduos da produção de vinho são representados por: resíduos orgânicos (bagaço de uva, contendo sementes e peles, caules e folhas), efluentes, emissão de gases (CO_2, compostos orgânicos voláteis, etc.) e resíduos inorgânicos (terra de diatomáceas, argila bentonítica, e perlita) (MUSEE; LORENZEN; ALDRICH, 2007). Sendo alguns desses resíduos citados na tabela 1 e compostos usualmente encontrados nestes subprodutos.

A valorização dos subprodutos se dá principalmente pela produção de fertilizantes do solo, bem como substrato de fermentação na produção de biomassa e de ração para gado (ARVANITOYANNIS; LADAS; MAVROMATIS, 2006; SRI HARSHA et al., 2013). No entanto, existem várias restrições para reutilizar estes materiais. Por exemplo, certos polifenóis presentes nos subprodutos são conhecidos por serem fitotóxicos e apresentar efeitos antimicrobianos durante a compostagem, prejudicando a sua utilização para esse fim. Quanto à sua utilização na alimentação animal, alguns animais mostram intolerância a certos componentes, como taninos condensados, que afetam negativamente a digestibilidade (GARCÍA-LOMILLO et al., 2014; GONZÁLEZ-CENTENO et al., 2014). Por isso, a valorização destes compostos fenólicos na aplicação em produtos farmacêuticos, cosméticos e indústrias de alimentos poderia constituir uma alternativa eficiente, rentável e favorável ao meio ambiente (MAKRIS; BOSKOU; ANDRIKOPOULOS, 2007).

Tabela 1 - Tipos de resíduos de vinificação

Resíduo de vinificação		Caracterização	Composição Química
Bagaço		É originado da prensagem das matérias-primas da vinificação, constituídas pelas partes sólidas das uvas e pelo mosto. Representa 12 a 15% em peso da matéria-prima inicial.	• Polifenóis • Polissacarídeos
Engaço		São os pedúnculos e ramificações da videira que sustentam as bagas de uva. Representam 2,5 a 7,5% em peso da matéria prima inicial.	• Ligninas • Polifenóis
Folhas		As folhas são grandes, alternas, pecioladas, cordiformes e com lóbulos dentados e pontiagudos. Ainda com poucas aplicações tecnológicas	• Terpenos • Polifenóis • Açúcares não redutores

Borra		São os resíduos formados na parte inferior dos tanques contendo vinho, após a fermentação, ou durante o armazenamento, bem como o resíduo obtido após a filtração ou centrifugação deste produto.	• Polissacarídeos • Polifenóis • Levedura • Matéria tartárica

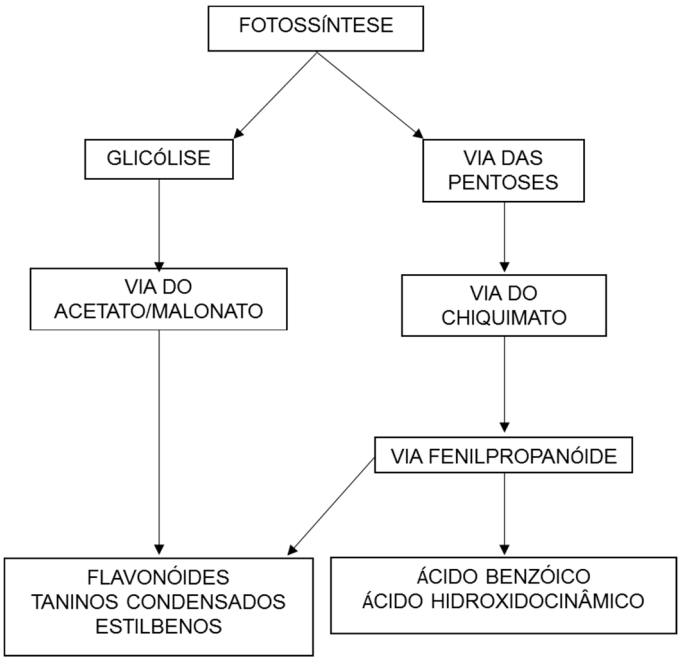
Fonte: (TEIXEIRA et al., 2014)

2.2 COMPOSTOS FENÓLICOS

Os metabolitos secundários possuem diferentes propriedades bioativas são encontrados predominantemente em frutas, legumes, raízes, folhas e semente desempenhando papeis fisiológicos e/ou morfológicos importantes no funcionamento e desenvolvimento das plantas em aspectos como estrutura, sistema de defesa, reprodução ou propriedades sensoriais (cor, amargor, adstringência e sabor) (ANASTASIADI et al., 2010). A concentração destes compostos depende de alguns fatores como técnicas de cultivo, cultivar, as condições de crescimento, processo de maturação, processamento e armazenamento, entre outros. Alguns fatores podem aumentar a presença destes como estresse, radiação UV, infecção por patógenos e parasitas, ferimento, poluição do ar e exposição a temperaturas extremas (BARCIA et al., 2014; GÓMEZ GALLEGO et al., 2012; ROCKENBACH et al., 2011a). Sendo os compostos fenólicos um exemplo destes.

Os compostos fenólicos são membros de uma classe de compostos naturais com grande interesse científico e terapêutico devido ao potencial anti-inflamatório, antiviral, antialérgico e antimicrobiano amplamente relatado na literatura (ARON; KENNEDY, 2008; BALASUNDRAM; SUNDRAM; SAMMAN, 2006). O termo “fenólico” ou “polifenólico” pode ser definido como sendo uma substância que tem em sua molécula com um ou mais núcleos aromáticos contendo substituintes hidroxilados e/ou seus derivados funcionais (ésteres, ésteres metílicos, glicosídeos e outros) (DIAS et al., 2016). Esses compostos são derivados de duas rotas sintéticas principais: a via do chiquimato e via do acetato. A Figura 1 demonstra genericamente a síntese desses compostos.

