Investigating Polyphenol Extracts Using Microwave Technology

A thesis presented in fulfilment of the requirements for the degree of

Doctor of Philosophy

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Thesis submitted to the University of Adelaide
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To my Husband Marek

and Daughters Marta and Monika

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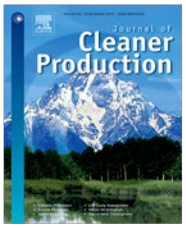

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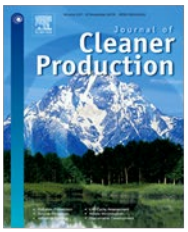

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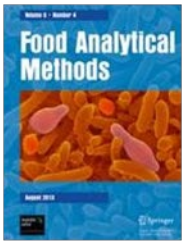

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Abstract

The objective of this thesis was to investigate phenolic extraction from grape skins using microwave technology and to add to the active research on phenolics in grapes. A research scholarship was awarded by the Grape and Wine Research and Development (known as Wine Australia) during 2010 – 2015.

Phenolics play a dual, friend or enemy, role in the wine and grape industry. Due to their antioxidant capacity, phenolics present in foodstuffs are understood to protect from chronic diseases caused by free radicals and lipid peroxidation and are widely used for nutritional and medicinal applications. On the other hand, that same antioxidant capacity is known to prevent efficient degradation in landfill of wine management waste (pomace/marc). Finally, phenolics affect the flavour, mouthfeel and colour of wines, and the modern wine industry is experimenting freely with the incorporation of phenolics in red and white wines to develop novel wines.

Grapes are a rich source of phenolics including those responsible for the skin colour. Grape extracts can be used for manufacturing nutraceuticals, including natural colourants, and functional foods, skincare and textile dyes as well as incorporated into Cleaner Production and Circular Economy (as opposed to the linear economy, where raw materials are used, products made, and wastes dumped) in waste management. The extracts can be obtained from primary biological material (in particular, when grapes are in over-supply) as well as from the post-winemaking waste (non-fermented pomace/post-fermentation marc), the latter helping in reducing the amount going to landfill.

There has been growing interest recently in finding more time and energy efficient and environmentally friendly methods of phenolic extraction. Microwave-assisted technologies offer such an opportunity. The extraction process can be optimized to achieve the maximal phenolic extraction for a particular cultivar. This study focusses on Australian commercial grape cultivars.

The thesis is structured as follows. Chapter 1 presents an extensive literature review on different extraction methods, including conventional and microwave ones.

Chapter 2 describes the microwave-assisted extraction (MAE) method, employing an industrial type of microwave reactor, which was compared with the conventional thermal extraction (CTE) method under the parameters producing the maximal phenolic extraction by each method. Multifactorial models were built to find optimal conditions to extract maximum total phenolics (TP) and colour (CIELAB chroma C^*) from separate white and red skin mixtures, each of six commercial cultivars collected at veraison and harvest. A high throughput Folin-Ciocalteu assay was developed for this study to measure TP. Adding novelty, and contrasting with the studies reviewed in Chapter 1, the microwave-assisted and conventional extraction methods were parallel-optimised by response surface methodology. The MAE and CTE protocols were set to minimise the method-to-method experimental error. It was found that the efficiency of the two TP extraction methods was comparable regardless of skin colour. The CTE provided more colour than MAE for white skins, and the same amount of colour for red skins. It was shown that the MAE method was 2.7-fold more energy efficient and 15-fold faster for a single extraction than the CTE method. The chapter discusses why the MAE methodology may be relevant for future trials at laboratory, pilot and production scales on the processing of

winemaking wastes (typically, up to 30% of the mass of crushed grapes) to reduce the landfill component. This approach would follow the Circular Economy tenets. This work has been published in Volume 251 online since 5th of Dec. 2019 by the *Journal of Cleaner Production*.

Chapter 3 compares the performance of the MAE and CTE methods in extracting TP and C* from the skins of the same individual cultivars as were used in the mixture extractions in the study in Chapter 2. The extractions were made under the optimal practical conditions for the skin mixtures. This comparison also included the spectral absorbance profiles at 280-600 nm. The variation in the phenolic extraction from the grape skins of individual cultivars collected at veraison and harvest were quantified for the first time in the literature (to the best of our knowledge). The average performance of extraction from individual skin collections was in agreement with the extraction expected for the mixture under the conditions set. Interestingly, it was noticed for the red skins only under the CTE method that the average TP extracted from individual cultivars (veraison and harvest combined) was 5% less than the TP obtained for the same red skins mixture, suggesting a potential synergy effect. The chapter presents a quantification of possible varietal and seasonal phenolic extraction. The results may be useful in oenology and industrial waste management. The manuscript was submitted on 23rd of Feb. 2020 to the *Journal of Cleaner Production* as a sequel to the previous study.

Chapter 4 investigates the applicability of a fast and simple attenuated total reflectance mid-infrared (ATR-MIR) spectroscopy method for the evaluation of TP and *C** of the white and red skin mixture extracts analysed in Chapter 2. This study was motivated by the observation that, although the Folin-Ciocalteu assay using 96-well plates and a liquid delivering robot was a high throughput method, it was still laborious and not likely to be attractive for the industry.

Partial least square (PLS1) regression models were built between the reference TP as well as *C**of the extracts and their raw and second derivative spectra. The full range of 4000-400 cm^{-1} and sub-ranges of 4000-1100 cm^{-1}, 1500-400 cm^{-1}, 1500-1100 cm^{-1} and 1457-1168 cm^{-1} were examined. (The last two ranges are used as phenolic fingerprint ranges.) It was found that the PLS1 models based on the second derivative of the 4000-1100 cm^{-1} sub-range provided the best results (fair classification) for both TP and *C**; hence these models are recommended for screening purposes. Moreover, some spectra ranges showed noticeable differences in absorption of phenolics and the compounds responsible for the colour of white and red skins. The study states that the ATR-MIR is worth considering for future trials in food industry and waste management. The manuscript has been accepted for publication on 6th of Jan. 2020 by *Food Analytical Methods*.

Chapter 5 presents the executive summary of the key research findings for this thesis, emphasising the novelty and potential benefits to manufacturing of nutraceuticals (including natural colourants), dietary supplements and non-alcoholic products, skincare, textile dyes, as well as to the wine industry and waste management. Future research directions are identified as including ATR-MIR assessment for quick optimization of the extraction process in industrial settings.

Papers Published or Submitted as Part of this Research

1. Kwiatkowski, M.; Kravchuk, O.; Skouroumounis, G. K.; Taylor, D. K. Response surface parallel optimization of extraction of total phenolics from separate white and red grape skin mixtures with microwave-assisted and conventional thermal methods. *Journal of Cleaner Production*. 2020, (online 5 Dec. 2019). Presented in Chapter 2

2. Kwiatkowski, M.; Kravchuk, O.; Skouroumounis, G. K.; Taylor, D. K. Total phenolics and colour in grape skin extracts of commercial white and red cultivars at veraison and harvest under microwave-assisted and conventional thermal extraction methods optimized for white and red grape skin mixtures. *Journal of Cleaner Production.* 2020, (submitted, 23 Feb. 2020). Presented in Chapter 3

3. Kwiatkowski, M.; Cozzolino, D.; Taylor, D. K. ATR-MIR spectroscopy predicts total phenolics and colour for extracts produced by microwave-assisted or conventional thermal extraction methods applied separately to mixtures of grape skins from white or red commercial cultivars. *Food Analytical Methods.* 2020, (accepted for publication 6 Jan. 2020). Presented in Chapter 4

Each of these manuscripts is presented in this thesis in either submitted or published form according to the instructions to authors of the relevant journal.

