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TESE DE DOUTORADO

DINÂMICA DE MATURAÇÃO DE UVAS DE UMA REGIÃO VITIVINÍCOLA EMERGENTE DE CLIMA TROPICAL DE ALTITUDE E SEU IMPACTO NO PERFIL FENÓLICO E NO PONTO IDEAL DE COLHEITA

Laura Oliveira Lago

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2025

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Tese apresentada ao Programa de Pós-graduação em Ciência e Tecnologia de Alimentos como um dos requisitos para obtenção do título de Doutora em Ciência e Tecnologia de Alimentos.

Orientadora: Prof. Dra. Juliane Elisa Welke

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RESUMO

A maturação das uvas exerce influência decisiva sobre a composição fenólica e sobre as características químicas e sensoriais dos vinhos. A dinâmica do amadurecimento da uva e seu perfil químico são altamente influenciados pelas condições climáticas. Nos últimos anos, as mudanças climáticas têm impulsionado a expansão da viticultura para regiões tropicais de elevada altitude, como uma estratégia de adaptação destinada a mitigar os impactos negativos do aquecimento global sobre a qualidade das uvas e dos vinhos tradicionalmente produzidos em áreas de clima temperado. O perfil fenólico das uvas é caracterizado por sua complexidade, tanto em termos do número de diversas estruturas químicas quanto de sua variação durante o amadurecimento. O objetivo deste trabalho foi avaliar a dinâmica de maturação de uvas tintas 'Malbec' e 'Cabernet Sauvignon' (*Vitis vinífera* L.) cultivadas em uma região vitivinícola emergente de clima tropical de altitude e investigar como ela influencia o perfil fenólico e a determinação do ponto ideal de colheita para a produção de vinhos de alta qualidade. As uvas de ambas as cultivares foram analisadas semanalmente ao longo de dez semanas a partir do *véraison*, o qual é definido como estágio que marca o início da maturação e envolve a mudança de cor e o acúmulo de açúcares nas bagas. Um método baseado em cromatografia líquida bidimensional abrangente acoplada a detector de arranjo de diodos e espectrometria de massas sequencial (LC × LC–DAD-MS/MS) foi desenvolvido e otimizado para caracterizar o perfil fenólico das uvas e vinho 'Malbec'. A elevada capacidade de resolução do método permitiu identificar 40 compostos fenólicos, incluindo antocianinas e flavan-3-óis, e demonstrou que as antocianinas atingem concentração máxima nas uvas colhidas entre as semanas 5 e 6 (W5-W6), seguida de declínio a partir da semana 7 (W7), estabilizando nas semanas 9-10 (W9-W10). Pela primeira vez, a LC × LC-MS/MS foi empregada para a elucidação de perfis fenólicos altamente complexos em uvas e vinhos. A maturação das uvas 'Cabernet Sauvignon' foi avaliada monitorando parâmetros físico-químicos, composição fenólica e atividade antioxidante. As uvas colhidas entre as semanas 6 e 9 apresentaram atributos favoráveis à vinificação. A acumulação de antocianinas, flavan-3-óis e flavonóis foi mais expressiva entre as semanas 6 e 7, período em que também

entre as uvas ‘Malbec’ e ‘Cabernet Sauvignon’ demonstra que diferentes cultivares, mesmo sob condições de cultivo semelhantes, podem apresentar diferenças na dinâmica de acúmulo, síntese e degradação desses compostos. O estudo reforça a importância da análise integrada de parâmetros físico-químicos, perfil fenólico e atividade antioxidante para a determinação do ponto de colheita e demonstra o potencial de técnicas analíticas avançadas na compreensão da qualidade enológica em regiões vitivinícolas emergentes do Brasil.

Palavras-chave: antocianinas; maturação; uva; cromatografia líquida bidimensional; fenólicos.

ABSTRACT

Grape ripening has a decisive influence on the phenolic composition and on the chemical and sensory attributes of wines. The dynamics of grape maturation and its chemical profile are strongly affected by climatic conditions. In recent years, climate change has driven the expansion of viticulture into high-altitude tropical regions as an adaptation strategy to mitigate the negative impacts of global warming on grape and wine quality traditionally associated with temperate climates. The phenolic profile of grapes is characterized by its complexity, both in terms of the diversity of chemical structures and their variation throughout ripening. The aim of this study was to evaluate the maturation dynamics of Malbec and Cabernet Sauvignon red grapes (*Vitis vinifera* L.) cultivated in an emerging high-altitude tropical wine region, and to investigate how ripening influences their phenolic profile and the determination of the optimal harvest point for producing high-quality wines. Grapes from both cultivars were analyzed weekly over a ten-week period starting at *véraison*, the stage marking the onset of ripening and involving color change and sugar accumulation in the berries. A new method based on the application of comprehensive two-dimensional liquid chromatography coupled to a diode array detector and tandem mass spectrometry (LC × LC–DAD-MS/MS) was developed and optimized to characterize the phenolic profile of Malbec grapes and wine. The high resolving power of the method enabled the identification of 40 phenolic compounds, including anthocyanins and flavan-3-ols, and demonstrated that anthocyanins reached their maximum concentration in grapes harvested between weeks 5 and 6 (W5–W6), followed by a decline from week 7 (W7), stabilizing between weeks 9 and 10 (W9–W10). For the first time, LC × LC–MS/MS was applied to elucidate highly complex phenolic profiles in grapes and wines. Cabernet Sauvignon ripening was evaluated by monitoring physicochemical parameters, phenolic composition and antioxidant activity. Grapes harvested between weeks 6 and 9 exhibited attributes favorable for winemaking. The accumulation of anthocyanins, flavan-3-ols and flavonols was most pronounced between weeks 6 and 7, a period that also corresponded to the highest antioxidant activity. The difference in the peak concentration of

anthocyanins between Malbec and Cabernet Sauvignon highlights that different cultivars, even under similar growing conditions, may display distinct dynamics of accumulation, synthesis and degradation of these compounds. Overall, the study reinforces the importance of integrating physicochemical parameters, phenolic composition and antioxidant activity when determining the optimal harvest time and demonstrates the potential of advanced analytical techniques to deepen the understanding of enological quality in emerging wine regions of Brazil.

Keywords: anthocyanins; ripening; grape; two-dimensional liquid chromatography; phenolics.

LISTA DE FIGURAS

Figura 1	Ilustração das fases fenológicas de uvas viníferas baseada na escala fenológica desenvolvida por Eichorn e Lorenz (1984) e adaptada por Coombe (1995).	25

LISTA DE TABELAS

Tabela 1	Estudos que avaliaram o efeito da altitude do vinhedo em características da uva e do vinho.	19

Sumário

1 INTRODUÇÃO 14
2 OBJETIVOS 16
2.1 Geral: 16
2.2 Específicos: 16
3 REVISÃO BIBLIOGRÁFICA 17
3.1 Viticultura de alta altitude 17
3.2 Fenologia da videira 24
3.3 Maturação fenólica e o ponto ideal para colheita de uvas 25
3.4 Compostos fenólicos e atividade antioxidante de uvas e vinhos 26
4 ARTIGOS 28
4.1 Artigo 1 – Evolution of anthocyanin content during grape ripening and characterization of the phenolic profile of the resulting wine by comprehensive two-dimensional liquid chromatography 28
4.2 Artigo 2 - Targeted metabolomics reveals the optimal harvest time of Cabernet Sauvignon grapes in an emerging tropical high-altitude region 61
5 DISCUSSÃO GERAL 97
6 CONCLUSÃO 99
REFERÊNCIAS 100

1 INTRODUÇÃO

O potencial produtivo de uma região para a viticultura é altamente influenciado pelo clima, que favorece o cultivo de uvas entre as latitudes de 40° e 50° no hemisfério Norte e 30° e 40° no hemisfério Sul. Consequentemente, o cultivo da videira ocorre tradicionalmente em regiões de clima temperado. No entanto, o conhecimento sobre a fisiologia da videira e os avanços das práticas vitícolas permitiram a expansão para regiões localizadas entre os trópicos, como áreas tropicais da Índia, Tailândia, Venezuela e a região Nordeste do Brasil (ANDRADE *et al*., 2024).

À medida que as bagas das uvas se desenvolvem e amadurecem, elas sofrem alterações no tamanho, composição química, cor, textura e sabor. A composição química da uva no momento da colheita é considerada o principal fator que afeta a qualidade do vinho, uma vez que as características organolépticas do vinho, como cor, adstringência, amargor e aroma estão diretamente associadas aos componentes químicos das uvas. Os compostos químicos da uva que contribuem para estas características são: compostos fenólicos (GUTIÉRREZ-ESCOBAR, ALIAÑO-GONZÁLEZ, CANTOS-VILLAR, 2021; TAN *et al*., 2024). Estes compostos estão distribuídos na casca, na semente e na polpa da fruta, estando presentes nas formas livre e ligada, e o seu conteúdo é produto do metabolismo da uva, que é influenciado pela cultivar da uva, pelas condições climáticas e pelas práticas vitivinícolas. As alterações nesses compostos são utilizadas para saber como eles se desenvolvem durante a maturação da fruta e como são afetados por fatores externos. Esta informação é crucial para ajudar os produtores de vinho a escolher a melhor data da colheita para obter um vinho com alta qualidade (ALLEGRO *et al*., 2021; ŠIKUTEN *et al*., 2021; TAN *et al*., 2024). Além de conhecer o perfil fenólico, parâmetros tradicionais, como porcentagem de sólidos solúveis (açúcar), pH, parâmetros de textura (dureza e firmeza) e cor, também são utilizados para determinar o estágio ideal de maturação da uva (COOMBE, 1995; BINDON *et al*., 2013).

As condições climáticas orientam o processo de desenvolvimento da videira, uma vez que este depende de temperatura, amplitude térmica,

radiação solar e disponibilidade de água adequadas ao longo do seu ciclo de crescimento, exercendo uma influência significativa na síntese e acúmulo de compostos fenólicos e açúcar na uva (BLANCQUAERT *et al*., 2019; DEIS *et al*., 2024; WANG *et al*., 2025).

A viticultura de alta altitude adquiriu enorme relevância na última década. A definição de viticultura de alta altitude depende do contexto regional e climático. Em regiões temperadas, vinhedos situados entre 500 e 600 metros acima do nível do mar são tipicamente classificados como tal, enquanto em regiões tropicais e subtropicais, apenas vinhedos acima de 800 metros são considerados de alta altitude, onde fatores ambientais influenciam o ciclo de crescimento da videira. A implantação de vinhedos em áreas de elevada altitude tornou-se uma estratégia de adaptação reconhecida para mitigar as consequências negativas do aquecimento global na qualidade da uva e do vinho. Isso é particularmente importante porque algumas regiões vinícolas podem se tornar extremamente quentes e secas, tornando a viticultura inviável, e regiões de alta altitude são caracterizados por um clima mais frio, pois a altitude modula o clima, principalmente a temperatura e a radiação solar que influenciam a bioquímica da uva (ARIAS *et al*., 2022; MANSOUR *et al*., 2022).

Estudos mostraram um efeito positivo da elevação da altitude no ciclo de crescimento e na composição química da fruta (ARIAS *et al*., 2022). Videiras de regiões de alta altitude exibem fases fenológicas mais longas, o que afeta o amadurecimento da uva e o tempo ideal de colheita (FALCÃO *et al*., 2010). Esse período prolongado pode permitir o acúmulo suficiente de compostos fenólicos na uva, contribuindo na produção de vinhos com maior intensidade de aroma e de cor (YUE *et al*., 2015; OLIVEIRA *et al*., 2019a; ARIAS *et al*., 2022).

Em relação ao Brasil, estudos explorando uvas e vinhos produzidos em regiões de alta altitude têm sido realizados nos últimos anos, como na região da Chapada Diamantina (800–1.200 m) na Bahia localizada no Nordeste brasileiro (OLIVEIRA *et al*., 2019a; OLIVEIRA *et al*., 2019b).

Localizado na sub-região do Agreste do Nordeste brasileiro, áreas do município de Garanhuns (08º53'25”S 36º29'34”W) (Pernambuco) têm despertado o interesse para a implantação de novas vinícolas. Essa região destaca-se por apresentar condições edafoclimáticas particulares, devido ao

fato de estar situado no planalto da Borborema em altitudes mais elevadas (841-1.000 m). Além disso, a proximidade com o oceano resulta na influência de frentes frias, maiores amplitudes térmicas e precipitações pluviométricas. O primeiro vinhedo dessa região foi instalado em 2018, sendo ocupada pelas cultivares Moscato Bianco, Cabernet Sauvignon e Malbec, escolhidas por terem se destacado em termos de aptidão e produtividade (SILVA *et al*., 2019).

2 OBJETIVOS

2.1 Geral:

- Avaliar a dinâmica de maturação de uvas cultivadas em uma região vitivinícola emergente de clima tropical de altitude e investigar sua influência sobre o perfil fenólico, bem como sobre a determinação do ponto ideal de colheita para a produção de vinhos de qualidade.

2.2 Específicos:

- Desenvolver um método baseado em cromatografia líquida bidimensional abrangente acoplada a detecção por arranjo de diodos e espectrometria de massas (LC × LC - DAD-MS/MS) para caracterizar o perfil de antocianinas de uvas Malbec ao longo da maturação e o perfil fenólico do respectivo vinho;

- Determinar o período ideal de colheita de uvas Cabernet Sauvignon através da avaliação de parâmetros físico-químicos, perfil fenólico e atividade antioxidante das uvas ao longo de 10 semanas de maturação (do *véraison* à colheita);

3 REVISÃO BIBLIOGRÁFICA

3.1 Viticultura de alta altitude

De acordo com a Organização Internacional da Vinha e do Vinho (OIV, do francês: *Organisation Internationale de la vigne et du vin*), “o *terroir* vitivinícola é um conceito que se refere a uma área em que se desenvolve o conhecimento coletivo das interações entre o ambiente físico e biológico identificável e as práticas vitivinícolas aplicadas, proporcionando características distintivas aos produtos provenientes desta área. *Terroir* inclui características específicas de solo, topografia, clima, paisagem e biodiversidade” (OIV, 2010). Uma vez que a viticultura é uma atividade humana, fatores humanos como decidir qual cultivar de uva plantar e quais práticas vitícolas e enológicas usar também fazem parte do *terroir*. Ele relaciona a qualidade e os atributos sensoriais do vinho com fatores ambientais (solo, topografia, clima) e práticas vitícolas em que as uvas são cultivadas (VAN LEEUWEN, SEGUIN, 2006).