Figura 1- Rotas sintéticas dos compostos fenólicos



Fonte: AJILA et al,2011 (adaptado)

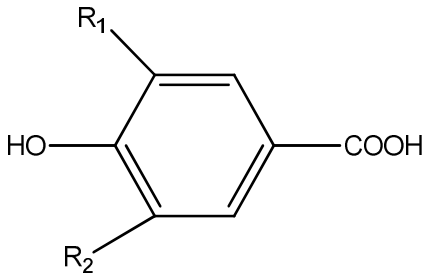
A atividade antioxidante dos compostos fenólicos é devido a capacidade de eliminar as espécies reativas, doar átomos de hidrogênio ou elétrons, ou quelar cátions metálicos. A estrutura dos compostos fenólicos é um fator determinante da sua atividade de captura de radicais e quelante, no caso dos ácidos fenólicos, por exemplo, a atividade antioxidante depende dos número e posição dos grupos hidroxila em relação ao grupo funcional carboxila. O potencial antioxidante dos ácidos fenólicos aumenta de acordo com o grau de hidroxilação, como é o caso do ácido gálico que é tetrahidroxilado. Nos flavanóides a estimativa do potencial antioxidante torna-se mais complicada devido à complexidade estrutural a qual pode ter diversos padrões de substituições possíveis nos grupos hidroxila podendo ser metilado, acilado ou sulfatado (BALASUNDRAM; SUNDRAM; SAMMAN, 2006).

As classes de compostos fenólicos abordados neste estudo foram alguns derivados do ácido benzóico e hidroxicinâmico, flavonóis, flavanóis e estilbenos. A tabela 2 elenca compostos e suas respectivas classes.

Tabela 2 – Principais compostos fenólicos encontrados nos resíduos de vinificação

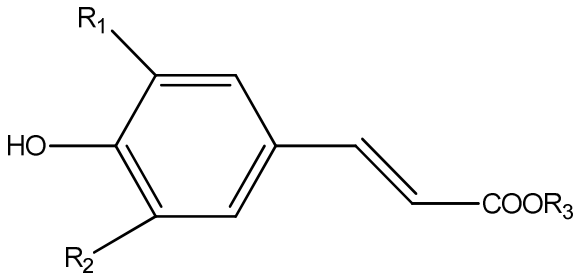
CLASSES/FÓRMULA ESTRUTURAL	**COMPOSTOS FENÓLICOS**	(continua)

Ácido Hidroxibenzóico



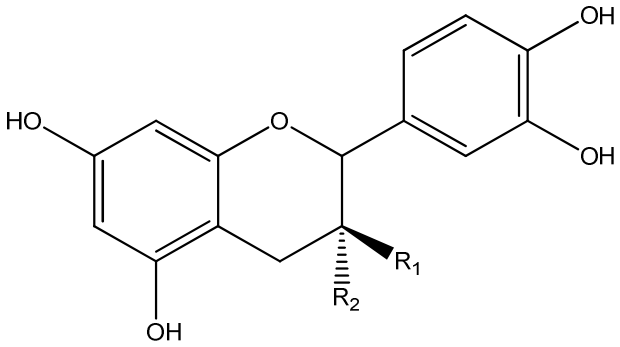
Composto	R_1	R_2
Ácido Gálico	OH	OH

Ácido Hidroxicinâmico



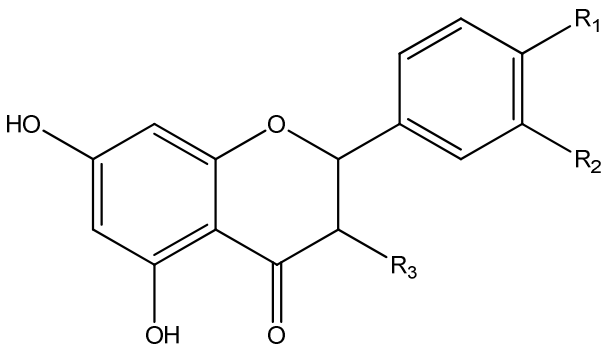
Composto	R_1	R_2	R_3
Ácido Cafeíco	OH	H	H
Ácido p-Coumárico	H	H	H
Ácido Ferúlico	OCH_3	H	H

Flavanóis



Composto	R_1	R_2
Catequina	OH	H

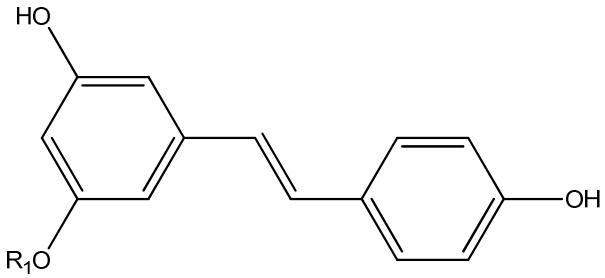
Flavonóis



Composto	R_1	R_2	R_3
Canferol	OH	H	H
Quercetina	OH	OH	H
Rutina	OH	OH	*

*Rutinose

Estilbenos



Composto	R_1
Resveratrol	H

2.3 AVALIAÇÃO DA ATIVIDADE ANTIRADICALAR

As moléculas orgânicas e os átomos que contém um ou mais elétrons não pareados, com existências independente, podem ser classificados como radicais livres. Essa configuração faz radicais livres moléculas altamente instáveis, com meia-vida curta e muito reativas. A presença dos radicais é crítica para a manutenção de muitas funções fisiológicas (RIBEIRO et al., 2005; VALKO et al., 2007).