This thesis has been prepared following the University of Adelaide specifications for a PhD thesis by publication format.

Papers Published in Conference Proceedings

Kwiatkowski, M.J., Skouroumounis, G.K., Taylor, D.K. 2011. Microwave technology improves extraction of polyphenols from the grape skin in comparison with a conventional method. *The grape and wine science symposium Crush 2011*, 28-30. Sept. 2011, Adelaide, South Australia.

Kwiatkowski, M.J., Skouroumounis, G.K., Taylor, D.K. 2011. The Winner is Microwave! (poster, awarded). *Agriculture, Food and Wine Research Day symposium*, 10 Nov. 2011, Adelaide, South Australia.

Kwiatkowski, M.J., Skouroumounis, G.K., Taylor, D.K. 2012. Microwave technology improves extraction of phenolics from the grape skins in comparison to conventional methods. *School of Agriculture, Food and Wine Postgraduate symposium*, 18-19. Sept. 2012, Adelaide, South Australia.

AWARD

Best Poster by A Postgraduate Student, Research Day 2011, School of Agriculture, Food and Wine Research Committee, University of Adelaide, presented to Mariola Kwiatkowski, George Skouroumounis and Dennis Taylor, *The Winner is Microwave!* Research Day, 10 Nov. 2011 (first author).

*Note: the name "**Mariola**" has been used alternatively to "**Maria Jolanta**" as it had been used in some publications prior to PhD and consequently in the three manuscripts written for this thesis.*

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Maria Jolanta Kwiatkowski

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Abbreviations

A	Absorbance
ACN	Anthocyanin
ATR	Attenuated-total reflectance
a.u.	Absorbance units
*C**	Chroma
CCRD	Central composite rotatable design
CTE	Conventional thermal extraction
DoE	Design of experiment
EtOH	Ethanol
FC	Folin Ciocalteu
FT	Fourier Transform
h	Harvest
GA	Gallic acid
GAE	Gallic acid equivalent
GS	Grape skin
HCl	Hydrochloric acid
LS	Liquid to solid ratio
M	Molar (moles/litre)
MAE	Microwave-assisted extraction
MeOH	Methanol
MIR	Mid-infrared
NIR	Near-infrared
nm	Nanometre
OIV	International Organisation of Vine and Wine
Rpm	Revolutions per minute
RSM	Response surface methodology
TA	Titratable acidity
TP	Total phenolics
TSS	Total soluble solids
UV	Ultra-violet
v	Veraison
VIS	Visible

Figures and Tables for Chapter 1.

"Science is an adventure of the whole human race to learn to live in and perhaps love the universe in which they are. To be part of it is to understand, to understand oneself, to begin to feel that there is a capacity within man far beyond what he felt he had, of an infinite extension of human possibilities…."

I.I. Rabi
1944 Nobel laureate physicist

(Adapted from "The Splendid Voyage. An Introduction to New Sciences and New Technologies" Pangratios Papacosta, 1986, Prentice Hall Press New York).

Note: I.I. Rabi was one of the first scientists in the US to work on the cavity magnetron, which is used in microwave radar and microwave ovens (adapted from Wikipedia).

Chapter 1 Introduction

1.1. Phenolics and Human Health

Free radicals (reactive oxygen species, ROS) in the human body contribute to DNA damage and lipid peroxidation, both of which are considered to be responsible for the development of chronic diseases, among them some forms of cancer and heart disease (Yousuf et al., 2016; Shama et al., 2014; Atanacković et al., 2012; Routray and Orsat, 2012; Ajila et al., 2011; Shahidi, 2009; Pace-Asciak et al., 1995; Frankel et al., 1993).

Phenolics are chemical structures containing a hydroxy-substituted benzene ring (Kennedy et al., 2006). The presence of that hydroxyl group (-OH) gives phenolics their anti-oxidant properties as the hydroxyl group is highly reactive towards the free radicals. After carbohydrates and acids, phenolics are the most abundant compounds in grapes (Margalit, 2004).

Phenolics are present in natural plant-based foods e.g. vegetables, fruits, grains and nuts, and when included in daily diet, they are believed to prevent and combat health problems. Phenolics play a major role in the chemical composition of foods and strongly impact their sensory properties. For example, tannins in wine are responsible for bitterness, astringency, mouth-feel and wine stability; anthocyanins improve wine colour and flavour, and increase mouth-feel (Parker et al., 2007; Vidal et al.; 2004a; Vidal et al., 2004b; Vidal et al., 2003).

In order to improve health as well as protect against diseases, there has been recently a growing interest in extracting phenolics from plant material to produce nutraceuticals and functional foods (Gul et al., 2016; Saltmarsh, 2015; Galanakis et al., 2013; Gil-Chávez et al., 2012; Shahidi, 2009). According to Gul et al. (2016), nutraceuticals are dietary supplements and food additives; they are produced from foodstuff, and can be taken in the form of tablets, capsules or solutions. Functional foods are those foods or dietary components which may be beneficial to our health and are modified by adding to them bioactive ingredients, also in the concentrated form. Food additives are of several types: preservatives, nutritional additives, as well as colouring, flavouring, texturizing and miscellaneous agents (Carocho et al., 2014). As artificial food colourants and flavours have been proven unhealthy (Saltmarsh, 2015; McCann et al., 2007), natural additives are preferred in food products, including wine (Saltman et al., 2015; Pina et al., 2012; Hogan et al., 2011, Rózek et al., 2010, Bridle and Timberlake, 1996; Jackman et al., 1987).

1.2. Chemistry of Grape Phenolics

Grapes are cultivated in various parts of the world and have been in human diet for thousands of years. They contain numerous phenolics of which content depends on variety, climate, soil, season and viticultural practices. Phenolics consist of two major groups: *flavonoids* (C_6-C_3-C_6) and *non-flavonoids*, with several sub-groups each (Margalit, 2004). The two *flavonoid* sub-groups are monomeric (coloured: anthocyanidins: if a moiety of sugar $C_6H_{12}O_6$ through oxygen is attached to position 3, they are called anthocyanins; and colourless: flavonols, flavones,

flavan 3,4-diols and flavan-3-ols) and polymeric (condensed tannins, also known as proanthocyanidins). The *non-flavonoid* sub-groups are hydroxycinnamic acids C_6-C_3, hydroxybenzoic acids C_6-C_1 and stilbenes (Margalit, 2004).

Phenolics also contribute to the colour of foods. Colour often indicates food quality and is related to the attractiveness of food to the consumer (Yousuf et al., 2016). Hence, the compounds responsible for the colour are present in skins. Anthocyanins are responsible for the berry skin colour of red grape cultivars. These compounds have been studied for many years; however, little is known which compounds are responsible for the colour of white skins (Keller, 2010 cited in Iland et al., 2011). Anthocyanins are pH dependent and they appear in four forms being in equilibrium (Margalit, 2004; Mazza and Brouillard, 1987). Anthocyanins exist only in the form of flavylium cations (AH^+) when pH is below 2. This form is of red colour when a moiety of sugar through oxygen is attached to position 3 or of yellow colour when hydrogen is in that position (Mazza and Brouillard, 1987). Due to proton transfer, the red flavylium cation (AH^+) changes into a blue quinoidal base (A). The other two forms, carbinol pseudobase (B) and chalcone (C), are colourless. They are both formed after hydration of the flavylium cation (AH^+). Malvidin 3-glucoside represents the most abundant anthocyanin in red grapes (Margalit, 2004; Mazza and Brouillard, 1987). All four forms are present in Malvidin-3-glucoside within the range of pH 0-6 (Mazza and Brouillard, 1987). The contribution of the flavylium cation (AH^+) is 100% at pH 0, 96% at pH 1.5, 9% at pH 3.5 and no contribution at pH 5. There are three equilibria points, when two forms have the same concentration, characterised by a p*K* values: p*K* 2.60 (the flavylium cation and carbinol pseudo-base), p*K* 3.50 (the flavylium cation and chalcone) and p*K* 4.25 (the flavylium cation and quinodal base) (Timberlake, 1980).