Durante o seu crescimento e desenvolvimento, a uva sofre alterações fisiológicas e bioquímicas que são responsáveis por induzir modificações em sua composição química. Portanto, avaliá-la ao longo do desenvolvimento é crucial para ajudar os produtores a escolher a melhor data da colheita, uma vez que os compostos químicos da uva responsáveis pela cor, adstringência, amargor, sabor e aroma dos vinhos, os quais afetam diretamente a qualidade da bebida, são compostos fenólicos e compostos voláteis (CONDE *et al*., 2007).

O cultivo da videira é altamente dependente do clima, pois o seu desenvolvimento requer temperatura, radiação solar e disponibilidade de água adequadas, influenciando o rendimento e a composição da fruta, que juntos determinam os atributos e a tipicidade do vinho (GARRIDO, BORGES, 2013; GONZÁLEZ-BARREIRO *et al*., 2015; GUTIÉRREZ-ESCOBAR, ALIAÑO-GONZÁLEZ, CANTOS-VILLAR, 2021). Essa estreita relação torna a viticultura extremamente vulnerável às mudanças climáticas (MOSEDALE *et al*., 2016).

A altitude é um fator importante a ser considerado no estabelecimento de um vinhedo uma vez que ela modula condições climáticas. Um aumento de 1 quilômetro na altitude produz uma diminuição de 6 ºC na temperatura média,

sendo que o mesmo aumento causa uma elevação de 10% nos níveis de radiação ultravioleta (UV) (KÖRNER, 2007; WHO, 2016). Um vinhedo de alta altitude é considerado entre 350-2900 m acima do nível do mar (MATEUS *et al*., 2001; XING *et al*., 2016; ARIAS *et al*., 2022).

A viticultura de alta altitude obteve grande importância nas últimas décadas devido ao seu potencial em produzir vinhos de alta qualidade em regiões mais quentes, seja pela natureza da sua geografia ou pelo avanço das mudanças climáticas, uma vez que o cultivo de vinhas em altas altitudes é uma estratégia eficaz para mitigar os impactos negativos do aquecimento global na qualidade das uvas e do vinho (ARIAS *et al*., 2022; MANSOUR *et al*., 2022). A Tabela 1 mostra os estudos que avaliaram o impacto da altitude em uvas e vinhos e seus principais resultados.

Tabela 1. Estudos que avaliaram o efeito da altitude do vinhedo em características da uva e do vinho.

Altitude (m)[a]	Região	Clima[b]	Amostra e características avaliadas	Principais resultados	Referência
635-671	Rivadavia, Mendoza, Argentina	Árido quente (BWh)	Vinho Malbec, perfil fenólico e sensorial	Vinhos de Tupungato tiveram as maiores concentrações de antocianinas, tirosol, *trans*-resveratrol e quercetina, enquanto os vinhos de Rivadavia tiveram as menores. Vinhos de Rivadavia foram positivamente associados aos atributos sensoriais herbáceo, tabaco, pimenta preta e doce. Vinhos de Maipú foram associados aos atributos frutas vermelhas, picante e uva passa.	Urvieta et al.(2018)
928	Maipú, Mendoza, Argentina	Semiárido quente (BSh)			
959-1051	Luján de Cuyo, Mendoza, Argentina	Semiárido quente (BSh)			
961-1100	San Carlos, Mendoza, Argentina	Semiárido frio (BSk)			
1006-1135	Tunuyán, Mendoza, Argentina	Semiárido frio (BSk)			
1240-1510	Tupungato, Mendoza, Argentina	Semiárido frio (BSk)			
950	Agrelo, Mendoza, Argentina	Semiárido quente (BSh)	Vinho Cabernet Sauvignon, perfil fenólico	Vinhos de Gualtallary apresentaram as maiores concentrações de antocianinas e *trans*-resveratrol, enquanto vinhos de Agrelo as menores. Vinhos de Agrelo apresentaram maiores concentrações de ácidos hidroxibenzóicos; vinhos de Altamira apresentaram maiores concentrações de ácidos hidroxicinâmicos; vinhos de Gualtallary apresentaram maiores concentrações de flavonóis. Não houve diferença significativa na concentração de flavanóis entre os vinhos provenientes de diferentes altitudes.	Muñoz et al.(2021)
1073	Pampa El Cepillo, Mendoza, Argentina	Semiárido frio (BSk)			
1085	Altamira, Mendoza, Argentina	Semiárido frio (BSk)			
1350	Gualtallary, Mendoza, Argentina	Semiárido frio (BSk)			
1100	Morro do Chapéu (Bahia)	Subtropical úmido (Cwa)	Vinho Syrah, perfil fenólico e sensorial	Vinhos de Bahia (1100 m) tiveram maior concentração de ácido succínico, fenóis totais, flavonóides, antocianinas totais, pigmentos totais e polimerizados, taninos monoméricos e oligoméricos, *trans*-resveratrol, antocianinas 3-O-glicosídeo e esterificadas com ácido *p*-cumárico e dímeros e trímeros de procianidinas e atributos sensoriais (tempero, acidez, amargor, adstringência, corpo e persistência) elevados. Os vinhos de Pernambuco (350 m) apresentaram maior concentração de flavonóis, ácidos tartárico e cítrico, taninos polimerizados e antocianinas 3-O-acetilglicosídeo e aromas frutado, floral, herbáceo e empireumático elevados.	Oliveira et al.(2019a)
350	Lagoa Grande (Pernambuco)	Semiárido quente (BSh)			
1100	Morro do Chapéu (Bahia)	Subtropical úmido (Cwa)	Uva Syrah, perfil fenólico, teor de ácidos orgânicos e açúcares	Uvas da Bahia (1100 m) tiveram maior concentração de ácidos málico e succínico no mosto e de antocianinas e taninos condensados na casca. Uvas de Pernambuco (350 m) apresentaram maior concentração de açúcares (glicose e	Oliveira et al.(2019b)
350	Lagoa Grande (Pernambuco)	Semiárido quente (BSh)			

				frutose) e ácidos tartárico e cítrico no mosto, de flavonóis, *trans*-resveratrol e antocianinas 3-O-acetilglicosídeo na casca e de taninos condensados nas sementes.	
965	Campos Novos, Santa Catarina	Subtropical úmido (Cfa)	Vinho Merlot, perfil volátil, fenólico, atividade antioxidante	Vinhos de Tangará apresentaram altas concentrações de ácidos hidroxicinâmicos, acetato de fenetila, cinamato de etila e γ-nonalactona e alta atividade antioxidante. Vinhos de Campos Novos apresentaram altas concentrações de *trans*-resveratrol, tirosol, flavonóis e ácidos hidroxicinâmicos, bem como dos ésteres isovalerato de etila, 2-metilbutanoato de etila e hexanoato de etila. Não foi possível definir claramente os principais constituintes químicos dos vinhos de Água Doce.	Arcari et al.(2021)
1059	Tangará, Santa Catarina	Subtropical úmido (Cfa)			
1300	Água Doce, Santa Catarina	Oceânico temperado (Cfb)			
774	Videira, Santa Catarina	Oceânico temperado (Cfb)	Uva Cabernet Sauvignon, fenologia e maturação da uva	São Joaquim A (maior altitude) e Videira (menor altitude) são os locais mais frios e mais quentes, respectivamente, de todos os estudados durante todos os eventos fenológicos. As uvas de Videira tiveram o menor período de maturação em comparação com as uvas de São Joaquim A que tiveram o maior.	Falcão et al.(2010)
960	Bom Retiro, Santa Catarina	Oceânico temperado (Cfb)			
1160	São Joaquim B, Santa Catarina	Oceânico temperado (Cfb)			
1350	Água Doce, Santa Catarina	Oceânico temperado (Cfb)			
1415	São Joaquim A, Santa Catarina	Oceânico temperado (Cfb)			
2150	Benzilan, Condado de Deqin, China	Subtropical de altitude (Cwb)	Uva Cabernet Sauvignon, perfil de flavonóides	As uvas de Shuori foram colhidas em 2009 e 2010, Benzilan em 2009 e Meilishi em 2010. A concentração de antocianinas e flavonóis variou em termos de altitude e safra, com amostras de uva da altitude mais alta (vinhedo Shuori) mostrando níveis mais altos desses flavonóides em comparação com as amostras de Benzilan e de Meilishi. A concentração de antocianinas das amostras de Shuori em 2010 foi maior do que em 2009, enquanto para flavonóis as uvas de Shuori colhidas em 2009 apresentaram níveis mais altos em comparação com as amostras de 2010. Em relação aos flavan-3-óis, na mesma safra houve pequenas diferenças em sua concentração entre as amostras de diferentes altitudes, enquanto houve uma grande diferença entre as estações de cultivo de 2009 e 2010. A maioria dos flavonóis e antocianinas foi influenciada mais pela altitude do que pela safra, enquanto para os flavan-3-óis o impacto da safra foi maior.	Xing et al.(2016)
2300	Meilishi, Condado de Deqin, China	Subtropical de altitude (Cwb)			
2900	Shuori, Condado de Deqin, China	Subtropical de altitude (Cwb)			
2282 e 2435	Condado de Danba, China	Subtropical de altitude (Cwb)	Vinho Merlot, atividade antioxidante, perfil fenólico, avaliação sensorial	Teor de fenólicos totais (TPC), teor de antocianinas monoméricas totais (TMAC), teor de compostos fenólicos individuais e atividade antioxidante aumentaram significativamente com a altitude. Os atributos sensoriais qualidade gustativa e harmonia aumentaram com a altitude.	Jin, Wu, Liu, 2017
2282,			Vinho	TPC, TMAC, teor de taninos, teor de	

2435 e 2608			Cabernet Sauvignon, atividade antioxidante, perfil fenólico, avaliação sensorial	compostos fenólicos individuais e atividade antioxidante aumentaram significativamente com a altitude. Vinhos produzidos a 2608 m apresentaram melhor intensidade e qualidade aromática, pureza, intensidade e qualidade gustativa e harmonia.	
2110	Xidang, Condado de Deqin, China	Subtropical de altitude (Cwb)	Vinho Cabernet Sauvignon, perfil volátil	Vinho de Xidang (2110 m) teve as maiores concentrações de ésteres, aldeídos e cetonas, o vinho de Dari (2249 m) teve os álcoois mais altos e o vinho de Jiunongding (2330 m) teve as maiores concentração de ácidos graxos. O número de compostos voláteis identificados aumentou com o aumento da altitude, enquanto a concentração total dos voláteis diminuiu, exceto para o vinho de Xidang.	Yue et al.(2015)
2249	Dari, Condado de Deqin, China	Subtropical de altitude (Cwb)			
2330	Jiunongding, Condado de Deqin, China	Subtropical de altitude (Cwb)			
2615	Adong, Condado de Deqin, China	Subtropical de altitude (Cwb)			
2788	Jiangpo, Condado de Deqin, China	Subtropical de altitude (Cwb)			
195	Província de Trento, Itália	Oceânico temperado (Cfb)	Uva Pinot Gris, fenologia da uva	Uvas Pinot Gris e Merlot, cultivadas em baixa altitude, apresentam menor avanço de fases fenológicas e maior encurtamento da estação de crescimento (brotação até data da colheita). Uvas Pinot Noir, Sauvignon Blanc e Chardonnay, localizadas em altas altitudes, tiveram um comportamento oposto.	Alikadic et al.(2019)
198			Uva Merlot, fenologia da uva		
339			Uva Chardonnay, fenologia da uva		
368			Uva Sauvignon Blanc, fenologia da uva		
470			Uva Pinot Noir, fenologia da uva		
100-150	Região do Douro, Portugal	Mediterrâneo de verão quente (Csa)	Uvas tintas Touriga Nacional (TN) e Touriga Francesa (TF), perfil de antocianinas e procianidinas	Uvas TN e TF cultivas em baixa altitude (100-150 m) tiveram concentração de antocianinas na casca menor do que as uvas TN e TF cultivadas em alta altitude (300-350 m e 250-300 m, respectivamente). O mesmo resultado foi observado nos vinhos produzidos a partir dessas uvas. Em relação ao perfil de procianidinas nas cascas e nas sementes, a uva TN apresentou maiores concentrações em baixa altitude (100-150 m) do que em alta (300-350 m), sendo que o mesmo foi observado para o vinho. Para a cultivar TF, maiores concentrações foram observadas nas uvas cultivas em alta altitude (250-300 m) e nos vinhos produzidos com elas.	Mateus et al.(2001)
300-350			Uva tinta Touriga Nacional (TN), perfil de antocianinas e procianidinas		
250-300			Uva tinta Touriga Francesa (TF), perfil de antocianinas e procianidinas		
100-150			Vinhos Touriga		