Os radicais livres podem ser definidos como espécies que contém um ou mais elétrons desemparelhados em orbitais atômicos ou moleculares, ou seja, que apresentam um número ímpar de elétrons em sua última camada eletrônica. Este elétron não pareado, geralmente, confere um grau de reatividade aos radicais livres. Nesta tentativa de se estabilizar quimicamente, os radicais livres podem ceder o elétron desemparelhado, oxidando-se ou receber um elétron, reduzindo-se, propiciando reações em cadeia que terminam alterando a conformação, a estrutura ou as funções de diversos componentes celulares (RIBEIRO et al., 2005).

As espécies reativas de oxigênio são radicais livres derivados do oxigênio e representam a classe mais importante de espécies de radicais gerados em sistemas vivos. Estas espécies são onipresentes e derivadas do metabolismo do oxigênio. Em condições fisiológicas do metabolismo celular aeróbico, o O_2 sofre redução tetravalente, com aceitação de quatro elétrons resultando na formação de H_2O. Durante esse processo são formados intermediários reativos como os radicais hidroxil, peroxil e superóxido (VALKO et al., 2007).

O radical superóxido é formado após a primeira redução do O_2 pela adição de um elétron, apesar dos efeitos danosos, o radical $O_2^{\bullet-}$, tem importância vital para as células de defesa e sem ele o organismo está desprotegido contra infecções causadas por vírus, bactérias e fungos. Sua formação ocorre de forma espontânea como por exemplo, na membrana mitocondrial, através da cadeia respiratória e por fagócitos ou fibroblastos durante o processo inflamatório (BARREIROS; DAVID, 2006).

Outro radical intermediário é o radical hidroxil, sendo um dos mais reativos com meia vida aproximada de 10^{-9} segundos, podendo oxidar qualquer estrutura biológica. Assim, quando produzido *in vivo*, o radical reage no sítio onde foi gerado. Sua principal via de formação é pela reação do peróxido de hidrogênio com metais de transição ou pela exposição à radiação ionizante, para se estabilizar promove a abstração de hidrogênios e/ou a adição de insaturações de moléculas próximas. Este radical pode modificar bases púricas e pirimídicas, inativar proteínas e iniciar a oxidação de ácidos graxos poliinsaturados das membranas celulares (BARREIROS; DAVID, 2006; VALKO et al., 2007).

Por fim, o radical peroxil é produto da abstração de um átomo de hidrogênio do grupo metileno das cadeias dos ácidos graxos poliinsaturados das membranas ou das partículas lipoprotéicas, formando um radical lipídico que reage com oxigênio produzindo radical peroxil, o qual na presença de um outro lipídeo ou outro doador de elétron, forma hidroperóxido lipídico e um outro radical lipídico. O hidroperóxido lipídico pode sofrer degradação catalisada por metais de transição e produzir ainda mais radicais reativos, como o radical peroxil ou o radical alcoxil que irão continuar a reação em cadeia e produzir, como por exemplo, o malondialdeído, o pentano e o etano.(VALKO et al., 2007)

Para limitar a produção dos radicais e impedir a indução de danos, defesas antioxidantes agem frente a produção contínua deles durante os processos metabólicos com os objetivos de equilibrar a formação e a remoção de espécies radicalares. O efeito prejudicial dos radicais livres ocorre quando eles estão em quantidade excessiva, ultrapassando a capacidade do sistema antioxidante de neutralizá-lo. Os compostos que podem supri-

mir a formação destas espécies podem ser: os antioxidantes (enzimáticos e não enzimáticos), os “varredores” de radicais livres e os quelantes. (BARREIROS; DAVID, 2006)

3 ARTIGO

Vitis vinifera L. cv Pinot noir pomace and lees as potential sources of bioactive compounds

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Vitis vinifera L. cv Pinot noir pomace and lees as potential sources of bioactive compounds

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Abstract

Food and agricultural industries generate substantial quantities of phenolic-rich by-products that could be valuable natural sources of antioxidants. The aim of this study was to identify and quantify the phenolic compounds and radical scavenging activities of two by-products (pomace and lees) from *Vitis vinifera* L. cv Pinot noir. We found a different distribution of phenolic classes (flavanols, flavonols, phenolic acids and stilbenes) and singular scavenging activity against free radicals (hydroxyl, superoxide and peroxyl radicals). The major class of phenolics in pomace was flavanols and in lees was flavonols, with catechin (117.9±2.5 μg g^{-1}) and quercetin (42.4±1.2 μg g^{-1}) being the most abundant individual compounds. We also found high potential on scavenging activity against superoxide radicals in pomace (80% of scavenging activity) and radical peroxyl (67% scavenging activity). These results show the possibility of using Pinot noir by-products as promising additives or as a source for the development of new products in different segments of the food and cosmetic industries.

Keywords: *Vitis vinifera* L. cv Pinot noir, By-product analysis, By-product composition, Radical scavenging activity, Flavanols, Catechin, Quercetin.

1. Introduction

Vitis vinifera L. production is widespread throughout the world, exceeding 69.9 million tons, 35.8 million tons of which are used to winemaking, resulting in 270 million hectoliters for consumption and industrial purposes (OIV, 2015). Natural extracts of winery by-product are known to contain a large variety of bioactive substances, called phenolic compounds (CRESPO; BRAZINHA, 2010). Chemically, phenolic compounds are defined as substances that have an aromatic ring with one or more hydroxyl substituent, including its functional groups. The antioxidant activity of these compounds depends on their structure, particularly the number and position of hydroxyl groups and the nature of the substitutions in the aromatic rings. There are about 8,000 different phenolic compounds that are divided into classes according to their chemical structure: phenolic acids, flavonoids, stilbenes and tannins (Lattanzio, 2013).