As it is stated in Iland et al. (2011), flavanols (flavan-3-ols) are represented by the monomers such as catechin, epicatechin, epicatechin gallate and epigallocatechin; tannins are the polymeric forms of flavan-3-ols. Flavonols, particularly quercetin, protect grapes against pathogens and environmental stresses. By absorbing light in the range of 280-330 nm flavonols protect the berry from damage. Hydroxycinnamic acids are the major phenolics in juice of white cultivars and contribute to their colour. Stilbenes, which include resveratrol and its derivatives, are the drivers behind the health benefits of the French paradox (Atanacković et al., 2012; Pace-Asciak et al., 1995; Frankel et al., 1993). They are synthesised in the grape berry when the vine is under stress, for example due to the infection caused by the *Botrytis cinerea* fungi (Iland et al., 2011; Romero-Pérez et al., 2001).

1.3. Grape Berry Development

Skin, seeds and flesh are the main tissues in grape berry. Their chemical composition depends on the grape variety and environmental conditions (climate, soil), season and viticultural practices. Phenolics are present in all three tissues - their composition in skins is similar to the composition in seeds except for (-) - epigallocatechin (Iland et al., 2011; Vidal et al., 2003).

Changes in size, skin colour and composition occur during the berry development (Kennedy, 2002). The berry development period consists of three stages including the two important time points: veraison and harvest. During the first stage from flowering to veraison (approximately 60 days) berries grow and different compounds are formed, among them tartaric, malic and hydroxycinnamic acids, as well as tannins and methoxypyrazines. At veraison, berries start

changing colour and berry ripeness begins. The second stage, called a lag phase, starts approximately 5-10 days prior to veraison. During this stage, berries reach at least half of their final size and continue to accumulate acids and tannins. At veraison, the third stage of berry development commences when sugars, glucose and fructose contents start to increase, and the contents of acids and tannins start to decline. Berries ripen, double in size and their skin softens. The reduction of malic acid is strongly correlated with climate, with a faster decline in warm regions (Kennedy, 2002). The content of benzoic and hydroxycinnamic acids, as well as of flavonols in grape skin, increases from veraison to harvest (Iland et al., 2011; Fernández de Simón, et al., 1993).

The compounds responsible for the skin colour are formed: anthocyanins in red grapes and non-anthocyanin compounds in white grapes (Iland et al., 2011). The content of anthocyanins gradually increases toward harvest. It is not yet known which specific non-anthocyanin phenolics are involved in the expression of skin colour of white cultivars - Keller (2010) stated that the yellow colour of their skins is due to the contribution of chlorophyll, carotenoids and flavonols (cited in Iland et al., 2011). Therefore, in this thesis the colour of red skins is discussed in terms of the total colour of the anthocyanin compounds formed during berry ripening; however, the colour of white skins is discussed in terms of the total colour of some phenolics, i.e. non-anthocyanin compounds and probably other non-phenolic compounds such as carotenoids and chlorophylls. The colour output in this thesis is of great importance to understanding the chemistry behind it, and to future research on grape skin colour, in particular of the white cultivars.

Grape skins account for 10-20 % of the grape berry weight, seeds for ~4%, juice 75% and stems ~5% (Margalit, 2004; Rankine, 2004; Singleton and Esau, 1969). Approximately 25% and 50% of phenolics are located in the skins of white and red cultivars, respectively (Singleton and Esau, 1969). The average TP content in skin, expressed as milligram of gallic acid equivalent per kilogram of grapes, is 0.90 g GAE/kg of white berries and 1.85 g GAE/kg of red berries, (Margalit, 2004; Singleton and Essau, 1969). Grape skin of white cultivars contains the highest TP content at veraison. For red cultivars, the TP content decreases during ripening until harvest, with the content of anthocyanins increasing during that period (Iland et al., 2011; Kennedy, 2002).

This thesis focuses on extracting phenolics and colour from grape skins, obtained from primary fruit. Note they can also be extracted from skins separated from bio-waste obtained from white and red vinification (pomace; non-fermented waste and marc: post-fermentation waste) (Kalli et al., 2018; Muhlack et al., 2018; de la Cerda-Carrasco et al., 2014; Lafka et al., 2007), as well as from non-alcoholic beverage production.

1.4. Cleaner Production and Circular Economy.

The Cleaner Production philosophy is based on choosing efficient processes which take into account minimisation of waste and emissions, along with the effective use of resources such as raw materials, energy, water and labour. Employing statistical analysis to assess the processes (such as extraction methods) helps to choose environmentally friendly and efficient methods (Almeida et al., 2013).

Valorisation of industrial waste, in particular agro-industrial ones, including post-winemaking pomace/marc (non-fermented/post-fermentation wastes), has become an environmental, economic and political issue recently in order to reduce landfill and its impact on the environment. New technologies have been sought, in emerging developments in hydrogen, carbon capture and storage, biofuels and waste-to-energy. Hence, recovering of compounds of commercial value and energy, and quick and efficient decomposition of the residue when processing biomass waste helps 'closing the loop' in the Circular Economy, which is an alternative to the linear economy, in which raw materials are used, product made and waste dumped (Ellen MacArthur Foundation, 2017; Geissdoerfer et al., 2017). Recently, by implementing the principles of the Circular Economy, a new and exciting almost-zero waste methodology has been reported by Sette et al. (2020), in which the waste from cider and wine industries were extracted to recover valuable compounds and then pyrolyzed/gasified to biochar.

Phenolics and colour can be extracted from plant material (e.g. skins, seeds, stems or whole grapes) or recovered from post-manufacturing wastes e.g., skins separated from the post-winemaking waste (pomace/marc) or whole pomace. Pomace/marc accounts for 10% to 30% of the mass of crushed grapes (Muhlack et al., 2018; Dwyer at al. 2014); it contains skins and seeds, sometimes stems, and it can be fermented (marc) or not fermented (pomace), depending on the winemaking protocol.

In Australia, wineries are obliged to follow strict production guidelines to avoid fines and penalties, by which they have to record the mass of pomace, its quality and methods of safe disposal (EPA, South Australia, 2016). Red winemaking involves skin contact during wine

maceration, while there is no skin contact involved in white winemaking - with some exceptions such as of rosé or some sparkling wines. Apart from anthocyanins, other monomeric phenolics can be recovered from pomace: stilbenes (e.g. resveratrol), flavonols (e.g. quercetin), and flavan-3-ols (e.g. catechin and epicatechin). Valorization of skins of specific cultivars offers high market value as their coloured compounds are sought-after by the manufacturers of functional foods and nutraceuticals including food natural colourants (García-Lomillo and González-SanJosé, 2017; Gul el al. 2016; Carocho et al., 2014; Galanakis, 2013), skincare (Taylor, 2016) or textile dyes (Bechtold et al., 2007). For example, a South Australian company Tarac Technologies, Barossa wine region (https://www.tarac.com.au/) until 2009 applied a 'closed loop' system by manufacturing commercially red skin extracts from pomace for wine colour enhancement. Another example, a family-owned French company which supplies the skincare industry with the grape seed extracts obtained from pomace (Taylor, V. 2016). Food colourants and phenolic products can be sourced from the grape waste (pomace/marc) as reported in Dwyer et al. (2014).

This research may be applicable for the future studies on phenolics and colour obtained from the skins of primary grape, whole grape as well as from the skins separated from wastes.