			Nacional (TN) e Touriga Francesa (TF), perfil de antocianinas e procianidinas		
300-350			Vinho Touriga Nacional (TN), perfil de antocianinas e procianidinas		
250-300			Vinho Touriga Francesa (TF), perfil de antocianinas e procianidinas		
1000 e 1500	Hadim, Cônia, Turquia	Semiárido frio (BSk)	Uva Ekşikara, perfil fenólico e atividade antioxidante	O conteúdo de antocianina monomérica detectado na baga e na casca foi maior na altitude mais alta (1500 m). Uma diminuição significativa foi observada nos níveis de tanino da casca e da semente com o aumento da altitude. Com o aumento da altitude, as quantidades de ácido clorogênico na baga e na semente diminuíram. Os flavonóis rutina e isorhamnetina-3-glicosídeo tiveram uma diminuição drástica em sua concentração na fruta inteira conforme a altitude aumentou. No entanto, seu conteúdo na casca e nas sementes não teve mudanças significativas como resultado da altitude mais alta. A atividade antioxidante da baga, casca e semente diminuiu significativamente com a diminuição da altitude.	Coklar, 2017
150	Kali Vrisi, região de Drama, Grécia	Subtropical úmido (Cfa)	Uva Merlot, características físico-químicas	Uvas de Nevrokopi (860 m) apresentaram significativamente bagas menores e maior teor de açúcar e acidez mais elevada em comparação às outras uvas. Uvas de Adriani tiveram significativamente mais acidez e maior peso e menor teor de açúcar do que uvas de Kali Vrisi.	Bouloumpasi et al.(2025)
150	Adriani, região de Drama, Grécia	Subtropical úmido (Cfa)			
190	Agora, região de Drama, Grécia	Oceânico temperado (Cfb)			
860	Nevrokopi, região de Drama, Grécia	Continental úmido de verão fresco (Dfb)			
150	Kali Vrisi, região de Drama, Grécia	Subtropical úmido (Cfa)	Vinho Merlot, teor fenólico e análise sensorial	Vinhos de Nevrokopi (860 m) tiveram significativamente maior teor fenólico do que os outros vinhos, com destaque para: procianidina B2, catequina, procianidina B1, epicatequina e ácido gálico (90% do total de fenóis).	
150	Adriani, região de Drama, Grécia	Subtropical úmido (Cfa)			

190	Agora, região de Drama, Grécia	Oceânico temperado (Cfb)
860	Nevrokopi, região de Drama, Grécia	Continental úmido de verão fresco (Dfb)

[a] m: metros acima do mar. [b] Classificação climática de Köppen-Geiger. TPC: teor de fenólicos totais (*Total phenolic content*); TMAC: teor de antocianinas monoméricas totais (*Total monomeric anthocyanin content*).

Os estudos apresentados na Tabela 1 demonstram que a altitude exerce influência decisiva na qualidade e nas características das uvas e vinhos. Como observado nas uvas Cabernet Sauvignon de Santa Catarina (FALCÃO *et al*., 2010), alta altitude retarda a maturação das uvas, fazendo com que as fases fenológicas sejam mais longas, o que pode prolongar o acúmulo de compostos fenólicos nas uvas (ARIAS *et al*., 2022). Vinhos produzidos acima de 1.000 m, como os Malbec de Tupungato (1.240-1.510 m) (URVIETA *et al*., 2018) e Cabernet Sauvignon de Gualtallary em Mendoza (MUÑOZ *et al*., 2021) e os Syrah da Bahia (1.100 m) (OLIVEIRA *et al*., 2019a) apresentaram maior concentração de compostos fenólicos, principalmente antocianinas, flavonóis e estilbenos. Além disso, o número de compostos voláteis e a atividade antioxidante aumentaram com o aumento da altitude, como evidenciado nos vinhos Merlot e Cabernet Sauvignon provenientes da China (YUE *et al*., 2015; JIN, WU, LIU, 2017).

De maneira geral as uvas apresentaram o mesmo comportamento, mas há diferenças no perfil fenólico entre casca e semente conforme a altitude, como nas uvas Syrah da Bahia (1.100 m) que tiveram maior concentração de taninos condensados na casca, enquanto que as uvas de Pernambuco (350 m) da mesma variedade apresentaram maior concentração de taninos condensados na semente (OLIVEIRA *et al*., 2019b). Contudo, variações regionais e entre cultivares, como no caso das uvas portuguesas Touriga Nacional (TN) e Touriga Francesa (TF) em que a TN mostrou mais taninos em baixas altitudes e a TF em altas altitudes (MATEUS *et al*., 2001), indicam que a

altitude não é um fator isolado, e seu impacto deve ser interpretado em conjunto com outros elementos do *terroir*.

3.2 Fenologia da videira

A fenologia é o estudo dos fenômenos naturais que ocorrem periodicamente em plantas, analisando sua relação com mudanças sazonais e climáticas. Em outras palavras, ela caracteriza as fases anuais do desenvolvimento vegetal, buscando entender as causas da variação no tempo desses eventos por meio da correlação entre condições climáticas e datas de ocorrência. Na viticultura, a fenologia foca no acompanhamento das etapas do ciclo de desenvolvimento da videira, desde a poda até a colheita. Esse conhecimento é essencial para selecionar locais e cultivares adequadas; planejar o manejo do vinhedo; organizar práticas agrícolas (poda, irrigação, colheita); e antecipar necessidades de mão de obra e equipamentos, tendo como objetivo final prever o comportamento da planta em diferentes ambientes, melhorando a eficiência e a qualidade da produção (KELLER, 2020).

Há diversos sistemas de classificação das fases fenológicas da cultura da videira. A escala proposta por Eichorn e Lorenz (1984) e modificada por Coombe (1995) é bastante utilizada: brotação (4), surgimento das primeiras folhas verdes; floração (19), surgimento de cachos de flores (inflorescências); frutificação (31), em que as flores fertilizadas transformam-se em pequenas bagas verdes; *véraison* (35), bagas começam a amadurecer, aumentar de tamanho e mudar de cor (de verde para amarelo nas uvas brancas; de verde para vermelho, roxo ou preto nas uvas tintas); colheita (38), uvas são colhidas quando atingem o ponto ideal de maturação para a produção de vinho (Figura 1).

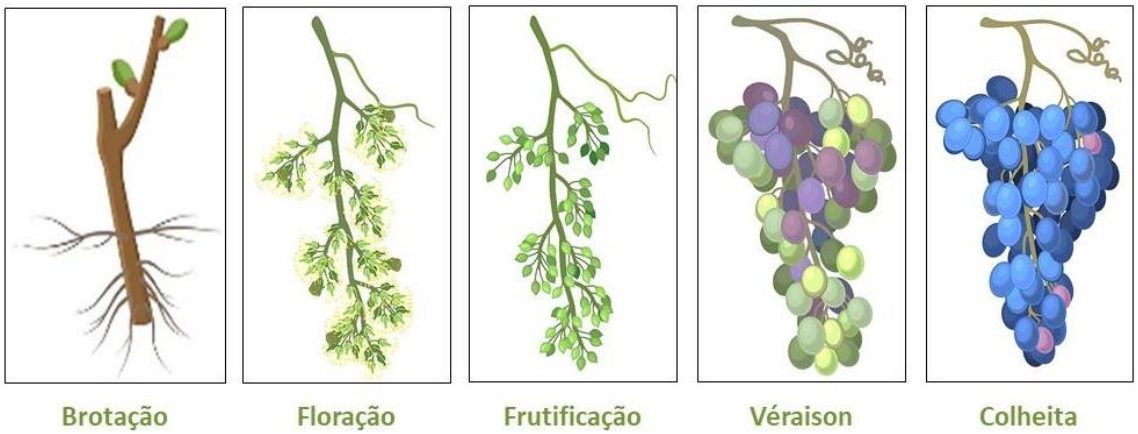


Figura 1. Ilustração das fases fenológicas de uvas viníferas baseada na escala fenológica desenvolvida por Eichorn e Lorenz (1984) e adaptada por Coombe (1995). Fonte: elaborado pela autora.

3.3 Maturação fenólica e o ponto ideal para colheita de uvas

A determinação do ponto ideal de colheita das uvas é um dos fatores de maior importância para a obtenção de vinhos de qualidade em qualquer região do mundo, uma vez que a composição química da uva muda durante a sua maturação, principalmente o teor de açúcares, compostos voláteis e compostos fenólicos (LIMA, 2010).

As antocianinas e proantocianidinas (taninos), compostos fenólicos presentes na casca das uvas tintas e sementes, respectivamente, são particularmente importantes para a qualidade dos vinhos, pois contribuem para características organolépticas do vinho, como cor (no caso dos tintos), corpo, adstringência e amargor. No caso dos vinhos tintos, os consumidores procuram vinhos com cor vermelha profunda, encorpados, taninos suaves e aroma frutado, sendo que este tipo de vinho só pode ser obtido a partir de uvas que atingiram a maturação fenólica completa. Uvas insuficientemente maduras têm menor capacidade de extração de antocianinas e proantocianidinas da casca e maior capacidade de extração de proantocianidinas das sementes, podendo produzir vinhos mais adstringentes e amargos (CANALS *et al*., 2005; CONDE *et al*., 2007; KONTOUDAKIS *et al*., 2011; ALLEGRO *et al*., 2021).

As características edafoclimáticas estão intimamente relacionadas ao desenvolvimento e maturação das uvas, condicionando a sua composição fenólica. A radiação solar, por exemplo, influencia na composição da fruta através de mecanismos fotossintéticos, regulando enzimas envolvidas na

síntese de compostos da uva, como a enzima fenilalanina amônia liase (PAL, do inglês: *phenylalanine ammonia lyase*) que atua na produção de fenóis e antocianinas. Temperaturas acima de 35 ºC durante a fase de maturação da uva podem prejudicar a composição fenólica e, consequentemente, a qualidade do vinho (SILVA, 2017). Em relação à pluviosidade, as concentrações de antocianinas tendem a serem maiores devido a uma menor incidência de precipitação (CHENG *et al*., 2014).

3.4 Compostos fenólicos e atividade antioxidante de uvas e vinhos

Os compostos fenólicos são substâncias formadas pelo metabolismo secundário das plantas, responsáveis pela pigmentação e adstringência e proteção da planta contra a radiação ultravioleta, parasitas e insetos. Sua estrutura compreende um ou mais anéis aromáticos ligados a um ou mais grupos hidroxila (-OH), além de poderem estar associados a grupos metila, moléculas de açúcar ou ácidos. Os compostos fenólicos são divididos em dois grandes grupos: flavonóides e não flavonóides. Os flavonoides é o grupo mais abundante, sendo divididos em cinco subclasses: antocianinas, flavanóis (monômeros (catequinas) e polímeros (proantocianidinas)), flavonóis, flavanonas e flavonas. Os não flavonóides possuem uma estrutura química mais simples que a dos flavonoides, sendo compostos principalmente por ácidos fenólicos (ácidos hidroxibenzóicos e ácidos hidroxicinâmicos) e estilbenos (resveratrol) (DURAZZO *et al*., 2019; LI, SUN, 2019; ALBUQUERQUE *et al*., 2021; GUTIÉRREZ-ESCOBAR, ALIAÑO-GONZÁLEZ, CANTOS-VILLAR, 2021). Eles são um fator chave na qualidade dos vinhos, pois determinam muitas de suas propriedades sensoriais, como cor, sabor, adstringência, amargor e contribuem para a estabilidade do vinho. Dentre esses compostos, as antocianinas e as proantocianidinas (também chamados de taninos condensados) são os mais importantes, além de serem os antioxidantes naturais presentes em maior concentração na uva e no vinho (CONDE *et al*., 2007). Esses compostos sofrem reações químicas durante a vinificação, como polimerização e condensação, resultando em pigmentos mais estáveis. Os produtos de condensação das antocianinas, por exemplo, são

menos sensíveis ao pH do que seus precursores e não sofrem descoloração (GARRIDO, BORGES, 2013; LI, SUN, 2019).

Antioxidantes são compostos que possuem a capacidade de inibir ou prevenir a oxidação através da inativação de radicais livres e redução do estresse oxidativo. O estresse oxidativo é o desequilíbrio do estado redox em que quantidades excessivas de espécies reativas de oxigênio (ROS, do inglês: *reactive oxygen species*) ou espécies reativas de nitrogênio (RNS, do inglês: *reactive nitrogen species*), como superóxido ($O_2^{\cdot -}$), hidroxil ($HO^{\cdot}$), hidroperoxil ($HO_2^{\cdot}$), peroxil ($ROO^{\cdot}$), óxido nítrico ($NO^{\cdot}$) e peroxinitrito ($ONOO^-$), superam a capacidade antioxidante (DAI, MUMPER, 2010; GULCIN, 2020). Os compostos fenólicos são potentes antioxidantes. Esse potencial antioxidante depende da estrutura química desses compostos, como o número e posições do grupo hidroxila (-OH), presença de ligação dupla (C2=C3) e conjugação com moléculas de açúcar ou outras substâncias nos anéis aromáticos. Os mecanismos pelos quais os compostos fenólicos exercem atividade antioxidante são: (1) eliminação de radicais livres, como ROS e RNS; (2) suprimir a formação de ROS/RNS por meio da inibição de enzimas ou quelar metais envolvidos na produção de radicais livres, como ferro e cobre; e (3) regular ou proteger a defesa antioxidante (DAI, MUMPER, 2010; CAMPOS, 2019).

A atividade antioxidante dos compostos fenólicos protege o vinho da oxidação (OLIVEIRA *et al*., 2011; ECHAVE *et al*., 2021). Diversos métodos foram desenvolvidos para avaliar a atividade antioxidante dos alimentos. Devido à diversidade da estrutura química e dos mecanismos pelos quais os compostos fenólicos exercem atividade antioxidante, este parâmetro tem sido avaliado com base em mais de um método (DAI, MUMPER, 2010; GULCIN, 2020).

4 ARTIGOS

4.1 Artigo 1 – Evolution of anthocyanin content during grape ripening and characterization of the phenolic profile of the resulting wine by comprehensive two-dimensional liquid chromatography

Este artigo é fruto do doutorado sanduíche (setembro de 2022 até fevereiro de 2023) realizado no Instituto de Pesquisa em Ciência de Alimentos (*Instituto de Investigación en Ciencias de la Alimentación*) da Universidade Autônoma de Madri, Espanha, sob a supervisão do Dr. Miguel Herrero.