Phenolic compounds exhibit a wide range of physiological properties, such as anti-allergenic, anti-artherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects. Moreover, they have been shown to scavenge reactive oxygen species (ROS) and electrolytes, inhibit nitrosation, chelate metals and may modulate the activity of some cellular enzymes (ARON; KENNEDY, 2008; BALASUNDRAM; SUNDRAM; SAMMAN, 2006; HELENO et al., 2014).

The wine industry produces a large amount of by-products and residues. These residues contain biodegradable organic matter, the disposal of which creates serious environmental problems. Over the past few years, the recovery of phenols from industrial residues is gaining considerable attention, since these compounds in the grape are only partially transferred to the wine and have antioxidant and biological activities (DEVESA-REY et al., 2011; GEORGIEV; ANANGA; TSOLOVA, 2014; KRZYWONOS et al., 2009; LIN et al., 2014). The food and agricultural processing industries also generate substantial quantities of

phenolic-rich by-products that could be valuable natural sources of antioxidants. The viticulture by-products are identified as grape stalk (GS), grape pomace (GP), grape marc (GM) and wine lee (WL). GP is comprised of the solid parts of grape and must, and is produced from the pressing of the raw material during wine production. As a pressing residue, GP represents about 12–15% of the initial weight from the raw material, containing carbohydrates, proteins and, in the seeds, a high content of lipids (DIMOU et al., 2015; FONTANA; ANTONIOLLI; BOTTINI, 2013). The chemical composition of GP is highly variable, depending on the nature of the caste, the process of wine production, moreover the atmospheric conditions surrounding the vegetation and the soil composition are crucial in increasing the concentration of these compounds. These aspects markedly influence the composition of the grapes, its condition systems, and its sanitary case at the moment of harvest, as well as determining the composition of the by-products (IORA et al., 2015).

Another by-product produced in large amounts is WL, also called as “heavy” or “light” lees (depending on the decanting step), defined as the residue that forms at the bottom of recipients containing wine, after fermentation, during storage or after authorized treatments. In addition, it is also defined as the residue obtained following the filtration or centrifugation of this product. Wine lees represent about 2–6% of the total volume of wine produced and mainly contain ethanol, tartaric acid and yeast cells. Due to the high quantities produced worldwide, WL became an ideal raw material for commercial production of tartaric acid and ethanol (BALASUNDRAM; SUNDRAM; SAMMAN, 2006; BARCIA et al., 2014; DIMOU et al., 2015; YU; AHMEDNA, 2013).

Publications on *Vitis vinifera* L. cv Pinot noir are scarce, with most studies reporting about the oenological quality and winery production (CAREW; CLOSE; DAMBERGS, 2015; GRIESSER et al., 2015; IACOPINI et al., 2008; SONG et al., 2015; TAUCHEN et al., 2015). Studies on the technological application of winemaking residues are also not often found in

the literature. Considering that recent studies suggest the use of these compounds as additives in different industry sectors, such as food (GARCÍA-LOMILLO et al., 2014; LAVELLI et al., 2014; SERRA et al., 2008) and cosmetic (GLAMPEDAKI; DUTSCHK, 2014), the aim of this study was to characterize the phenolic compounds and evaluate the antiradical activity of the lees and pomace from *Vitis vinifera* cv. Pinot noir in order to use these materials as potential antioxidants in food and pharmaceutical industries. Lees and pomace obtained from the wine industry were characterized by UHPLC-ESI-MS/MS for quantitative assaying of phenolic acids, flavanols, flavonols and stilbenes (*trans*-resveratrol). The radical scavenging activity of dry material of lees and pomace was examined for the most reactive oxygen species ($HO^{\bullet}$, $ROO^{\bullet}$ and $O_2^{\bullet -}$).

2. Materials and methods

2.1. Chemicals

Chemical analytical-grade standards (purity ≥95%) of catechin, apigenin, chrysin, luteolin, galagin, fisetin, kaempferol, myricetin, quercetin, quercitrin, rutin, caffeic acid, ferulic acid, gallic acid, p-coumaric acid, *trans*-cinnamic acid, vanillic acid and *trans*-resveratrol were obtained from Sigma-Aldrich (St. Louis, MO, USA). Methanol formic acid, acetic acid (Sigma–Aldrich; St. Louis, MO, USA) and acetonitrile (Panreac; Castellar del Vallès, Barcelona, SPN) were LC-MS grade**.** Xanthine, xanthine oxidase, 2'-7'-dichlorofluorescein diacetate (DCFH-DA), Folin-Ciocalteu reagent and sodium carbonate were obtained from Sigma–Aldrich (St. Louis, MO, USA). Aqueous solutions were prepared with ultra-pure water from a Milli-Q Synergy UV system (Merck Millipore, Darmstadt, Germany).

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123 2.2. Sample preparation

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125 The press residue (considered as GP) and WL of Pinot noir were supplied by Velho
126 Amâncio winery, located in Santa Maria, RS, Shouthern Brazil. The samples were from
127 Campaign 2014. The GP and solid phase of WL were dried in an air-circulation oven (Ehret,
128 Emmendingen, Germany) for 12 h at 50 ºC, the ground and stored under refrigeration until
129 analysis. Phenolic compounds were extracted as described by Gómez-Alonso et al. 2007, with
130 modifications. Exactly 20 g of by-product was measured and homogenized with 150 mL of a
131 mixture of Ethanol/H_2O/HCOOH (50:48.5:1.5, v/v) in a blender for 2 min and then centri-
132 fuged at 2500 g for 15 min. The extracts were kept at 4 ºC until needed.