1.5. Extraction Methods

Various methods are employed for extracting phenolics. From the economic point of view, it is vital to use effective and efficient extraction methods which are also environmentally friendly (Almeida et al., 2013). The process of phenolic extraction from skins is complex due to the involvement of many factors, such as the type of solvent, temperature, time, liquid to

solid ratio, pH and stirring. Those factors, in particular temperature, impact the process of breaking the link between phenolics and the cell wall (Pinelo et al. 2006).

There are conventional and non-conventional methods of extraction (Routray and Orsat, 2012; Ajila et al., 2011; Ignat et al., 2011). The conventional thermal extraction (CTE) method is commonly used; it is based on maceration of skins by a solvent in a capped vessel immersed in a water bath at a chosen temperature and for a chosen time - the example shown in Figure 1 was used in this thesis. The advantage of this method is its simplicity. When optimizing a CTE method, Spigno et al. (2007) reported that more phenolics were extracted from a red grape marc using 30% rather than 20% of an aqueous ethanol solution, at 60 ^{o}C rather than at 45 ^{o}C, and for an extraction time of no longer than 20 hours, to avoid phenolic degradation. For the same reason, phenolics must also be protected from light at all times (e.g. by covering the water bath with a black plastic box, Figure 1).

Figure 1. Ratek WB4 water bath (Ratek Instruments Pty Ltd, Australia). The black box was used to eliminate light (School of Agriculture, Food and Wine, University of Adelaide).

Non-conventional extraction methods have been developed to shorten the extraction time and improve the energy efficiency, to reduce the amount of solvent, and hence the cost of extraction, as well as lowering the risk of phenolic degradation. Commonly used novel methods are: microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), supercritical fluid extraction (SFE) and accelerated solvent extraction (ASE) (Routray and Orsat, 2012). As the previous research work by Li et al. (2011) was focussed on the extraction of phenolics from the mixture of the grape seeds using the MAE method, for this thesis the grape skins were chosen employing MAE, along with CTE methods.

Microwaves are electromagnetic radiations with a frequency from 0.3 to 300 GHz (wavelength λ 0.3-30 cm, or wavenumber 3-0.03 cm^{-1}), located between the radio-waves (at wavelength from 0.01 cm to 100 km) and infrared ranges (wavenumber of 400-0 cm^{-1} far-infrared, 4000-400 cm^{-1} mid-infrared and 14285-4000 cm^{-1} near-infrared) (Stuart et al., 1996). Industrial and domestic microwave ovens operate at 2.45 GHz in order to avoid interferences with radio communications (Wang and Weller, 2006; Kaufmann and Christen, 2002). Electric and magnetic fields are perpendicular to each other and the electric field causes heating through dipolar rotation and ionic conduction. The dipoles rotate within molecules of solvent and of the solids; that rotation is the source of energy releasing the compounds into the solution, i.e. extract. MAE offers a rapid delivery of energy to a total volume of solvent and solid plant matrix, with subsequent efficient and homogeneous heating of the solvent and solid matrix. Because water within the plant matrix absorbs microwave energy, cell disruption is promoted by internal superheating, which facilitates desorption of chemicals from matrix into the polar solvent, hence improving the recovery of bioactive compounds. Also, the higher the dielectric constant of the solvent, the better the solvent is in MAE (Kaufmann and Christen, 2002).

Microwave ovens can be monomode or multimode cavities (Routray and Orsat, 2012). Monomode cavities are used in focused (closed) types of ovens, while the multimode are used in open systems. Compared with domestic ovens, industrial ovens (reactors) allow for much better control of temperature, time, pressure and stirring. It is worth noting that domestic microwave ovens are not recommended for research purposes due to a lack of control of temperature and pressure. In addition, Kaufmann and Christen (2002) stated that domestic microwaves should not be considered for research purposes because applying microwave energy to highly flammable solvents may cause hazards and reproducibility may be poor due to lack of homogeneity of the microwave field.

An industrial type monomode reactor, of which an example is shown in Figure 2, was used in this thesis. The temperature was controlled (± 0.1 ºC) using a Maxus WX801700 air compressor (Campbell Hausfeld, USA) coupled to the microwave reactor. The computer attached to the microwave allowed monitoring the extraction process by watching the pressure, temperature and power charts as well as stirring. The total time of microwave work consisted of three phases: the ramping time (less than 1 minute) after which the chosen extraction time was counted, followed by cooling time (less than 2 minutes).

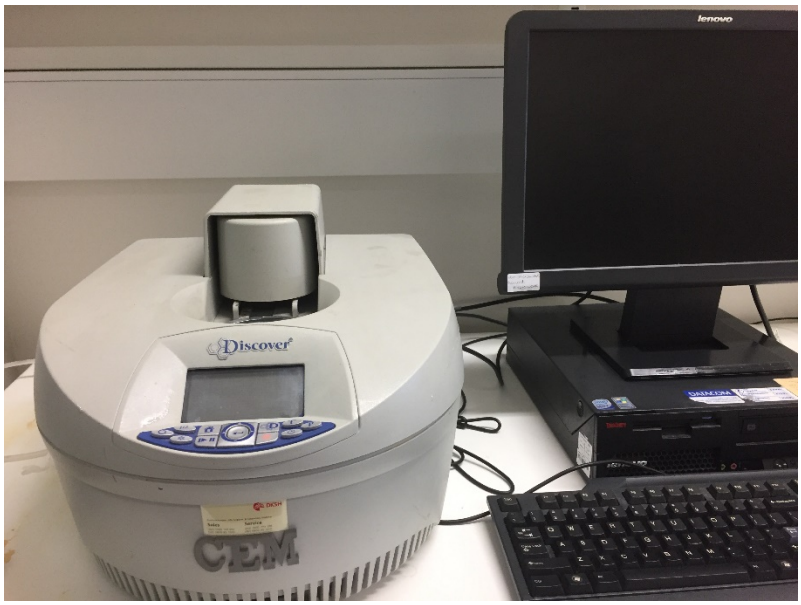

Figure 2. Industrial microwave reactor CEM Discover S-Class (CEM Co., USA)

(School of Agriculture, Food and Wine, University of Adelaide).

1.6. Design of Experiment and Response Surface Methodology.

In 1926 Sir Ronald A. Fisher became the "father" of the application of statistical principles in the design of experiment (DoE) of agricultural field trials. He has left legacy to the agriculture researchers, in particular to those who worked for CSIRO in the Waite Campus at the end of his life.

The design is built on three fundamental components of experiments (Kuehl, 2000):

1) **Local control** of field conditions for the reduction of experimental error,
2) **Replication** as a means to estimate experimental error variance,
3) **Randomisation** for valid estimation of experimental error variance.

In order to explain the importance of DoE to fulfil the given goal of this thesis, the DoE used is Chapter 2 shown as an example. As the goal of this thesis was to find the optimal conditions for maximising the total phenolic (TP) content extracted from grape skins under a novel extraction method such as MAE, to be compared with a CTE method. The models were built by employing chosen factors at different levels each. The choice of factors was made after considering what could affect the response variable (in this thesis TP and colour CIELAB chroma C^*) of the grape skin extracts. The treatment represented the levels chosen for each factor and consisted of a minimum of three replications of an experimental unit.

The DoE consisted of four phases:

1) preliminary screening,
2) advanced screening,
3) response surface (RS) optimisation,
4) methods' validation.

The results of the previous phase were taken into account in the following phase. The two goals of the preliminary screening were: 1) to identify which factors affected the response variable most, and 2) to evaluate and compare the robustness of the two extraction methods. The effect of a factor was defined as a change in the measured response caused by a change in the level of that factor (Kuehl, 2000). Factors, which were difficult to control for their minimum return, or baseline factors that needed to be fixed at a certain level to enable the process, were identified and set at levels most practical for the extraction process. Important factors, which could be optimized for the maximum return, were identified for the next stage of screening.