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Evolution of anthocyanin content during grape ripening and characterization of the phenolic profile of the resulting wine by comprehensive two-dimensional liquid chromatography

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Abstract: The typical phenolic profile in grapes is characterized by its complexity both in terms of number of diverse chemical structures and their variation during ripening. Besides, the specific phenolic composition of grapes directly influences the presence of those components in the resulting wine. In this contribution, a new method based on the application of comprehensive two-dimensional liquid chromatography coupled to a diode array detector and tandem mass spectrometry has been developed to obtain the typical phenolic profile of Malbec grapes cultivated in Brazil. Moreover, the method has been demonstrated to be useful to study how the phenolic composition in grapes evolved during a 10-week ripening period. Main detected compounds in grapes and in the wine derived from them were anthocyanins, although a good number of polymeric flavan-3-ols were also tentatively identified, among other compounds. Results show how the amount of anthocyanins present in grapes was increased during ripening up to 5–6 weeks and then decreased towards week 9. The two-dimensional approach applied was demonstrated to be useful for the characterization of the complex phenolic profile of these samples, involving more than 40 different structures and has the potential to be further applied to the study of this important fraction is different grapes and wines systematically.

Keywords: phenolic compounds, grape, wine, two-dimensional liquid chromatography, LC × LC

1.Introduction

Phenolic compounds are a group of natural bioactive molecules found mainly in vegetables and fruits, including grapes and their by-products such as wine [1]. They are produced in grapes in response to environmental stress, like predation, attack by microorganisms, UV light levels and water deficit. Their composition and content in wine is greatly influenced by grape variety and maturity stage, technological practices to which grapes are exposed, type of yeast and bacteria used in the alcoholic and malolactic fermentation, respectively, and contact with solid parts of the grape during maceration [2–4].

Polyphenols are essential in the quality of wine, especially red ones, contributing to sensory properties (color, flavor, astringency and bitterness). Besides, they have been described to have antioxidant, anti-inflammatory, cardioprotective and anti-hypertensive activity and positive effects on human microbiota composition and functionality. Thus, different previously presented studies have been focused on the determination of these compounds [3, 5, 6].

They are characterized by having an enormous structural variability, ranging from simple phenolic molecules to highly polymerized compounds, and are mostly found in conjugated forms with mono- and polysaccharides, that can be linked to different positions within the polyphenolic structure. This usually makes the profiling of these compounds a laborious task [3, 7]. The analytical approach most-frequently adopted to identify these bioactive compounds is liquid chromatography (LC). Nevertheless, if the polyphenolic content of the analyzed sample is very complex, the typically used one-dimensional LC (1D-LC) might not be sufficient for obtaining enough resolution [8, 9]. On this matter, the utilization of multidimensional approaches, such as comprehensive two-dimensional liquid chromatography (LC × LC), considerably enhances the separation potential since it takes advantage of the combination of two independent separation mechanisms to effectively boost the available resolving power as well as to produce a powerful increase on peak capacity [10] . One of

the key features of this technique is that the whole sample is analyzed through two separation mechanisms (dimensions). Consequently, compounds that are not resolved in a single separation may be further separated using a second separation. Due to the excellent resolving power provided, LC × LC has been demonstrated as a really useful tool for different purposes in food analysis [11–13]. As grapes and wines are considered very complex samples in terms of polyphenols composition, different works have explored the possibility of using different LC × LC coupling for their analysis, including for the identification of polyphenols in wines [14,15], grapes [16], grape juices [17] and winemaking by-products, such as seeds [18] or grapevine canes [19]. Among the different couplings of separations mechanisms that can be combined, the use of hydrophilic interaction chromatography (HILIC) and reversed phase (RP) conditions in the first (^{1}D) and second (^{2}D) dimensions, respectively, has shown an excellent potential for polyphenols analysis [20, 21]. This coupling is characterized by a high degree of orthogonality, as separations in the two dimensions are based on non-correlated mechanisms. However, important issues make its application challenging, mainly the solvent strength mismatch produced between the solvents used as mobile phases in the ^{1}D and those used in the ^{2}D [22]. In this regard, different modulation procedures have been proposed to limit this problem and improve the peak shapes and resolution in the ^{2}D separations. Among them, the use of focusing modulation or the use of a dilution step of the ^{1}D effluent before being injected in the ^{2}D have been shown as good strategies to reduce the solvent incompatibility effect [22, 23].

In this context, the aim of this article is to develop a new LC × LC method that combines a HILIC separation in the ^{1}D with a RP-based separation in the ^{2}D coupled to diode array detector (DAD) and tandem mass spectrometry (MS/MS) to characterize the anthocyanin profile of Malbec grapes with different degrees of ripeness and the phenolic profile of wine produced from them to study the composition and evolution of polyphenols during grape maturation as well as the final composition after winemaking. To the best of our knowledge, this is the first report in which a dedicated LC × LC method is applied to study the evolution of secondary metabolites over time in foods.

2.Materials and methods

2.1.Samples and chemicals

Grape (*Vitis vinifera* L.) samples from the variety Malbec were collected from a vineyard from Garanhuns (Pernambuco, Brazil) between December 2021 and February 2022. This cultivar was chosen because it stood out in terms of aptitude and productivity in this region. Grape samples were collected weekly (during 10 weeks) from the phenological stage number 35 of the vine (beginning of ripening; berries beginning to color and softening) [24], totalizing 10 samples: W1, W2, W3, W4, W5, W6, W7, W8, W9 and W10.

The wine was produced with grapes harvested at W9, as this is the usual practice adopted by wineries in this Brazilian region. The red wine was prepared in the Oenology Laboratory of Embrapa Semiárido in the city of Petrolina, Pernambuco. Potassium metabisulfite, commercial yeast *Saccharomyces cereviseae* var. bayanus Maurivin PDM (0.20 - 0.30 g L^{-1}), Gesferm® ammonium phosphate fermentation activator (around 0.20 g L^{-1}) and Everzym color® pectinolytic enzyme (0.01 - 0.03 g L^{-1}) were used as oenological adjuvants. After destemming and crushing the grapes, alcoholic fermentation and solid-liquid maceration were carried out at 24 ± 2 °C. Subsequently, spontaneous malolactic fermentation was performed at 17 ± 1 °C, until the complete transformation of malic acid into lactic acid, evidenced by paper chromatography (around 30 days), and cold tartaric stabilization (0 °C) for ten days, and with the addition of 0.4 g L^{-1} of Stabigum® (E414 Gum arabic + E353 metatartaric acid). Bottling was carried out in dark bottles of 750 mL, with prior correction of the free sulfur dioxide content to 50 mg L^{-1}. The bottles were stored for 30 days in a horizontal position in a wine cellar at 16 ± 1 °C and humidity around 60% for the aging of the wine in the bottle.

HPLC grade methanol, acetic and formic acid were purchased from VWR Chemicals (France). Acetonitrile (LC grade) was supplied by Carlo Erba Reagents (France). Water employed was Milli-Q grade obtained from a Millipore system (Billerica, MA).

2.2. Extraction of phenolic compounds from grape and wine

The phenolic compounds were extracted according to a previously published method [25], with some modifications. Grape samples were lyophilized (Liotop® L101, Vitória, Brazil) at -58 ± 2 °C and 6.7 ± 1 Pa for around 96 h to obtain samples with moisture of 1.5 ± 0.2%. The extraction of phenolic compounds from the lyophilized samples W1-W10 (0.5 g in 50 mL falcon tubes) was performed using 5 mL of a methanol:water solution (8:2, *v/v*) acidified with 0.35% formic acid, vortexing for 3 min followed by centrifugation (3000 rpm, 4 °C, 5 min) on a Rotina 380R centrifuge (Hettich, Tuttlingen, Germany). The described procedure was repeated 5 times. Supernatants were pooled and evaporated with nitrogen. The sample was reconstituted with 1 mL of methanol. The extracts were then filtered through a nylon syringe filter (0.45 µm) and placed in 3 mL HPLC vials. Extraction procedure was performed in triplicate. The phenolic compound extraction from wine was carried out by using 5 mL of the sample and 5 mL of a methanol:water solution (8:2, *v/v*) acidified with 0.35% formic acid. The mixture was vortexed for 3 min and evaporated with nitrogen. The sample was reconstituted with 1 mL of methanol. Extraction procedure was performed in triplicate.

2.3. LC × LC analyses

2.3.1. Instrumentation

LC × LC instrumentation consisted of an Agilent 1200 series liquid chromatograph (Agilent Technologies, Santa Clara, CA) equipped with an autosampler. Additionally, a LC pump (Agilent 1290 Infinity) was coupled to perform the second dimension (^{2}D) separation. ^{1}D and ^{2}D were connected by an electronically-controlled two-position ten-port switching valve (Rheodyne, Rohnert Park, CA, USA) acting as a modulator equipped with two identical 50 µL or 100 µL injection loops. Modulation time of the switching valve was 1 min or 1.5 min as indicated below. For the introduction of the make-up flow when necessary, an additional Agilent 1200 series pump controlled using a 1200 Instant Pilot (Agilent) was employed. A diode array detector (DAD) was coupled after the second dimension in order to register every ^{2}D analysis. Signals at 280, 254 and 550 nm were monitored and UV–Vis spectra from 190 to 550 nm

were registered using a sampling rate of 20 Hz. Besides, an Agilent 6320 ion trap mass spectrometer (MS) equipped with an electrospray interface working under positive ionization mode was coupled in series using the following conditions: dry temperature, 350 °C; dry gas flow rate, 12 L min^{-1}; nebulization pressure, 345 Pa; mass range, *m/z* 90–1200 Da; ultra scan mode (26,000 *m/z*/s). Auto MS(n) was performed for acquiring tandem MS/MS spectra. The flow eluting from the ^{2}D column was split before entering MS instrument to ca. 0.7 mL min^{-1}. The LC data were elaborated and visualized using LC Image software (version 1.0, Zoex Corp., Houston, TX). For method optimization, different columns were tested in the ^{1}D and in the ^{2}D. The used columns, as their main characteristics are included in Table S1.

2.3.2. LC × LC separation conditions

The final LC × LC separation conditions were obtained after optimization, as described below, involving the following conditions:

i)^{1}D separation: ZIC–HILIC column (150 × 1 mm, 3.5 µm, Merck, Darmstadt, Germany) eluted using acetonitrile (A) and 10 mM ammonium acetate, pH 5 (B) at 25 µL min^{-1} applying the following gradient: 0 min, 5% B; 20 min, 25% B; 30 min, 30% B; 45 min, 35% B; 50 min, 40% B; 80 min, 55% B; 85 min, 5% B; 100 min, 5% B. In all cases, the injection volume was 10 µL.

ii)^{2}D separation: C18 column (Ascentis Express C18, 50 × 4.6 mm, 2.7 µm, Supelco, Bellefonte, CA) eluted using water (0.1% formic acid, A) and acetonitrile (B) at 3.0 mL min^{-1} with the following 1.5 min repetitive gradients: 0 min, 5% B; 0.3 min, 5% B; 0.8 min, 30% B; 0.9 min, 40% B; 1 min, 90% B; 1.2 min, 90% B; 1.21 min, 5% B. This gradient was repeated during the whole LC × LC analysis time. To match the duration of each ^{2}D separation, the modulation time set for the switching valve actuation was 1.5 min.

Moreover, an additional make-up flow was introduced to reduce the relative solvent strength at the exit of the ^{1}D using a T-piece at a flow rate of 75 µL min^{-1}. The make-up flow was composed of water. When the make-up flow was employed, the final separation conditions used in the ^{2}D involved the same column and mobile phases as above stated, although the elution conditions

were adjusted as follows: 0 min, 5% B; 0.1 min, 15% B; 0.3 min, 20% B; 0.8 min, 35% B; 0.81 min, 5% B; using a flow rate of 2.5 mL min^{-1}. This gradient was repeated during the whole LC × LC analysis time. To match the duration of each ^{2}D separation, the modulation time set for the switching valve actuation was 1.0 min. Besides, the sampling loops employed under this set-up had an inner volume of 100 µL.

Other columns tested in both dimensions are described in Section 3.

2.3.3. Theory, peak capacity, orthogonality

To estimate the main figures-of-merit of the developed 2DLC approaches, different calculations were carried out, specifically to provide values or peak capacity and orthogonality.

Peak capacity (n_c) was calculated for each dimension starting from the basic equation shown below (1):

$$n_c = 1 + \frac{t_G}{\bar{w}} \quad (1)$$

where t_G is the gradient time and w is the average peak width. For both dimensions, the average peak width was obtained from no less than 10 representative peaks selected along the analyses. Peak broadening factor <β> has been proposed to correct ^{1}D peak capacity in LC × LC systems, so that the potential deleterious effect of undersampling of the ^{1}D effluent is considered. This parameter was calculated as follows:

$$^{1}n_{c,\,corrected} = \frac{^{1}n_c}{\sqrt{1 + 0.21\left(\frac{t_S}{^{1}\sigma}\right)^2}} \quad (2)$$

where t_s is the sampling time and $^1\sigma$is the average width of ^{1}D peaks as standard deviation in time units before modulation. With this information, the overall LC × LC peak capacity was calculated as follows:

$$^{2D}n_{c,\,theoretical} = {}^1n_c \times {}^2n_c \quad (3)$$

This eq. (3) simply follows the so-called product rule. To have a more realistic value, and to correct possible effects from modulation and undersampling, practical peak capacity was calculated as follows (eq. (4)):

$$^{2D}n_{c,\,practical} = \frac{{}^1n_c \times {}^2n_c}{\sqrt{1 + 3.35 \times \left(\frac{{}^2t_c \times {}^1n_c}{{}^1t_G}\right)^2}} \quad (4)$$

Where ^2t_c is the ^{2}D separation time cycle (equal to the modulation time). It has to be noted, that this calculation already considers <*β*>.