133 The total content of phenolic compounds (TPC) in the extracts was measured by the
134 Folin-Ciocalteu method (LIN; TANG, 2006). This method is based on the change in color
135 caused by reduction of the Folin reagent by phenolates in the presence of sodium carbonate.
136 An aliquot of 100 µL of the extract was added to 2.8 mL of deionized water containing 100
137 µL of Folin reagent, followed by 2 mL of 2% sodium carbonate. The samples were incubated
138 for 30 minutes at room temperature, protected from light. The absorbance was measured at
139 740 nm in a Hewlett-Packard HP8452A spectrophotometer (Palo Alto, CA, USA). Gallic acid
140 was used as a standard calibration curve (from 0.0 to 0.8 mg mL^{-1}) and the results were ex-
141 pressed as gallic acid equivalents (mg GAE/g of dry matter). All analyses were made in tripli-
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149 2.3. Radical scavenging assays

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151 The radical scavenging activity was measured by the generation of three different free
152 radicals. Hydroxyl radical scavenging activity was assayed using the method described by
153 Zhao et al. (2006), based on the generation of $HO^{\bullet}$ by the fenton reaction. The radical scav-
154 enging activity against peroxyl radicals ($ROO^{\bullet}$) was assayed using the method described by
155 Amado et al. (2009), which is based on the thermic decomposition of 2,4 dichlorofluoroscein.
156 The scavenging activity against superoxide anion ($O_2^{\bullet-}$) was performed using an HPX/XOD
157 system following the procedure described by Zhao et al. (2006), with some modifications. All
158 assays were made in triplicate and calculated by the following equation:

159 $$\text{Radical scavenging activity (\%)} = [1 - (S - S_B)/(C - C_B)] \text{ X } 100$$

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161 2.4. Quantification of phenolic compounds by UHPLC-ESI-MS/MS

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163 Analyses were based on the method described by Faccin et al. (2015) and carried out
164 in an Agilent 1260 chromatograph (Agilent technologies, Palo Alto, CA, USA) equipped with
165 Agilent 6420 triple quadrupole mass analyzer with an ESI source (gas flow of 11 L min^{-1},
166 nebulizer of 30 psi, capillary voltage of ± 2.4 kV and gas temperature of 250 °C), which was
167 set to multiple reaction monitoring (MRM) mode. A C18 Zorbax SB, Rapid Resolution HD
168 column (1.8 µm, 2.1×50 mm) was used in the separations. An injection volume of 5 µL was
169 used in all chromatographic runs.

170 The solvents in the mobile phase were 0.1% acetic acid in water (solvent A) and ace-
171 tonitrile (solvent B) with the following gradient elution program: 0.0–0.10 min 8% B; 0.10–
172 3.45 min 8% B; 3.45–6.90 min 54.1% B; and 6.90–9.00 min 100% B as the column washing
173 step. A post-run time of 4 min was used for re-equilibration of the column. The mobile phase

flow-rate was 0.8 mL/min and the temperature of column was set at 40 ºC. Phenolic compounds were identified by retention time and mass spectra compared to standards. The extracted samples were subjected to a cleanup process using solid-phase extraction (SPE) prior to the chromatographic analysis in order to isolate the phenolic compounds. For this procedure, Phenomenex Strata C18 cartridges were used, which were conditioned with 6 mL of methanol:acetic acid 0.2% (1:1; v/v), then equilibrated with 6 mL of 0.1% acetic acid. Afterwards, 2 mL of the sample was applied to the cartridges with a flow rate of 2 mL/min. The cartridges were washed with 2 mL of acetic acid 0.2%. Elution was carried out with 2 mL of methanol.

3. Results and discussion

3.1. Total phenolic content and radical scavenging activity

Total phenolic content of pomace (90.21 mg GAE g^{-1} dry matter) was almost 3-fold higher than in lees (30.86 mg GAE g^{-1} dry matter), as shown in Table 1. This difference on content should contribute to the lower scavenging activity of WL compared to GP, considering that $O_2^{\bullet -}$ scavenging activity in WL was nearly half of the GP scavenging activity. These results are similar to those previously published, which also found high phenolic content and radical scavenging activity in GP (BURIN et al., 2014; LORRAIN et al., 2013). However, the dynamics of the transference of phenolic compounds from pomace to must and/or also during the extraction process may change the distribution of antioxidants classes, modifying the susceptibility for radical scavenging activities (BARCIA et al., 2014; GÓMEZ GALLEGO et al., 2012; ROCKENBACH et al., 2011b).

3.2. Quantification of phenolic compounds by UHPLC-ESI-MS/MS

By applying the developed UHPLC-ESI-MS/MS method, successful separation, identification and quantification was achieved for catechin (flavanol); kaempferol, myricetin, quercetin, quercitrin and rutin (flavonols); caffeic acid, ferulic acid, gallic acid, p-coumaric acid, acid and vanillic acid (phenolic acids), and *trans*-resveratrol (stilbene). The separation pattern is represented by the total ion chromatogram of GP (Figure 1) and WL (Figure 2) showing the major peaks for the main phenolic compounds in the studied winery by-products. Quantification was based on signal-response after the extraction of each ion transition in MRM mode. The calibration curves were constructed with standard addition using eight levels of increments in triplicate. All curves showed good linearity ($r^2 > 0.9934$) in the studied concentration ranges (Table 2). The lowest LOD and LOQ corresponded to quercitrin (0.5 µg L^{-1} and 1.7 µg L^{-1}, respectively), while the highest LOD and LOQ were observed for myricetin (130.3 µg L^{-1} and 434.3 µg L^{-1}, respectively).