The aim of the advanced screening was to find which factors from those identified in the preliminary screening impacted significantly the response variable and in what direction. The factors under investigation were set at two levels: low and high, determined by the experimenter with reference to efficiency and safety. In our experiments, for example, five factors were identified in the preliminary screening to be used in the advanced screening for each extraction method. The full factorial design for five factors at two levels each (2^5) would require 32 samples to be prepared and analysed for each extraction method, separately for red and white cultivars. Aiming at triplicates of extraction, the full factorial design would require 384 samples. The choice of a fractional factorial (half-factorial) instead of the full factorial design was justified here. The number of samples to be prepared and analysed was reduced from 384 to 192 in a five-factor two-level fractional factorial resolution V design (Kuehl, 2000). The main effects and two-factor interactions of the five factors selected for advanced screening were still modelled efficiently but with half as many samples. The design was employed to narrow down the multi-factorial space to be prepared for the next step of maximising the response variable. Fewer factors were selected for the next stage, response surface (RS) optimisation (three factors were selected for each extraction method in this thesis).

For the methods' optimisation, RS methodology coupled with a central composite rotatable design (CCRD), shown in Figure 3, was chosen in this thesis to fit second-order models to find the optimal conditions for three factors at which the maximum of the response variable could be obtained following the steepest ascent path (Kuehl, 2000).

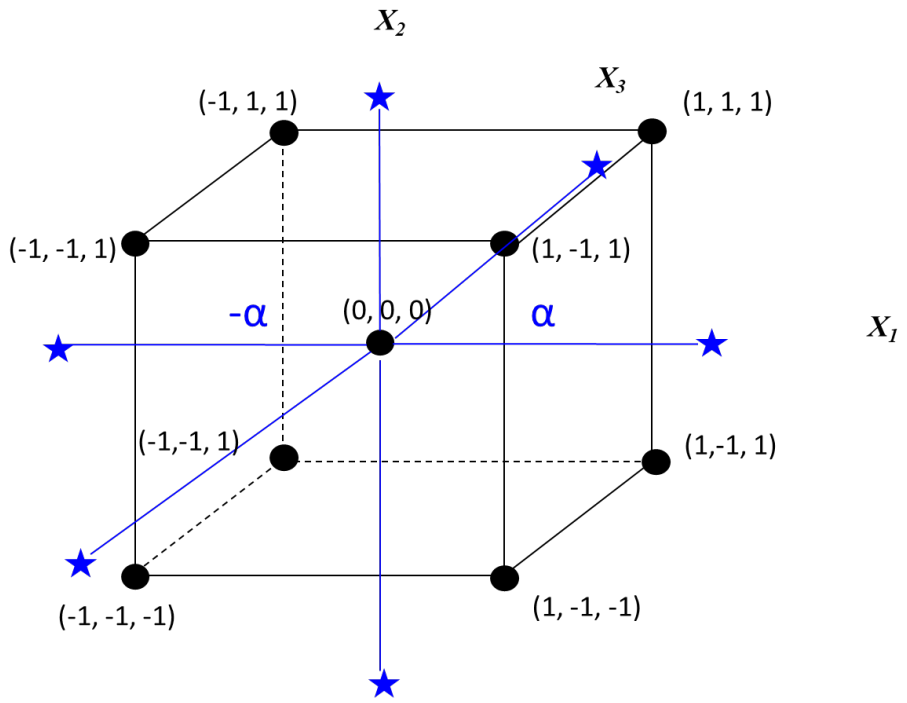

Figure 3. Central composite rotatable design for three factors at two levels each.

The three coordinates denote the factors in the design: X_1, X_2 and X_3. Each factor is presented at two levels: low (coded -1) and high (coded 1). The central point is added at the mid-level of each factor. Six additional points are added outside the design cube to allow an extension by the factor α (often set at 1.68) beyond the lower and upper levels of each factor. In this optimisation design the second-order response surface based on three factors (found significant in the previous phase) and their interactions is examined as b presented by Eq. (1):

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 \quad (1)$$

in which X_1, X_2, X_3 are three factors, $b_0,\ldots,b_{33}$ are regression coefficients and Y is the response variable.

Both extraction methods required to have their efficiency validated in an independent completely randomized experiment. The repeatability of the extractions under both methods was to be checked by conducting three consecutive extractions in a completely randomised design: for each extraction, the same portion of solids was to be treated with a portion of solvent.

For comparison of two extraction methods in terms of their efficiency, a percentage error was used as described by the equation for the response variable; in this study TP by Eq. (2) and C^* by Eq. (3):

$$\text{Percentage error} = (|TP_{CTE} - TP_{MAE}| / TP_{CTE}) * 100 \quad (2)$$

$$\text{Percentage error} = (|C^*_{CTE} - C^*_{MAE}| / C^*_{CTE}) * 100 \quad (3)$$

1.7. Analytical Methods.

1.7.1. Determination of Total Phenolic Content.

Since 1965 (Singleton and Rossi, 1965), the Folin-Ciocalteu (FC) assay utilizing cuvettes has been used as one of the standard antioxidant capacity (AOC) methods to measure the TP content in natural products (Prior et al., 2005). As presented in Tables 1 and 2, many researchers have used this method to measure TP in plant extracts; some researchers used high performance liquid chromatography (HPLC), mass spectrometry (MS) or liquid chromatography-mass spectrometry (LC-MS). There are some spectral methods based on the absorbance measurement at 280 nm for total phenolic measurement developed by Iland et al. (2004) for grape homogenate extracts and by Somers and Evans (1977) for young red wines, but both methods are very laborious.

The Folin-Ciocalteu assay by Singleton and Rossi (1965) was modified by Waterhouse (2001). The latter was used by Li et al. (2011) to analyse the TP content in grape seed mixture extracted employing MAE and RS-methodology, and was also used in the preliminary and advanced screenings phases of this thesis. The Waterhouse TP assay (2001) is very laborious and time consuming. Although a fractional factorial design was used to reduce the number of samples, still a large number of samples was required to be analysed for TP. Hence, the Waterhouse TP assay (2001) in a cuvette had to be modified. As described in detail in Chapter 2, a high throughput Folin-Ciocalteu utilizing a 96-well plate assay was developed, specifically for this thesis, to determine the TP content in the grape skin extracts. That already high throughput assay was further modified by adapting it for robot dispensing of all liquids (Figure 4) to speed up the work.

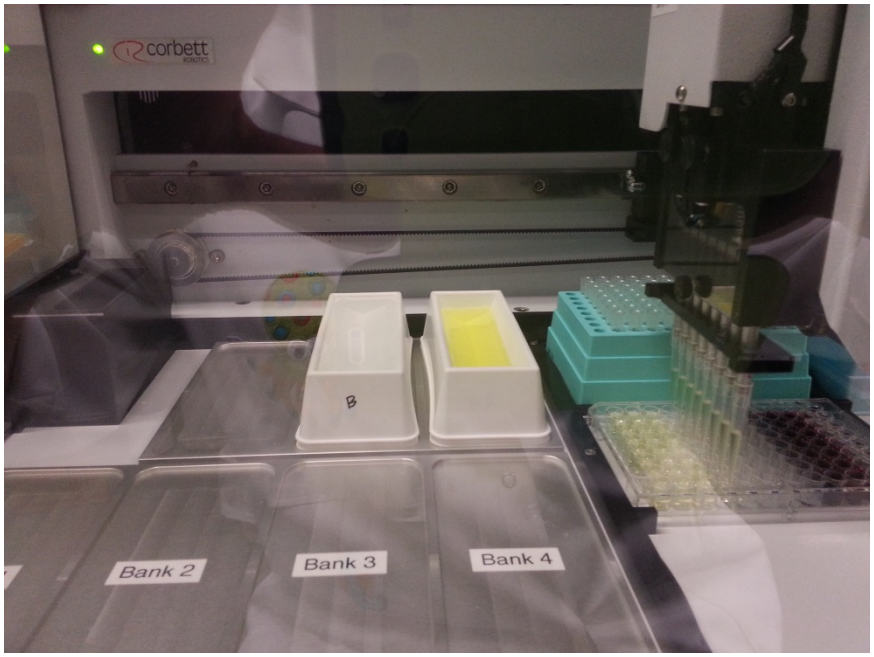

Figure 4. Liquid dispensing robot **(**Corbett Robotic Systems Pty Ltd., Australia)

(School of Agriculture, Food and Wine, University of Adelaide).