To estimate the orthogonality (A_0) degree of the system, the approach based on asterisk equations was followed [26]. Briefly, this method considers the spread of peaks along the four imaginary lines crossing the ^{2}D space forming an asterisk. More practical details can be found elsewhere [19, 26].

2.4. Conventional one-dimensional LC

Conventional one-dimensional LC analyses of the grape and wine samples were performed using an Agilent 1200 series liquid chromatograph (Agilent Technologies, Santa Clara, CA) equipped with an autosampler. The method used was based on a previously reported [27], with some modifications. Briefly, a C18 column (column (150 × 2.1,3 µm, Phenomenex, Germany) was employed. The mobiles phases employed consisted of water (0.1% formic acid, solvent A) and acetonitrile (0.1% formic acid, solvent B), eluted at 0.2 mL min^{-1} using the following gradient: 0 min, 5% B; 5 min, 15% B; 30 min, 50% B; 35

min, 65% B; 38 min, 100% B. The detection was performed using a DAD, recording the signals at 280, 490 and 550 nm and the injection volume was 10 µL.

3.Results and discussion

3.1. Optimization of separation

Under 1DLC conditions, the separation of phenolic compounds is commonly performed in reversed phase mode using C18 columns [27]. In order to evaluate the potential of conventional 1DLC and the eventual need for LC × LC for the separation of these complex samples, both, the wine and a grape sample (W9) were analyzed using a conventional C18 column. As can be observed in Fig. S1, the complexity of the phenolic compounds pattern present in the samples did not allow obtaining a complete separation of the compounds. Both chromatograms were characterized by the presence of large humps containing many coeluted compounds without baseline separation. This fact clearly pointed to the need for LC × LC separations.

In this context, an online comprehensive 2DLC method, as orthogonal as possible was targeted for the analysis of the grape and wine samples included in the present study. The final aim was to find the analytical conditions allowing a proper separation of the phenolic fraction present in these complex samples. Red grape and wine phenols are believed to be composed of a heterogeneous mixture of phenolic acids, flavan-3-ols, flavonols and anthocyanins [28]. For this reason, to obtain the complete phenolic profile of these samples, analytical methods with sufficient resolving power are needed, as previously demonstrated. In this regard, the use of LC × LC combining the use of a HILIC-based separation in the ^{1}D together with a C18 column in the ^{2}D has already been shown to provide appropriate levels of separation power and it is characterized by a high degree of orthogonality [21, 20]. Consequently, this combination was the one selected for the characterization of grapes and wine polyphenols in the present research.

Initially, the optimization of the separation conditions in each dimension was carried out individually. To do that, a selected grape sample (harvested at week 10, W10) was used as model sample and injected using conventional

one-dimensional LC emulating the conditions that should be employed later on in the 2DLC method. Table S1 shows the columns screened for the ^{1}D separation. The aim of this screening was to test the capabilities of different stationary phases for the separation of the polyphenols, including diol, ZIC-HILIC, and silica particles. For each column, a separate optimization was performed. Fig. S2 shows the chromatograms obtained after optimization for each tested column. As can be observed, the best results were obtained using the ZIC-HILIC column as it provided the best compromise between sample distribution (number of separated peaks and wide of those) and analysis time. The other tested columns also provided separations that could be suitable for the LC × LC method, as the sample was widely distributed, although at the expense of significantly longer analysis times. Besides, the sample was just separated in 2–3 big humps, increasing the chance for coelutions in the final 2DLC method. These separations using diol and silica stationary phases could not be further improved, even after thorough optimization. Consequently, the ZIC–HILC column was selected for the LC × LC analyses.

An important aspect considered is that the minimum possible flow rate providing a good distribution along the separation space for a given reasonable analysis time was considered optimum. For the ^{2}D, and based on our previous experience, two stationary phases (C18 and PFP) were tested, both packed inside a short column and in the form of partially porous particles. The use of this type of particles allows a good resolution with lower back-pressures produced. For the optimization, a total analysis time of 1.5 min was established as target time to allow the separation and re-conditioning of the column using very high flow rates (> 2.0 mL min^{-1}). In Fig. S3, a comparison of the performance of the two tested columns under optimum separation conditions is shown. As can be observed, the C18 column provided an increased separation power with respect to the PFP column, showing a significantly higher number of separated peaks. In this latter case, an increase in the flow rate employed with the aim to decrease the total analysis time did not improve the separation of the two big humps obtained.

Although the conditions shown in Fig. S3 are not actually produced under a 2DLC analysis, bearing in mind that here the whole sample is being injected,

the obtained separations provide a clear idea of the potential of the column to be applied to the studied samples. In spite of this, it is common that further optimization or gradient tweaks are needed once the system is run in LC × LC mode.

Once the individual conditions were selected for each dimension, the HILIC × RP method was implemented. To do that, the same grape sample used during optimization (W10) was injected into the system. The modulation time employed in the switching valve was 1.5 min and it was equipped with two identical 50 µL sampling loops. Taking into consideration the optimum flow rate determined in the ^{1}D (25 µL min^{-1}), each transfer filled 75% of the total volume available in the loops, assuring that loss of sample was not produced. The separation obtained after coupling and minor adjusting of the ^{2}D gradient profile is shown in Fig. 1A. As it can be observed, although the separation of a good number of components was obtained, very intense peaks were eluted grouped at the beginning of the ^{2}D separations (ca. t_R = 10 s). For this reason, it was decided that further modification of the ^{2}D gradients should be necessary to have a better utilization of the available ^{2}D separation space.

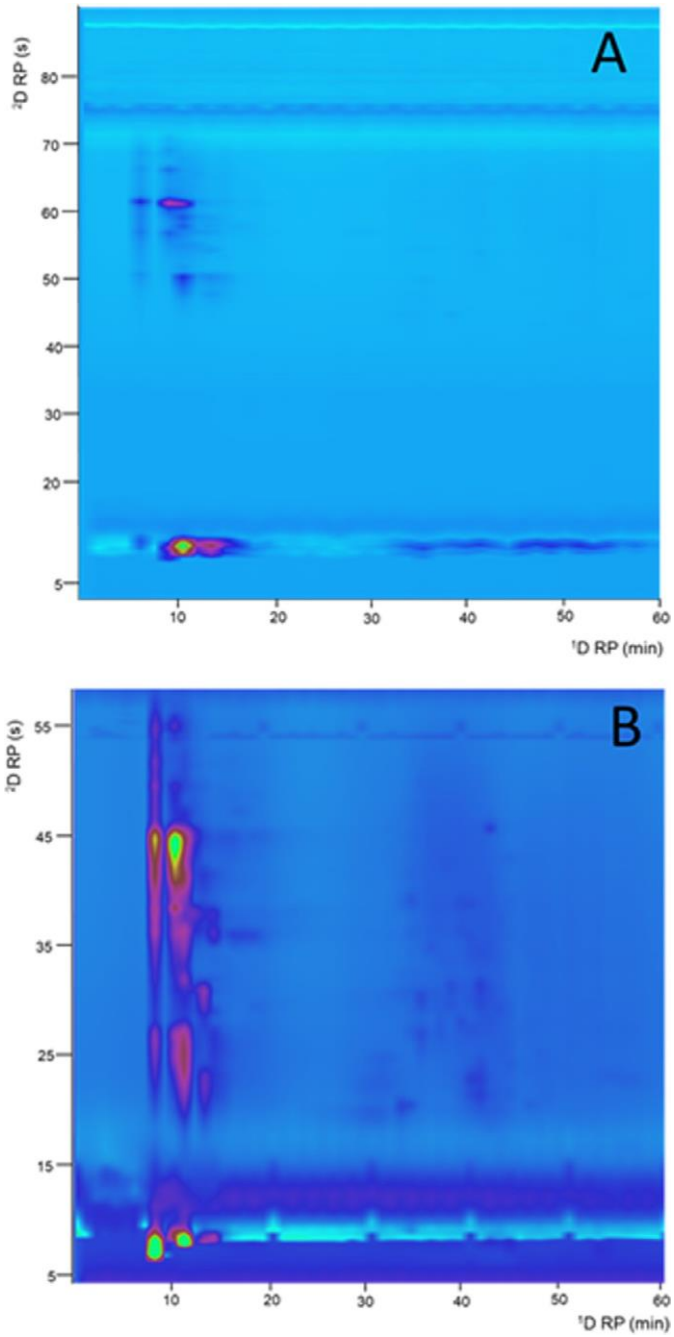


Fig. 1. 2D plots (280 nm) obtained for the polyphenolic profiling of a grape sample (W10) under the optimized HILIC × RP conditions using direct transfer between dimensions **(A)** and after the introduction of a dilution step **(B)**. Separation conditions as explained in the text.

Different gradients and modifications of the composition of the mobile phases were studied. A summary of the findings observed is illustrated in Fig. S4. However, none of the tested approaches allowed a significant improvement in the separation of this area. The elution of a high number of components at the beginning of the ^{2}D separations was most likely due to the solvent strength mismatch that typically occurs between HILIC and RP separations. The problem

could be minimized to some extent thanks to the fact that only 75% of the sampling loop volume was used to store and transfer the ^{1}D effluent, being the rest filled with ^{2}D mobile phase. In any case, as this solution was clearly insufficient, the introduction of a dilution step using an appropriate make-up flow was studied. In this case, an additional pump was used to reduce the overall solvent strength of each fraction transferred. The used make-up flow was composed of water. As the aim of the use of this approach is to reduce the fraction solvent strength, water is considered the most-favorable case for the subsequent RP separation in the ^{2}D. Moreover, based on previous findings, a 3:1 make-up solvent-to-fraction ratio was considered appropriate. As the ^{1}D flow rate employed was 25 mL min^{-1}, the make-up flow was introduced at 75 mL min^{-1}. Consequently, the sampling loops installed in the modulator were changed to an increased inner volume of 100 µL. By using this approach, not only the separation in the ^{2}D improved significantly, but also it was possible to carry out a re-optimization of the gradient employed to reduce to total ^{2}D analysis time to 1.0 min, which improves the undersampling effect of the ^{1}D separation. After implementation of the 1.0 min repetitive gradients into the HILIC × RP protocol, the obtained ^{2}D separation was significantly improved, as illustrated in Fig. 1B. As it can be inferred from the comparison established in Fig. 1, the addition of the make-up flow was demonstrated to enhance the retention in the ^{2}D, avoiding the early elution and partial coelution of a great part of the transferred components (as shown in Fig. 1A).

The improvement achieved in terms of resolution and better use of the available separation space are translated into higher peak capacity values and orthogonality values when the make-up flow is employed, as can be seen in Table 1 (1106 *Vs* 1095). However, this is a good example of the limitations often encountered when using peak capacity as an estimation of the performance of the method. As can be observed, the theoretical peak capacity (not considering any correction in terms of degree of orthogonality) obtained for the optimum set-up (with make-up flow) was only marginally better than the peak capacity obtained without make-up flow. However, it is clearly visible from Fig. 1 that this latter set-up produced a significantly worse use of the available ^{2}D space (Fig. 1A) as a result of the elution problems above commented.

Nevertheless, the higher gradient time available when make-up flow was not employed (modulation time 1.5 min) compared to the optimum set-up (modulation time 1.0 min) as well as the fact that a lot of peaks coeluted in a narrow band around $^{2}t_R$ = 10 s caused that the $^{2}n_c$ value was higher under non-optimum conditions (Table 1). Overall, these results demonstrate how, although peak capacity may be a useful parameter to compare the performance of 2DLC methods, these values should not be considered alone when describing the figures-of-merit of the analytical approach, as they can led to a clear misjudgment.

Table 1. Peak capacity and orthogonality values calculated for the separations corresponding to a grape sample (W10) under optimum LC × LC conditions.

		Without make-up flow	With make-up flow
^{1}D	$\overline{w}$ (min)	2.34	1.51
	$^{1}n_c$	27	40
	$<\beta>$	2.19	2.19
	$^{1}n_c$ corr.	17	26
^{2}D	$\overline{w}$ (s)	1.41	1.41
	$^{2}n_c$	65	43
LC x LC	Modulation time (min)	1.5	1
	^{2}Vinj (loop volume) (µL)	50	100
	Z_1	0.62	0.77
	Z_2	0.97	0.89
	Z_-	0.80	0.83
	Z_+	0.82	0.96
	A_0 (%)	63	74
	$^{2D}n_c$ theoretical	1095	1106

3.2. Characterization of the polyphenolic fraction of grapes and wine

Once the HILIC × RP method was optimized, it was applied to the analysis of 10 grape samples of different maturation degree (week 1 to week 10), as well as to the analysis of the wine prepared from the samples harvested at week 9. Thanks to the application of this novel method, the evolution of the polyphenols present in the samples during grape maturation could be followed, as well as their transformation as a result of the winemaking procedure. To assign the separated compounds, the information provided by the DAD as well as the MS and MS/MS characteristic fragmentation patterns was used, and compared to the values found in the literature.

Table 2 summarizes the information collected for each peak, as well as the tentative identification reached for the wine sample. Besides, Fig. 2 shows the ^{2}D contour plot obtained for the phenolic profile present in the wine. As it can be observed at first sight comparing Figs. 2 and 1 B, the overall phenolic composition in the wine was significantly more complex than the one present in the grape samples.

Table 2. Identification parameters of phenolic compounds determined by LC x LC-DAD-MS/MS using the optimized HILIC × RP method.