According to the results presented in Table 2, the predominant phenolic compound in GP was (+)-catechin. The average concentration of this flavanol was 117.9±2.5 µg g^{-1}, representing the majority compound found in this by-product by using the proposed method. Beyond the presence of catechin, GP showed a characteristic peak in the full scan mode corresponding to (−)-epicatechin 3-*O*-gallate molecule, having a $[M-H]^-$ of *m/z* 441. Therefore, it is probably related to the presence of this proanthocyanidin in the GP extracts, as already found by (Del Rio et. al 2011). However, the quantification was not possible due to the absence of (−)-epicatechin 3-*O*-gallate as a standard substance in the proposed and validated method (see section 2.4). Therefore, the identification of (+)-catechin in the SRM mode and (−)-epicatechin 3-*O*-gallate in the full scan mode by MS/MS detection can be used as chemical markers for proanthocyanidins and anthocyanins, as already reported in other works for other

plant extracts (Rockenbach et al. 2011a, Wu and Prior 2005, Li and Deinzer 2006, Pati et al. 2006). The main compound within the screened flavonols was myricetin 4.2±2 µg g^{-1}, followed by gallic acid (3.9±0.3 µg g^{-1}) as phenolic acid and *trans*-resveratrol (1.0±0.1 µg g^{-1}) as stilbene. These results are in agreement with other work reporting high amounts of catechin and gallic acid in *Vitis vinifera* L. grape pomaces (DE LA CERDA-CARRASCO et al., 2015; IORA et al., 2015; JARA-PALACIOS et al., 2014). Moreover, myricetin has been also identified in other *Vitis* species, as reported by Ramirez-Lopez and DeWitt (2014) in *Vitis aestivalis* pomace. Other flavonols and phenolics acids were found in pomace, but in lower concentrations, agreeing with previous work (DE LA CERDA-CARRASCO et al., 2015; IORA et al., 2015; JARA-PALACIOS et al., 2014).

In grape pomace, the occurrence of the compounds described here corroborate the results published in other works, where catechin (Kammerer et al. 2004, Rockenbach et al. 2011a, 2011b, Antoniolli et al. 2015, Fontana et al. 2016), quercetin (Kammerer et al. 2004, Rockenbach et al. 2011a, 2011b, Antoniolli et al. 2015, Fontana et al. 2016), ferulic acid (FONTANA; ANTONIOLLI; BOTTINI, 2016), gallic acid (Kammerer et al. 2004, Rockenbach et al. 2011a, 2011b, Antoniolli et al. 2015, Fontana et al. 2016), p-coumaric acid (FONTANA; ANTONIOLLI; BOTTINI, 2016) and *trans*-resveratrol (Kammerer et al. 2004, Rockenbach et al. 2011a, 2011b, Antoniolli et al. 2015, Fontana et al. 2016) have been described as majority phenolic compounds. However, these works were not dealing necessarily with the same grape caste. Furthermore, the climatic variables, soil and cultivation conditions as well as the different extraction techniques can lead often to a discrepant quantitative comparison. In addition, no data were found in the literature for comparing the occurrence of vanillic acid in grape pomace. Beyond the majority compounds identified in grape pomace, proanthocyanidins are also expected to be present at high levels, since is it has been considered a rich source of flavan-3-ols as reported elsewhere (Kammerer et al. 2004, Rockenbach et al.

2012). In this work, (+)-catechin and (−)-epicatechin 3-*O*-gallate were identified in GP extracts, which permit us to infer that the studied GP extracts have proanthocyanidins as majority and bioactive compounds, as also reported elsewhere. (Rockenbach et al. 2011, Wu and Prior 2005, Li and Deinzer 2006, Pati et al. 2006). Other proanthocyanidins could not be detected or identified in our work, since the proposed method was not developed and validated from screening specifically of this phenolic compound class. Furthermore, the comparison of our work with other developed LC-MS/MS methods (Rockenbach et al. 2011, Wu and Prior 2005, Li and Deinzer 2006, Li and Deinzer 2007, Pati et al. 2006, Tian et al. 2005) is not always possible, since these methods make use of different ionization sources (i.e. APCI), mobile phase composition or ionization conditions.

In contrast, WL showed a different distribution of phenolic compounds. The major flavonol was found to be quercetin (42.4±1.7 μg g^{-1}), followed by kaempferol and myricetin (9.6±0.6 μg g^{-1} and 7.8±3.5 μg g^{-1}, respectively). Phenolic acids were also found in high amounts, caffeic and vanillic acids (5.7±0.1 μg $g^{-1}$and 3.8±0.3 μg g^{-1}, respectively). However, the higher skin/seed ratio and the transfer of phenolics from solid matter to must during maceration/fermentation can increase and change the occurrence of certain polyphenols in different by-products (DE LA CERDA-CARRASCO et al., 2015; ROCKENBACH et al., 2011b).

3.3 Application of grape by-products

Considering that grape constitutes one of the main fruit crops in the world, its by-products from winemaking are considered a bioresource available on a large-scale. They can be also referred as ‘‘antioxidant dietary fibers’’, since grape contains both phenolics and dietary fibers. Thus, grape by-products could be proposed as food components, which can play

274 an important role in prevention of human diseases and enhance the nutritional value of food-
275 stuffs (Amaya-Cruz et al. 2015, Beres et al. 2016).

276 Recent studies have shown a growing interest in the incorporation of the aforemen-
277 tioned residues in various food products in order to increase the durability, improve organo-
278 leptic properties and nutritional value. Thus, Mildner-Szudlarz et al. (2015) used powdered
279 Pinot Noir pomace for the partial replacement of wheat flour in the preparation of muffins.
280 Besides the increased concentration of antioxidants and decreased Nε-(1-Carboxymethyl)-L-
281 lysine formation, a great acceptance in the sensorial analysis was achieved. Similarly, Tseng
282 and Zhao (2013) proposed the fortification of yogurt and salad dressing with Pinot Noir pom-
283 ace in order to increase the nutritional value and durability of the final product. The results
284 showed an increase in the fiber content, moreover reduced peroxidation lipid indicating the
285 possibility to increase the product lifetime.