1.7.2. Determination of Colour

The colour of skin extracts is determined by compounds such as anthocyanins in red skins (Iland et al., 2011), and non-anthocyanin compounds including some groups of phenolics, presumably flavonols, and non-phenolic carotenoids and chlorophylls in white skins (Keller, 2010 (cited in Iland et al., 2011). As Table 1 shows, the anthocyanin content in red skin extracts is often analysed using laborious and expensive techniques such as HPLC, MS and HPLC-MS.

Commonly used in the wine industry (OIV, 2018a) is the spectral method of colour measurement developed by Ribéreau-Gayon, Pontallier and Glories (1983) for red wines. The Ribéreau-Gayon et al. (1983) method is based on reading of the absorbance at 420, 520 and

620 nm for the colour stability, and the sum of all three absorbance values for colour index. This method was employed by Amendola et al. (2010) for the colour measurement of the extracts obtained from whole red pomace, and by de la Cerda-Carrasco et al. (2014) for the extracts from whole white and red pomaces. Similar methods based on absorbance measurements at 420 nm and 520 nm were developed for wines and grape homogenate extracts by Iland et al. (2004) and Somers and Evans (1977) for young red wines.

The spectral CIELAB (1976) method was established by Commission Internationale de l'Eclairage (CIE) in 1976, and since 1978 it has been recommended for the wine industry by the Office International de la Vigne et du Vin (OIV) for the measurement of wine colour. The CIELAB algorithm is based on the visible part of the electromagnetic spectrum in the range of 350-750 nm. CIELAB is a quick method, which does not require diluting samples, in contrast to the methods of Ribéreau-Gayon et al. (1983) or Iland et al. (2004).

CIELAB uses a three-dimensional colour space (Figure 5) (ASTM, 2000; OIV, 2018b; Ohno, 2000) The typical ranges of CIELAB coordinates are 0 (black) to 100 (white) for L^* (luminance), -100 (green) to 100 (red) for a^*, and -100 (yellow) to 100 (blue) for b^*. The values are measured by the spectrophotometer at a chosen illuminant (daylight D65 chosen in this thesis) and a standard observer angle (10-degree chosen in this thesis). From the coordinates the chroma C^* is calculated using the Pythagoras' equation, $C^* = (a^{*2} + b^{*2})^{0.5}$ as the length of the vector on the (a^*, b^*) plane, complemented with hue h = tan-1(b^*/a^*). In this thesis chroma was chosen as the parameter describing the total colour of the white and red skin extracts because it combines their redness, greenness, yellowness and blueness. In the case of white skin extracts, the highest contribution to chroma comes from b^*, and in the case of red

extracts a^* is the highest contributor. Liang et al. (2011) used chroma C^* to measure the colour of grape skins (not the skin extracts) mostly of red, and of some white, cultivars collected at harvest and found it to be correlated with the total anthocyanin content measured by HPLC only for red skins; C^* was moderately negatively correlated with anthocyanin content (Pearson correlation coefficient r = - 0.55). To the best of our knowledge, there have been no studies using chroma C^* to determine the colour of the white grape skin extracts, whereas Kuck and Noreña (2016) used chroma of skin extracts of Brazilian red grapes extracted under a CTE method.

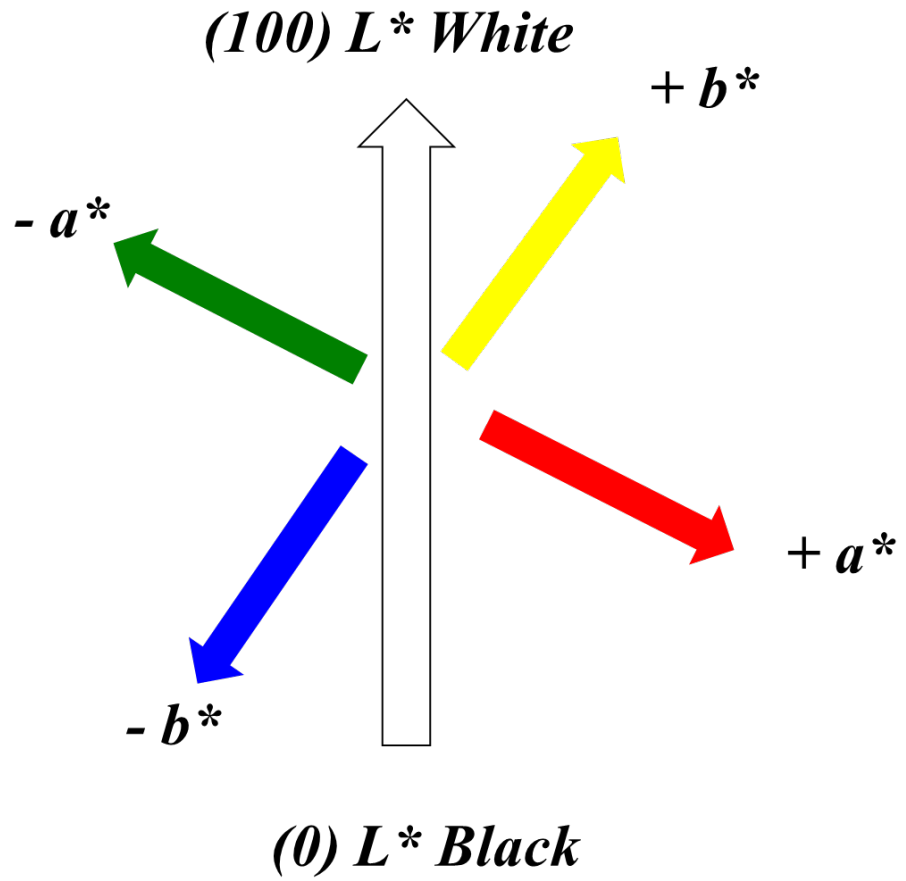

Figure 5. CIELAB colour space.

1.7.3. Prediction of Total Phenolic Content and Colour by ATR-MIR spectra

An investigation was undertaken on the feasibility of infrared spectroscopy (IR) for further speeding up the measurement processes for TP and *C** when analysing a large number of grape skin extracts in this thesis. The choice of vibrational spectroscopy in the assessment of phenolics in this thesis has been confirmed by the recent reports of Teixeira dos Santos et al.

(2017) and Machado and Domínguez-Perles (2017), recommending vibrational spectroscopy for phenolic measurement in the wine industry, including by-products.