Peak	Total t_R (min)	t_R ^2D (s)	λ abs	$[M + H]^+/M^+$	Main fragments	Proposed identification
1	6.13	7.60	280, 530	495.3	332	NI
2	6.41	24.55	276, 526	493.7	331	Malvidin-O-hexoside
3	6.56	35.55	286, 530	535.7	331	Malvidin-O-acetylhexoside
4	6.63	38.20	290, 530	581.6	547, 419, 401, 331, 293, 251	NI
5	6.73	43.95	284, 530	639.6	331	Malvidin-O- p-coumaroylhexoside
6	6.84	50.30	286, 530s	611.3	287	Cyanidin-O-dihexoside
7	8.13	8.05	276, 528	493.5	331	Malvidin-O-hexoside
8	8.26	15.75	272	535.1		Malvidin-O-acetylhexoside
9	8.39	23.50	280, 526	493.4	331	Malvidin-O-hexoside
10	8.41	24.65	280, 526	493.4	331	Malvidin-O-hexoside
11	8.44	26.40	282, 528	493.4	331	Malvidin-O-hexoside
12	8.53	31.50	292	535.1	331	Malvidin-O-acetylhexoside
13	8.60	35.85	298, 268	535.9	331	Malvidin-O-acetylhexoside
14	8.63	38.10	290			
15	8.73	43.90	284, 525	638.6	331	Malvidin-O-p-coumaroylhexoside
16	8.79	47.45	262			
17	10.15	8.70	272, 524	465.7	303	Delphinidin 3-hexoside
18	10.38	22.80	282, 300 s, 324	493.6	331	Malvidin-O-hexoside
19	10.60	36.05	260	536.1	331	Malvidin-O-acetylhexoside
				595.8	332	Malvidin dipentoside
20	10.70	41.85	284, 526	640.8	331	Malvidin-O-p-coumaroylhexoside
21	12.60	36.10	260, 356	493.2	331	Malvidin-O-hexoside
				535.6	331	Malvidin-O-acetylhexoside
22	29.50	30.15	275, 325s	609.6	447, 301	Peonidin-6-coumaroylhexoside
23	33.33	20.05	278	579.3	427, 409, 291	Procyanidin dimer
24	35.52	31.25	280, 310s	501	371, 259	NI
25	38.65	38.80	304, 514	707.4	399	p-Coumaroyl vitisin A
26	44.13	8.05	280, 328 s, 530	365.5	203, 275, 347	NI
27	42.32	19.70	300 s, 328	595.5	577, 443, 427, 291, 247	(Epi)gallocathechin-(*epi*)catechin
28	42.33	20.25	280, 310	475.3	456, 311	NI
29	42.40	24.20	274, 506	561.2	399	Vitisin A
30	42.51	31.05	280	797.1	365, 617	NI
31	43.59	35.20	280, 305s	562.1	399	NI
				603.5	399	Malvidin-3-(6-acetyl)glucoside-pyruvic acid
32	44.34	20.10	280	579.2	427, 409, 291	Procyanidin dimer
33	44.38	22.70	276	867.1	579, 577, 409, 715	Procyanidin trimer
34	45.46	27.80		867.1	697, 579, 577,425	Procyanidin trimer
35	50.32	19.45	278	138.8	-	p-Hydroxybenzoic acid
36	50.49	29.25	280	493.8	331	Malvidin-O-hexoside
37	52.27	16.45	280	680.1	497, 467, 305	NI
38	52.38	22.60	278	867.2	579, 449, 409	Procyanidin trimer
39	56.35	21.00	275	428.7	409, 247, 229	NI
40	56.41	24.75	280	465.3	303	Delphinidin-hexoside

*NI, not identified; s, spectral shoulder.

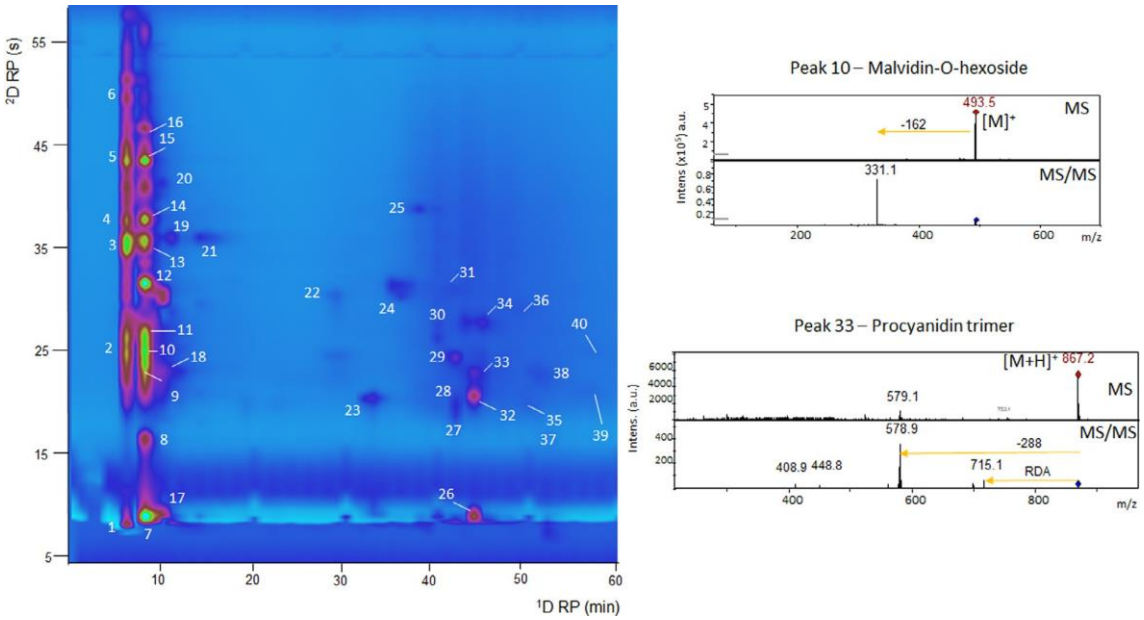


Fig. 2. Two-dimensional HILIC × RP plot (280 nm) corresponding to the Malbec wine sample analyzed under optimum conditions with the addition of make-up flow during the modulation. For peak identification, see Table 2. For detailed separation conditions, see text.

Most of the detected phenolic compounds belonged to anthocyanins. As it can be observed in the ^{2}D plot shown in Fig. 2, the separation obtained was clearly formed by two groups of components. The first one, eluting earlier from the ^{1}D was mainly composed of the anthocyanins, whereas the second group, eluting from 30 min comprised other phenolic compounds. Among anthocyanins, malvidin derivatives were the most abundant. Different malvidin-hexoside derivatives were detected thanks to their characteristic molecular $[M]^{+}$ ions at *m/z* 493.7 that provided a specific product ion at *m/z* 331 corresponding to the aglycone, after the neutral loss of the sugar moiety ($[M-162]^{+}$). Fig. 2 also shows the typical MS and MS/MS spectra for this kind of compounds (peak 10). The same fragmentation pattern was observed for malvidin-coumarylhexoside and malvidin-acetylhexoside. Apart from malvidin, other anthocyanins corresponding to delphinidin-hexoside (peaks 17 and 40) and to peonidin-6-coumaroylhexoside (peak 22) were also tentatively assigned, thanks to the detection of their typical molecular ions and the appearance of fragments compatible with their corresponding aglycones upon fragmentation.

The polyphenolic composition was also composed of vitisin A and p-coumaroyl vitisin A (peaks 29 and 25, respectively), with molecular ions at *m/z*

561.2 and 707.4, respectively. In these cases, the occurrence of fragment ions at *m/z* 399 ($[M-162]^+$ and ($[M-162-146]^+$, respectively) allowed the peaks' assignment, in agreement with those fragments reported in the literature [29]. The remaining tentatively identified compounds were flavan-3-ols. The 2D separation allowed grouping this family of compounds in the medium-last part of the analysis. In this group, diverse procyanidin dimers and trimers were detected. As can be observed in Table 2, the fragment ions detected matched the expected fragmentation pattern for both dimers (*m/z* 579) and trimers (*m/z* 867). Specifically, two peaks corresponding to procyanidin dimers were detected (peaks 23 and 32), and assigned thanks to the detection of their molecular ions as $[M+H]^+$ together with fragment ions at *m/z* 427 and 409 generated thorough a retro Diels-Alder fission and its loss of water, respectively [30], as well as a fragment corresponding to the flavan-3-ol monomer (*m/z* 291). In turn, peaks 33, 34 and 38 presented a $[M+H]^+$ ion at *m/z* 867.1. The fragmentation pattern obtained was compatible with that of procyanidin trimers. In fact, fragment ions at *m/z* 715 were present, as a result of a retro Diels-Alder fission of the heterocyclic ring, as well as others at *m/z* 579 resulting from the quinone methide cleavage of the interflavonoid bond (Fig. 2) [30].

Although simple monomeric flavan-3-ols were not detected in this sample, a derivative with *m/z* 595.5 and characteristic fragment ions at *m/z* 577, 433, 427 and 290 was detected, corresponding to (*epi*)gallocatechin-(*epi*)catechin dimer (peak 27). A single phenolic acid, *p*-hydroxybenzoic acid (peak 35) was also tentatively identified. Other compounds were also detected and are summarized in Table 2, although an identification could not be reached.

3.3. Evolution of grape polyphenols during ripening

In this work, besides the optimization of a HILIC × RP method for the characterization of the phenolic profile of grapes and wine, the use of the enhanced separation power achieved by this new method was used as a direct application in a food quality strategy consisted of the study of grape anthocyanidins evolution along the post-harvest ripening stages. The phenolic profile clearly evolved with the advancement of the degree of ripeness of the grapes (W1 to W10), as shown in the 2D plots of Fig. S5. As above mentioned,

anthocyanins were the main compounds detected in grape and wine samples. This fact can be seen in Fig. 3, which shows the DAD 2D plots of the sample W9 acquired at 280 nm (common wavelength for phenolic compounds) and 520 nm (specific wavelength for anthocyanins). Thanks to the specificity of 520 nm for the detection of anthocyanins, it is possible to distinguish the anthocyanins from the rest of the phenolic compounds. In this way, it is clear that the anthocyanins were grouped in the first 20 min of the analysis with a much higher intensity than the rest of the separated phenolic compounds. For this reason, the evolution of the main anthocyanins was evaluated during the ripening of the grapes using the peak volume obtained in DAD.

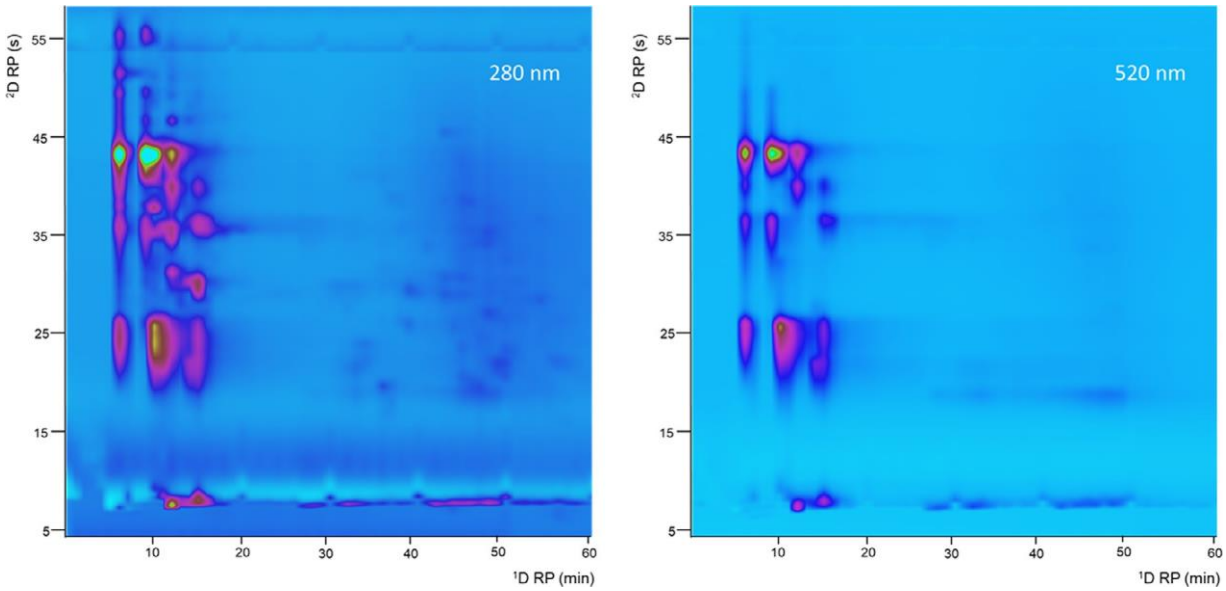


Fig. 3. 2D plots at 280 nm and 520 nm obtained under the optimum HILIC × RP separation conditions for the grape sample W9 with the addition of make-up flow during the modulation. Separation conditions as in Fig. 2.

The identification of the selected anthocyanins can be observed in Table S2, whereas the assignment of each peak is shown in Fig. S5. Fig. 4 graphically shows the evolution of the individual anthocyanins and the behavior of each of them along the 10 weeks. Interestingly, most of the anthocyanins showed a similar trend; their initial content is low (especially at W1 and W2) and then grows to a maximum at W5-W6, followed by a decrease of these compounds from week 7 and stabilization in weeks 9 and 10. These results agree with the

outcomes reported by Blancquaert *et al*. [31] who showed a maximum anthocyanin concentration at the berries state which corresponds to the period between the *veraison* and the maturation of the fruit which can last five to eight weeks depending on the variety and climatological conditions. This period characterized by the maximum concentration of anthocyanins in grapes also matches the higher concentration of sugars in the berry, known as sugar ripeness and in consequence, phenolic ripeness. This fact is due to sugars may regulate the synthesis pathways of these phenolic compounds [31].