286 Wine lees also represent a source of bioactive compounds, although there are few
287 studies involving its application in the food market up to date. The addition of the light frac-
288 tion of wine lees in ice cream proposed by Hwang et al. (2009) can be considered as one of
289 the few applications found in the literature.

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291 ## 4. Conclusion
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293 This study showed that grape pomace is a rich source of phenolic compounds, which
294 can be used for technological purposes as a natural antioxidant product or an additive. In addi-
295 tion, grape pomace showed the highest content of total phenolics and radical scavenging ac-
296 tivity, being catechins (proanthocyanidins) the majority compounds found in the extracts. The
297 winemaking by-products obtained from *Vitis vinifera* L. cv Pinot noir can be considered as a
298 potential source of antioxidants, thereby allowing innumerous technological applications in

other industrial sectors. Herein, there are studies on the use of these by-products in patisseries, such as the manufacture of cookies, muffins and breads, for improving their quality and durability (DAVIDOV-PARDO et al., 2012; MILDNER-SZKUDLARZ et al., 2015; PENG et al., 2010). Additionally, winemaking by-products have been also investigated as antioxidant products in asphalts used in pavements (CALABI-FLOODY; THENOUX, 2012), since products rich in phenolic compounds lead to the diminution of asphalt oxidative aging.

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475 Table 1 Phenolic content and scavenging activity in winemaking by-products of *Vitis vinifera*
476 L. cv Pinot noir (n=3)

Analysis	GP	WL
Total phenolic content (mg GAE/g dry matter ±SD)	90.2 ±4.7	31.9 ±2.4
Hydroxil scavenging activity (%±SD)	61 ±2.8	59 ±4.2
Superoxide scavenging activity (%±SD)	80 ±1.7	41 ±0.9
Peroxil scavenging activity (%±SD)	75 ±3.0	67 ±2.7

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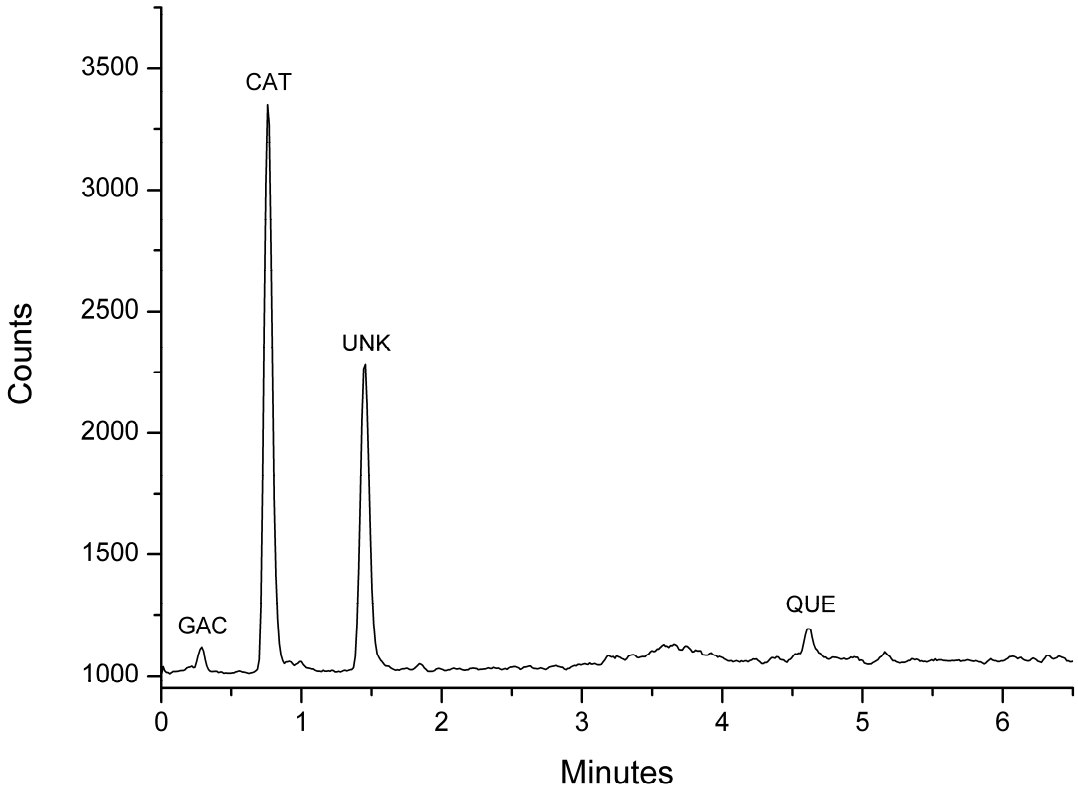
Table 2. Retention time (t_R), ESI-MS/MS data, analytical parameters and phenolic concentration of winemaking by-products of *Vitis vinifera* L. cv Pinot noir (n=3)