Infrared spectroscopy (spectra ranges specified in Section 1.5) is based on the vibrations of the atoms of molecules, and it can be used in gaseous, liquid and solid samples (Stuart et al., 1996). Its simplicity, robustness, cost effectiveness, and status as environmentally friendly (Dambergs et al., 2015; Cozzolino, 2014) were considered when deciding to include infrared spectroscopy as a measurement method in this thesis. Near-infrared spectroscopy (NIR, 13400-4000 cm^{-1}) was efficiently used previously for in-cuvette analysis of compositional parameters in wine and grape homogenate extracts (Cozzolino et al., 2011; Cozzolino et al., 2008a; Cozzolino et al., 2008b; Janik et al., 2007; Cozzolino et al., 2004). In addition, wine compositional parameters were analysed using non-destructive in-bottle NIR measurements (Cozzolino et al., 2007). Jara-Palacios et al. (2016) employed NIR hyperspectral imaging to assess 27 individual phenolics in seeds, skins and stems separated from white grape pomace.

However, for this thesis, mid-infrared spectroscopy (MIR, 4000-400 cm^{-1}) was chosen over NIR, because the former could deliver sharper spectral peaks and offered no limitations regarding low concentrations of the analyte (Cozzolino, 2014; Cozzolino, 2011). Lu and Rasco (2012) reported advanced quality in MIR analyses. Fragoso et al. (2011) measured the total phenolic and anthocyanin contents (by measuring absorbance at 280 nm and 520, respectively) and condensed tannin (using precipitation method with methyl cellulose) of red grape homogenate extracts using Fourier Transform (FT) MIR spectra in the region of 2989-979 cm^{-1}. Attenuated total reflectance MIR (ATR-MIR) was used by Musingarabwi et al. (2016) to investigate a number of compositional parameters, but not phenolics, of Sauvignon Blanc grape

homogenate extracts at various stages of berry development. The same technique was successfully employed in the measurement of various compositional parameters, excluding phenolics, of commercial white and red wines (Cozzolino et al., 2011), phenolics and other compositional parameters in Shiraz wines (Ristic at al., 2016) and in white grape juices (Shah et al., 2010). As none of these studies have reported the use of ATR-MIR spectroscopy to evaluate the TP content and CIELAB chroma *C** of grape skin extracts, this thesis investigates the feasibility and benefits of ATR-MIR spectroscopy to measure TP and *C** of white and red grape skin mixture extracts separately (Chapter 4). Hence, ATR-MIR scans were performed during the advanced screening, optimisation and methods' validation phases in the first study described in Chapter 2.

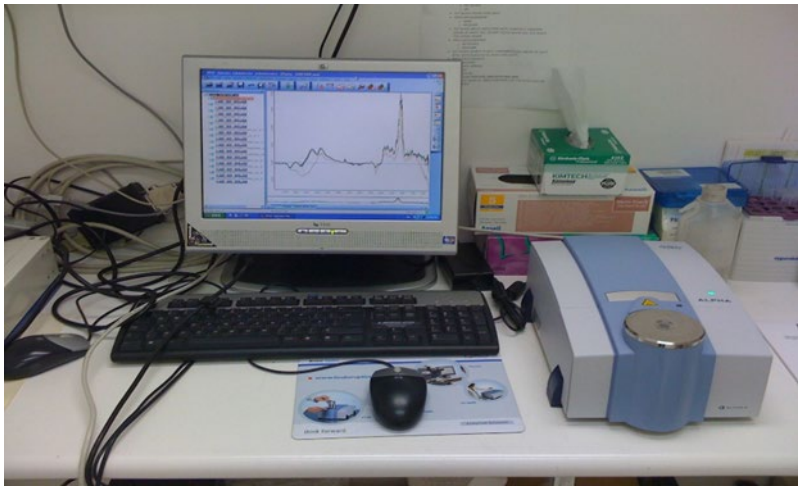

Figure 6. ATR-MIR Bruker Alpha instrument (Bruker Optics GmbH, Ettlingen, Germany) (Image provided by Dr Daniel Cozzolino, School of Agriculture, Food and Wine, University of Adelaide).

1.7.4. Determination of Total Soluble Solids and pH.

A digital refractometer was utilized to measure the total soluble solids (°Brix) of the juice samples corresponding to the grape samples of which the skins were prepared for the MAE and CTE methods. Prior to extraction, the pH of each sample was measured using a pH meter (Eutech Instruments) and adjusted to the pH as specified by the DoE.

1.8. Comprehensive Review of the MAE and CTE Research Studies on Phenolic and Colour Extraction from Grape Skin.

A comprehensive literature search was conducted on Web of Science; Table 1 presents most relevant and representative examples of studies describing the use of the MAE and CTE methods in extracting phenolics and colour from the skins of white and red grape cultivars collected from veraison to harvest, at harvest only, as well as from skins separated from pomace/marc. It is difficult to compare the extraction efficiency across multiple studies, as their settings vary greatly, with only a few comparing MAE and CTE methods, employing RS optimisation (to one only method), which prohibits estimating the full potential of the extraction methods. Other complications include the variability in the choice of grape cultivars, sampling and skin collection protocols. Some representative research studies are discussed in detail below.

Casazza et al. (2010) compared TP extracted using MAE and CTE methods, both non-optimised for maximum TP, from skins separated from marc after white vinification of a single

red grape cultivar of Pinot Noir. The same source of skins as in Casazza et al. (2010) was utilised by Casazza et al. (2012) to investigate TP extraction using an RS-optimized CTE method, concluding that maximum TP content could be obtained after a laborious and long extraction time of 19 hours, making it not practical for studies with a large number of samples.

Ghassempour et al. (2008) conducted a comparison between RS-optimised MAE and non-optimised CTE methods for the extraction of a range of anthocyanins from skins of the Iranian *Vitis vinifera* cv. Shahani grapes. The authors reported that MAE was more efficient than CTE as it required shorter time and less solvent.

Liazid et al. (2007) investigated the stability of 22 phenolic compounds extracted from the skins of two red and one white cultivar under MAE not RS-optimised. The authors reported that if the extraction temperature exceeded 100 ºC (with some exceptions like the benzoic acid and derivatives of cinnamic acid groups stable up to 150 ºC, coumarins and flavonols up to 125 ºC) it might cause the degradation of phenolics. A well-designed but limited study by Liazid et al. (2011) centred only on anthocyanin content and identification from skins of a single red cultivar, Tintilla de la Rota employed a RS-optimised MAE method (based on a fractional factorial design employing six factors at two levels). Key findings indicated that maximum anthocyanin levels could be attained in a short extraction time (5 min.) whilst temperatures above 60 ºC should be avoided due to anthocyanin degradation. The findings of Liazid et al. (2007) and Liazid et al. (2011) were taken into account when designing the experiments for this thesis.

Peralbo-Molina et al. (2012) compared phenolic extraction and identification from white and red skins isolated from pomace after alcohol distillation of different cultivars by employing MAE and CTE methods. Unfortunately, neither of these methods were optimised, again casting doubt on whether maximum TP extraction levels could be concluded. Pinelo et al. (2005) optimized a CTE method employing the RS methodology based on three factors at three levels each to extract optimal phenolic levels from skins separated from pomace of white and red grapes of Garnacha (Grenache). The authors reported that methanol was the most efficient solvent in extracting phenolics in comparison with ethanol and water. For this thesis the aqueous ethanol solutions were chosen as solvents in extracting phenolics and colour from grape skins under MAE and CTE due to the fact that the higher the dielectric constant of the solvent the more effective the MAE extraction is. Although the dielectric constant for ethanol is lower (24.3) than for methanol (32.6) (Kaufmann and Christen, 2002), ethanol as less toxic was chosen for this thesis. Adding water to the solvent is beneficial as water has the highest the dielectric constant (78.5) in comparison to other solvents, hence strong absorption of microwave energy in breaking bonds between phenolics (in particular anthocyanins as water soluble phenolics) and cell wall of grape skin.