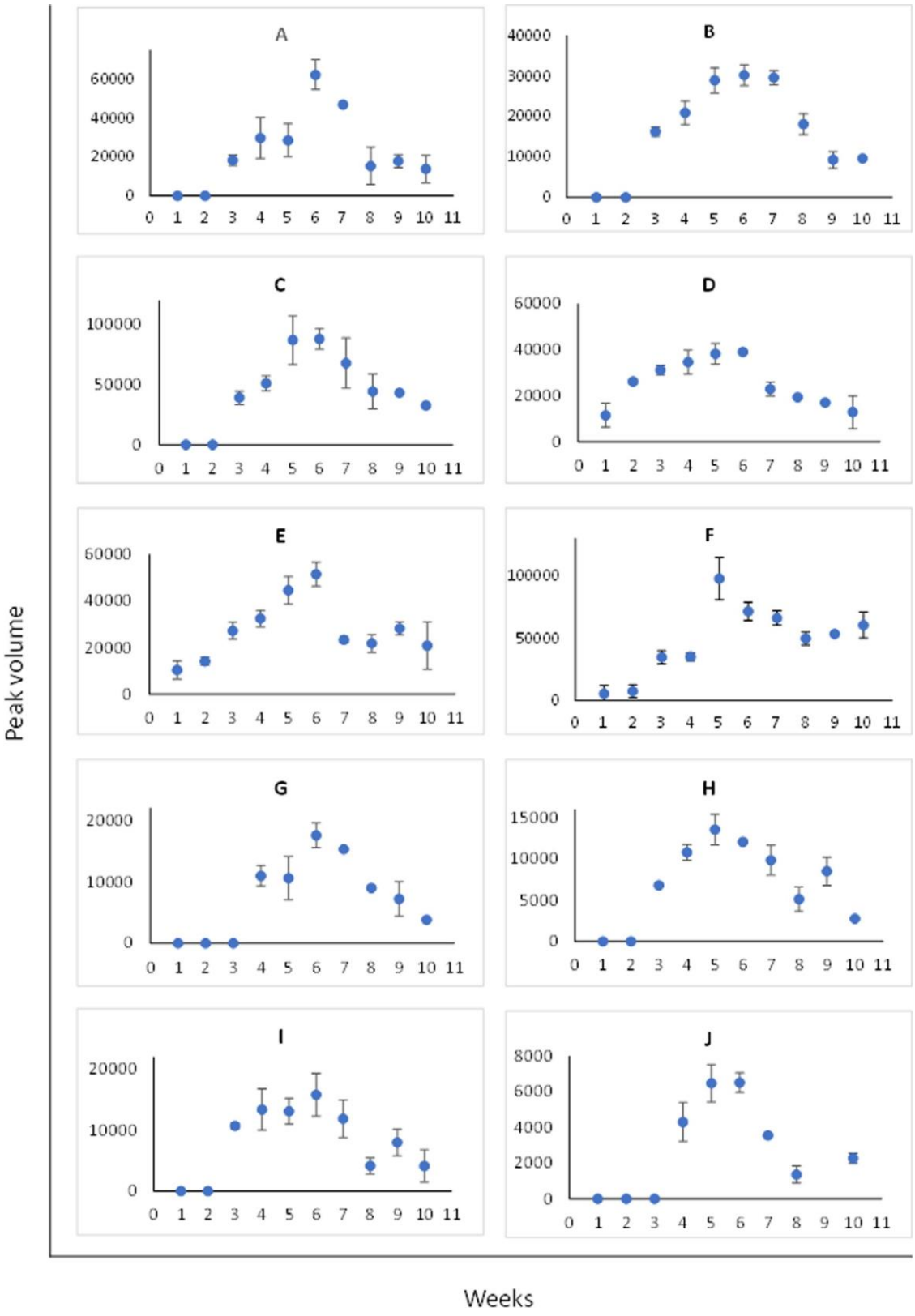


Fig. 4. Concentration trend of the main anthocyanins found during grape ripening over a period of 10 weeks (W1-W10). IDs of each graph (A-J) correspond to the identified compounds represented in Fig. S5 and Table S2.

The reduction of the anthocyanidin content after the seventh week of ripeness may be related to degradation reactions of anthocyanins in advanced stages that occur due to the exposure of grapes to high temperatures, as observed in tropical viticulture regions [32]. Therefore, considering the high anthocyanin content and its relationship with the higher sugar content in the grapes, the elaboration of wines from grapes harvested in week 6 might be advantageous for the wine quality, since the higher concentration of anthocyanins is related to greater color intensity, as well as greater color stability during wine storage. Moreover, sugar content is related to the quality of the fermentation, although other parameters related to fruit ripeness may strongly impact on the selection of the harvest time like the presence and concentration of tannins or aroma compounds like acetate esters, aldehydes or alcohols [33].

4.Conclusions

The optimization of a new HILIC × RP method coupled to DAD and MS detectors has allowed obtaining the phenolic profiles of different grape samples involving diverse ripeness degree as well as of the wine derived from them. Different stationary and mobile phases and gradients have been tested in both dimensions, and diverse modulation procedures have been evaluated. Moreover, the final analytical conditions after final columns combination selection were also re-optimized and fine-tuned. The new method provided with a resolving power significantly enhanced compared to conventional one-dimensional approaches. Thanks to the combination of both used detectors, a good number of characteristic anthocyanins and flavan-3-ols have been tentatively identified in the wine. Most of the detected anthocyanins were composed of malvidin derivatives, being this aglycone the most abundant compound in the wine sample. Moreover, the method was useful to acquire the specific trends followed by selected polyphenols during grape ripening along 10 weeks. Results showed that anthocyanins tend to accumulate during the first 5–6 studied weeks, and then their concentration decrease towards weeks 9–10 of ripening. These findings helped define the ideal period for harvesting Malbec grapes grown in a tropical viticulture region in order to take advantage of the greater potential of anthocyanins that impart color to wines.

Overall, this methodology further demonstrates how the application of LC × LC can be a valuable tool for the analysis of very complex food samples in spite of the obvious higher complexity compared to one-dimensional methods.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Miguel Herrero reports financial support was provided by Spain Ministry of Science and Innovation.

CRediT authorship contribution statement

Laura Oliveira Lago: Investigation, Methodology, Writing – original draft. **Pawel Swit:** Investigation, Methodology, Writing – review & editing. **Mairon Moura da Silva:** Resources, Methodology. **Aline Telles Biasoto Marques:** Resources, Methodology. **Juliane Welke:** Conceptualization, Methodology, Funding acquisition, Supervision, Writing – review & editing. **Lidia Montero:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Miguel Herrero:** Conceptualization, Methodology, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

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Supplementary materials (Artigo 1)

Table S1. Columns used for the optimization of the HILIC × RP method used in the present research.

Column, stationary phase	Dimensions	Particle size and morphology	Manufacturer
First Dimension			
ZIC-HILIC	150 × 1.0 mm	3.5 µm, 100 Å	Merck (Darmstadt, Germany)
Luna silica	150 × 1.0 mm	3 µm, 100 Å	Phenomenex (Torrance, CA)
Lichrospher diol-5	150 × 1.0 mm	5 µm, 100 Å	HiChrom (Reading, UK)
Second Dimension			
Ascentis express C18	50 × 4.6 mm	2.7 µm partially porous	Supelco (Bellefonte, CA)
Kinetex PFP	50 × 4.6 mm	2.6 µm partially porous	Phenomenex (Torrance, CA)

Table S2. Identification proposed for the anthocyanins evaluated in the grape samples during ripening for a 10-week period.

ID	Rt ^{1}D (min)	Rt ^{2}D (s)	Total Rt (min)	M+/[M+H]$^{+}$	Main fragments	Identification
A	6	24.45	6.41	493.3	331	Malvidin-O-hexoside
B	6	35.85	6.60	535.3	331	Malvidin-O-acetylhexoside
C	6	43.4	6.72	535	331	Malvidin-O-acetylhexoside
D	10	23.5	10.39	493.4	332	Malvidin-O-hexoside
E	10	25.6	10.43	520.8	317	Petunidin-O-acetylhexoside
F	10	43.6	10.73	638.6	331	Malvidin-O-p-coumaroylhexoside
G	15	21.75	15.39	483.4	NA	
H	15	24.5	15.41	493.9	331	Malvidin-O-hexoside
I	16	36.4	16.61	493.5	331	Malvidin-O-hexoside
J	18	35.9	18.60	611.9	303	Delphinidin-O-p-coumaroylhexoside

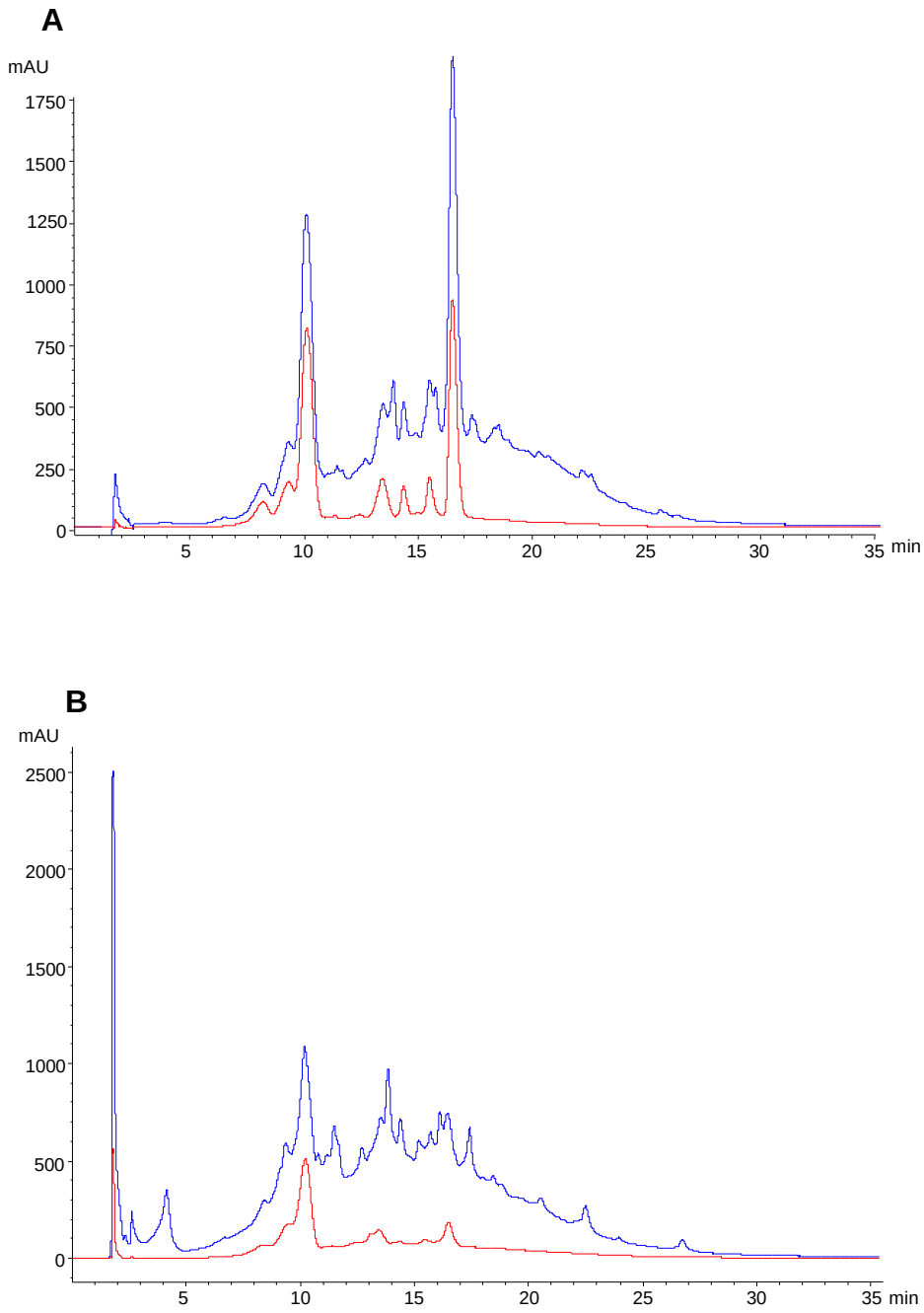


Figure S1. C18-RP 1DLC analysis of grape W9 (A) and wine (B). Blue: separation acquired at 280 nm; red: separation acquired at 520 nm.

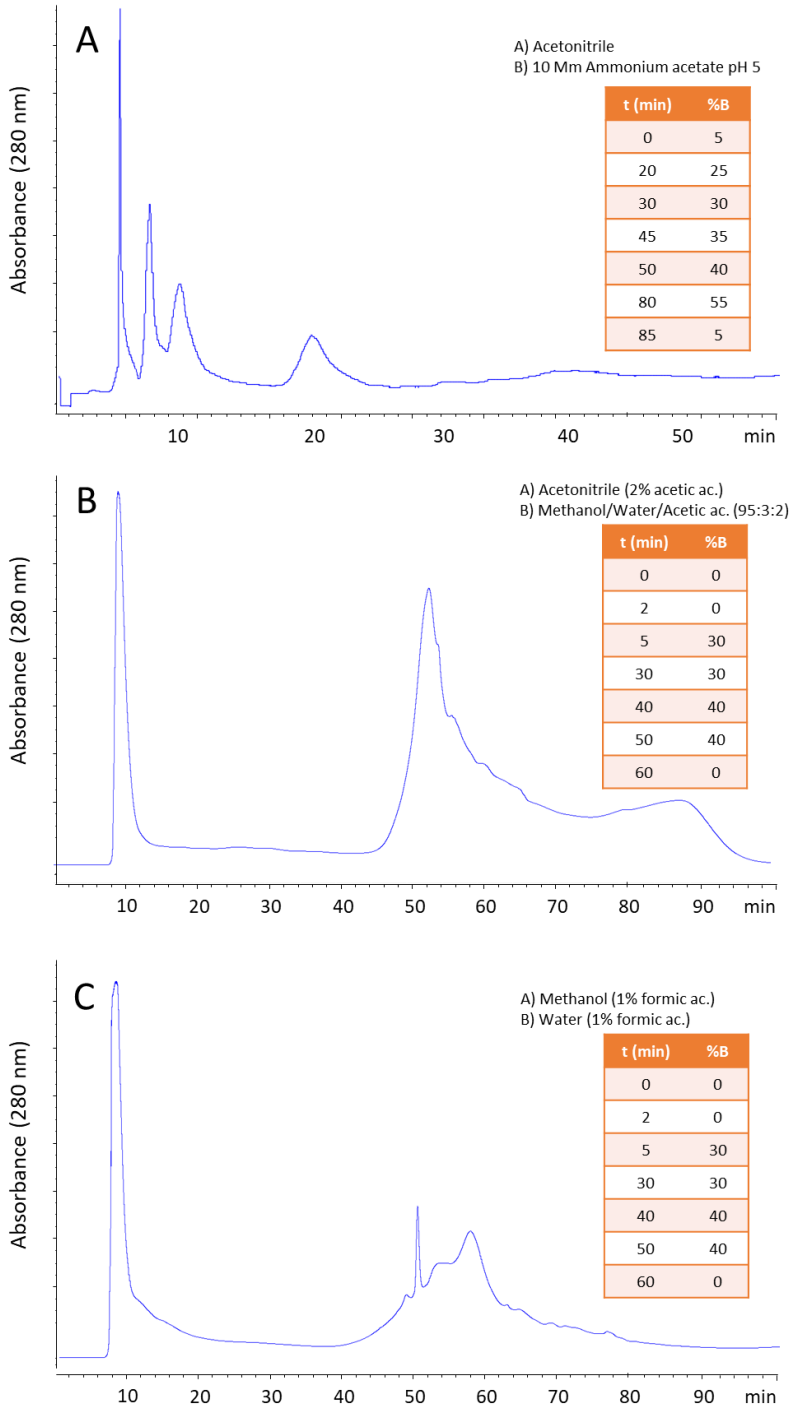


Figure S2. Chromatograms (280 nm) obtained for the HILIC separations obtained from a grape sample after optimization in a Zic-HILIC (A), diol (B) and silica (C) columns. Separation conditions are shown in inserts.