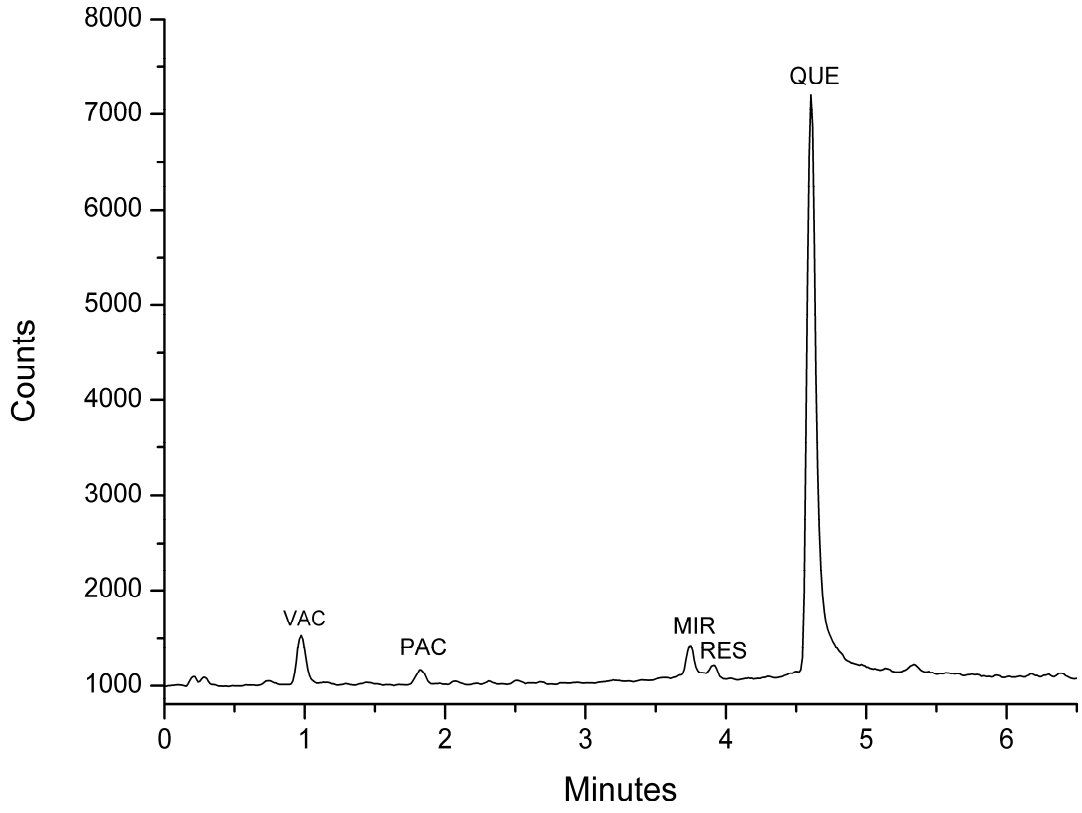
Compounds	t_R (min)	Precursor ion $[M-H]^-$	Product ion (m/z)	LOD (µg L^{-1})	LOQ (µg L^{-1})	Linear range (µg L^{-1})	GP (µg g^{-1})	WL (µg g^{-1})
Flavanols								
Catechin	0.76	289.1	245.1	7.5	25.1	100.1–250.1	117.9 ±2.5	4.3 ±0.4
Flavonols								
Kaempferol	5.332	285.0	239.0	33.1	110.3	118.6–1185.5	ND	9.6 ±0.6
Myricetin	3.718	317.0	150.9	130.3	434.3	1375.9–4586.3	4.2 ±2.0	7.8 ±3.5
Quercetin	4.581	301.0	151.1	7.7	25.8	51.4–257.2	2.6 ±0.2	42.4 ±1.7
Quercitrin	3.644	447.1	301.1	0.5	1.7	3.8–19.2	0.2 ±0.01	0.2 ±0.01
Phenolic acids								
Caffeic acid	0.978	179.0	135.0	3.8	12.8	38.3–127.7	<LOQ	5.7 ±0.1
Ferulic acid	2.491	193.1	134.1	8.3	27.6	59.3–296.4	0.3 ±0.2	1.1 ±0.2
Galic acid	0.286	169.0	125.1	7.7	25.6	76.7–230.2	3.9 ±0.3	1.1 ±0.1
ρ-coumaric acid	1.812	163.0	119.1	2,0	6.5	14.0–56.0	0.4 ±0.1	1.8 ±0.1
Vanillic acid	0.974	167.0	152.2	15.1	50.2	98.3–491.4	1.7 ±0.3	3.8 ±0.3
Stilbenes								
Resveratrol	3.881	227.1	185.2	4,0	13.5	14.5–144.7	1.0 ±0.1	3.0 ±0.1

Figure 1. LC-ESI-MS chromatograms showing the detection of major peaks in grape pomace.



GAC = Gallic acid, CAT = Catechin, QUE = Quercetin

Figure 2. LC-ESI-MS chromatograms showing the detection of major peaks in wine lees.



VAC = Vanillic acid, PAC = p-Coumaric acid, MIR = Myricetin, RES = *trans*-Resveratrol, QUE = Quercetin

4 CONCLUSÃO

- As técnicas empregadas para caracterização e quantificação permitiram atingir os objetivos desse trabalho.

- Conseguiu-se traçar o perfil dos compostos fenólicos constituintes dos resíduos de vinificação por LC-MS, onde determinou-se: catequina, canferol, mircetina, quecetina, quercitrina, ácido cafeíco, ácido ferúlico, ácido gálico, ácido p-coumárico, ácido vanílico e resveratrol.

- Dos resultados obtidos, o bagaço apresentou distribuição dos compostos diferente da borra, apresentando em maior quantidade ácido gálico e quercitina, respectivamente.

- A atividade antiradicalar se mostrou maior no bagaço do que na borra possivelmente devido a diferença na concentração de fenólicos e constituintes da matriz.

- Fica evidente a importância de estudo envolvendo a análise da composição química dos subprodutos oriundo da indústria vinífera, a fim de possibilitar o maior conhecimento sobre seus constituintes e com isso promover a utilização como fonte alternativa de compostos fenólicos.

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