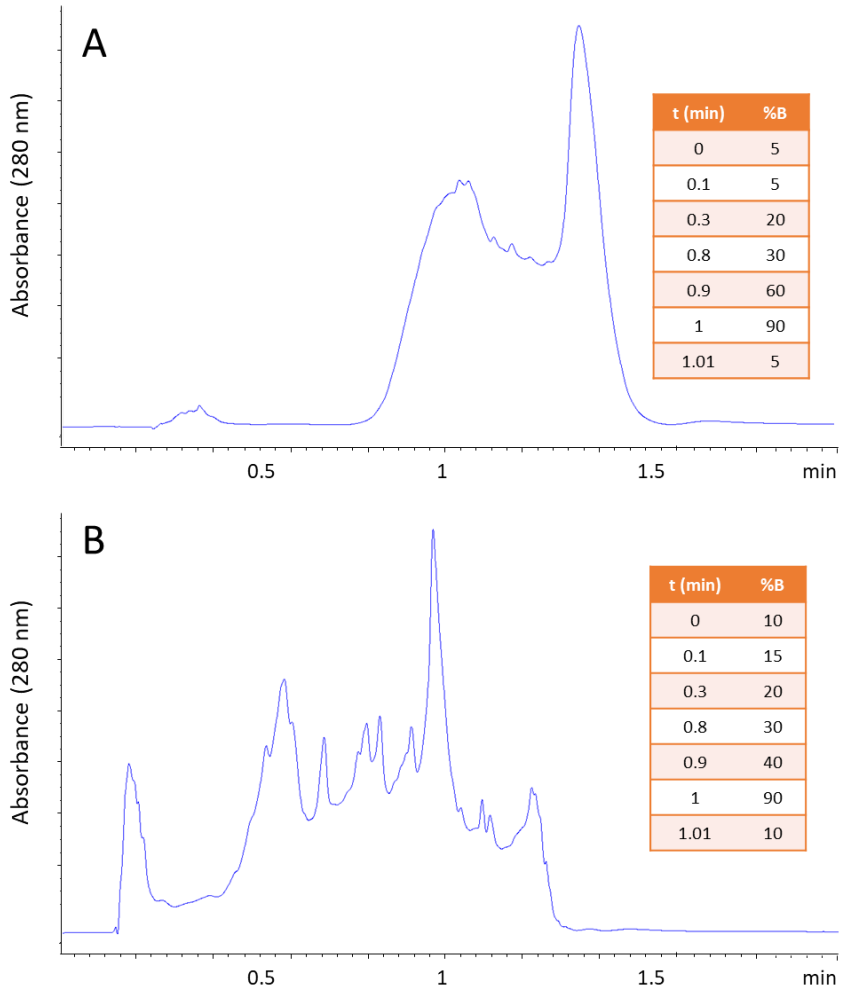


Figure S3. Chromatograms (280 nm) obtained for the RP-based separations obtained from a grape sample after optimization in a PFP (A) and C18 (B) columns. Separation conditions are: flow rate 2 ml min^{-1} (Panel A) or 3 ml min^{-1} (panel B); elution with water (0.1% formic acid, solvent A) and acetonitrile (solvent B) as mobile phases. Specific gradients were shown in the respective chromatogram.

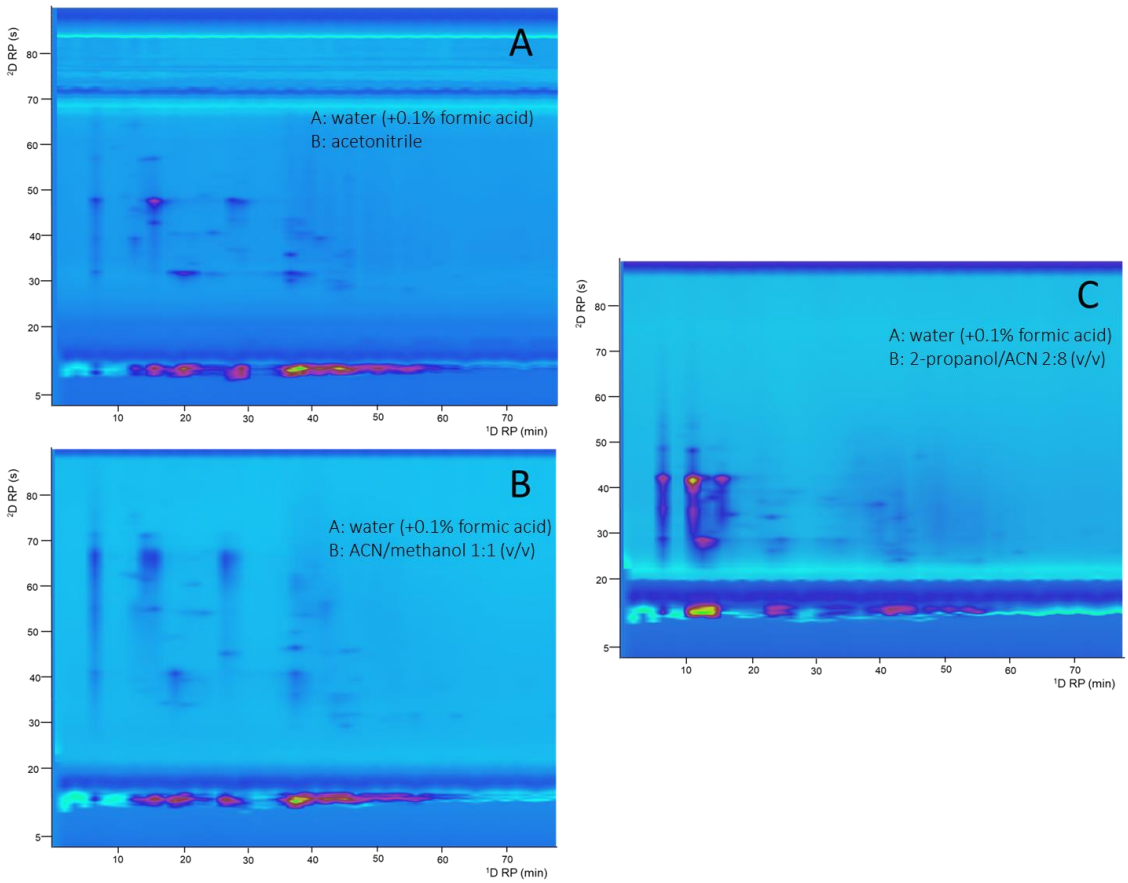


Figure S4. 2D contour plots from the polyphenolic fraction of a grape sample (W10) obtained using different gradients and mobile phases composition in the second dimension, as indicated.

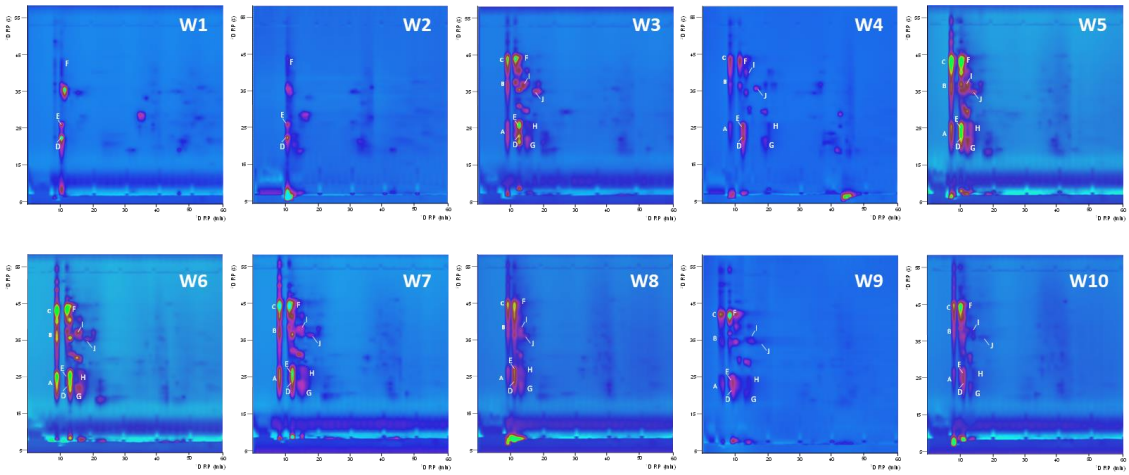


Figure S5. 2D plots obtained under the optimum separation conditions for the grape samples of different ripening degree (W1 to W10).

5 DISCUSSÃO GERAL

Este trabalho foi desenvolvido em parceria com a Embrapa Semiárido que tem se dedicado à realização de pesquisas com o objetivo de identificar tecnologias enológicas que contribuam para o desenvolvimento e fortalecimento da vitivinicultura no semiárido do Nordeste brasileiro.

No primeiro artigo (item 4.1), foi desenvolvido um método abrangente de cromatografia líquida bidimensional (LC × LC, do inglês: *two-dimensional liquid chromatography*) que combinou uma separação por cromatografia de interação hidrofílica (HILIC, do inglês: *hydrophilic interaction chromatography*) na primeira dimensão (^{1}D) com uma separação em fase reversa (RP, do inglês: *reversed phase*) na segunda dimensão (^{2}D), acoplado a um detector de arranjo de diodos (DAD, do inglês: *diode array detector*) e espectrometria de massas em tandem (MS/MS, do inglês: *tandem mass spectrometry*), para caracterizar o perfil fenólico de uvas tintas Malbec cultivadas no Brasil colhidas em diferentes estágios de maturação e o perfil fenólico do vinho produzido a partir delas. O objetivo principal foi usar o método desenvolvido para analisar a evolução do conteúdo de compostos fenólicos durante o amadurecimento das uvas Malbec para definir o período ideal para a realização da colheita.

Os resultados demonstraram que o método bidimensional desenvolvido permitiu a caracterização detalhada do perfil fenólico de uvas e vinho Malbec, sendo significativamente superior às abordagens unidimensionais convencionais. A elevada resolução da LC × LC permitiu identificar diversas antocianinas e flavan-3-óis e monitorar seu comportamento ao longo de 10 semanas de maturação. Observou-se que o conteúdo de antocianinas nas uvas aumentou até um máximo nas amostras W5-W6, seguido por uma diminuição nas semanas subsequentes.

No artigo 2 (item 4.2), foram avaliadas as mudanças nas características físico-químicas, no perfil fenólico e na atividade antioxidante durante a maturação de uvas Cabernet Sauvignon cultivadas em um vinhedo localizado na cidade de Garanhuns (Pernambuco) com o objetivo de identificar o momento ideal de colheita das uvas. As uvas colhidas entre as semanas 6 e 9 (G6-G9) apresentaram parâmetros físico-químicos favoráveis à vinificação,

6 CONCLUSÃO

Ao integrar os resultados obtidos com Malbec e Cabernet Sauvignon, este trabalho evidencia que a maturação das uvas em regiões tropicais de altitude não apenas influencia profundamente o perfil fenólico e a qualidade potencial do vinho, como também exige acompanhamento contínuo e criterioso. A identificação do ponto ideal de colheita, que correspondem ao período entre as semanas 5 e 6 para a cultivar Malbec e entre as semanas 6 e 7 para a cultivar Cabernet Sauvignon, contribui diretamente para a definição de estratégias de manejo e para a tomada de decisão na viticultura. Esse conhecimento valoriza as particularidades climáticas da região e amplia o potencial para a produção de vinhos de alta qualidade, ao permitir que a colheita seja realizada no momento de máxima concentração de antocianinas, compostos essenciais para a intensificação da cor dos vinhos. Além disso, os achados reforçam a importância de também monitorar as características físico-químicas e a atividade antioxidante das uvas durante a maturação para a tomada de decisão final.

Importante destacar que a diferença no pico de concentração das antocianinas entre as uvas Malbec e Cabernet Sauvignon demonstra que diferentes cultivares, mesmo sob condições de cultivo semelhantes, podem apresentar diferenças na dinâmica de acúmulo, síntese e degradação desses compostos.

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