In the last two decades researcher have been trying to find ways of reducing the landfill to add value to post-winemaking waste, in particular with a focus of using grape skin extracts, hence the need to find efficient extraction methods, in manufacturing nutraceuticals, supplements and functional foods. Caldas et al. (2018) optimised a CTE method for maximising TP when investigating the TP content recovered from skins separated from a Brazilian red pomace after sparkling wine production; the design was based on two factors (solid to liquid ratio and ethanol concentration) at 5 levels each. Then, the optimal conditions found for maximum TP under CTE (50% ethanol and solid to liquid ratio of 1:10) were employed in MAE and ultrasound-

assisted (UAE), none of them optimised for maximum TP. Although it was reported that UAE performed better in comparison with MAE under those fixed conditions, the authors did not optimised MAE and UAE methods for maximising TP.

The skins isolated from pomace of seven white cultivars, including Chardonnay and Riesling, were used for phenolic extraction under a CTE method non-optimized for maximum TP by Sri Harsha et al. (2014) and demonstrated that recovering phenolics from by-products could have potential health benefits in manufacturing new functional foods, in particular for diabetic and elderly people. Also, a non-optimized CTE was employed by Moncalvo et al. (2016) in recovering phenolics from skins separated from the pomace/marc of white and red cultivars, among them Chardonnay, Pinot Noir and Nebbiolo, and of red cultivars by Ky and Teissedre (2015) among them Grenache and Shiraz. Non-optimized CTE methods of phenolic extraction from skins collected at harvest were employed for white and red cultivars by Katalinić et al. (2010) and Pantelić et al. (2016), and for red cultivars such as Shiraz, Cabernet Sauvignon and Merlot by Arnous and Meyer (2008) and the last two cultivars were the research subjects of Lorrain et al. (2011). The above-mentioned commercial grape cultivars are among these choses for this thesis.

Changes from veraison to harvest in TP content extracted under CTE not optimized for maximum TP extraction have been investigated by several authors. Tian et al. (2019) noted a seasonal decrease in TP: 2.7 mg GAE/g of berry at veraison to ~1.5 mg GAE/g of berry at harvest, in the skins of Sauvignon Blanc. Geană at al. (2016) reported that the TP content in skins for Merlot and Cabernet Sauvignon slightly decreased, and for Pinot Noir slightly increased from veraison to harvest. An increase in TP and anthocyanin (ACN) content from veraison to harvest was reported by Dokoozlian and Kliewer (1996) for skins of Pinot Noir and

Cabernet Sauvignon; the same effect was found by Fournand et al. (2006) in skins of Shiraz, and by Locatelli et al. (2016) in skins of Nebbiolo, all under non-optimised CTE. However, none of these studies considered MAE as an alternative method of extraction.

As Table 1 demonstrates the broad range of applying MAE and CTE in phenolics, anthocyanins and colour extraction from grape tissues, just a few examples of application of these two methods as shown in Table 2 in extracting phenolics from skins of other than grape plant material such as peanut, hazelnut and citrus mandarin.

No MAE or CTE studies have been found utilizing the same protocols to parallelly optimise the five factors of interest: ethanol concentration, the liquid to solid ratio, temperature, extraction time and pH, separately and in for MAE and CTE in order to maximise TP extraction levels. Additionally, given that the colour of skin extracts is impacted by phenolics, it was vital to find the optimal conditions for colour extraction from grape skin.

1.9. Aims of this Thesis.

There are three studies presented in sequel in this thesis:

1) In **the first major study** (Chapter 2) microwave-assisted and conventional thermal extraction methods were developed; both methods were parallelly optimised using response surface (RS) methodology to maximise the total phenolic (TP) content extracted separately from white and red skin mixtures, each mixture containing skins of berries of six commercial grape cultivars collected at veraison and harvest. Additionally, the same RS models built for the two TP extraction methods were utilized to evaluate optimal conditions to maximise the

colour of skin extracts, represented by CIELAB chroma *C**. The design of experiment (DoE) consisted of four phases: preliminary and advanced screenings, optimisation and model validation of each of the MAE and CTE methods. A Folin Ciocalteu high-throughput method was specifically developed for this study to evaluate the TP content.

2) The goal of **the second study** (Chapter 3) was to apply both extraction methods under the optimal practical conditions found in the first study to assess the TP content and colour *C** of the extracts obtained individually from the skins of the six white and six red commercial cultivars collected at veraison and at harvest over one vintage and partially used in Study 1. The choice to utilize both methods from the first study, instead of optimising them for TP and *C** for individual cultivars, was to make the cultivar comparisons under the same conditions and check for possible method-by-cultivar interaction effects in comparison to the TP content in extracts from skin mixtures. It was also important to quantify the variation one would expect between varieties at different time-point of berry development and to identify which extraction method consistently produces higher TP and *C**.

3) The aim of **the final study** (Chapter 4) was to investigate the feasibility of attenuated total reflectance mid-infrared (ATR-MIR) spectroscopy to quantify the TP content and colour *C** of the white and red grape skin mixture extracts obtained during the first study. The TP content and *C** were predicted using partial least square (PLS1) regression models built between the chemical data for the skin mixture extracts obtained during the advanced screening, optimisation and validation of the MAE and CE methods and the corresponding ATR-MIR spectra of those extracts.

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Table 1. A review of MAE and CTE research studies on phenolic extraction from grape skins peeled from berries or separated from pomace/marc.

Cultivar	Skin origin	Extraction method	Investigated factors	DoE Optimisation	Target extract	Analytical method	Reference
Iranian cultivar Shahani	Red skins	MAE	MeOH, EtOH (0.1% 12N HCl) LS (2.5-10 mL/g GS) Temperature (60-80 ºC) Time (2-10 min)	RS-optimized	ACN	HPLC & LC-MS/MS	Ghassempour et al. (2008)
		CTE	MeOH (0.1% 12N HCl) LS (25 mL/g GS) Time (48 h) Temperature (rt)	Non-optimized			
Pinot Noir	Red skins from pomace after white vinification	MAE	MeOH under nitrogen LS (5 mL/g DW) Temperature (110 ºC)	Non-optimized	TP	FC	Casazza et al. (2010)
		CTE	EtOH or MeOH Temperature (rt) Time (19 h)	Non-optimized			
		CTE	EtOH LS (3.3-10 mL/g DW) Time (9-29 h) Temperature (rt)	RS-optimized	TP	FC	Casazza et al. (2012)
Tintilla de Rota	Red skins	MAE	MeOH (50-80%) Stirring (on/off) Temperature (50-100 ºC) Time (5-20 min) Power (100-500 W)	RS-optimized	ACN	HPLC	Liazid et al. (2011)

			LS (12.5-25 mL/g GS)				
Red Globe (table cultivar) Napoleon	Red skins	MAE	MeOH (50% aqueous) 1 mL of stock solution in 20 ML MeOH Temperature (50-175 ºC)	Non-optimized	ACN	HPLC	Liazid et al. (2007)
Moscatel	White skins		Power (500 W) Time (20 min)				
Chinese Kyoho cultivar	Red skins	MAE	EtOH 95% with 1.5 M HCl (85:15) Energy density (20-40 W/mL) Citric acid (0.2-0.6 mol/L) LS (15-20 mL/g GS) Power (400-800 W) Time (20-60 s)	RS-optimized	ACN	HPLC-MS	Li et al. (2012)
Bordo	Red skins	CTE	Aqueous citric acid 2%, (w/v) LS 1:3 (w/v) Time (20 h) Temperature (22 ºC)	Non-optimized	TP Colour of powders ACN	FC CIELAB Spectral A520,700nm	Kuck and Noreña (2016)
57 red and 21 white cultivars	White and red skins	CTE	Formic acid/ H_2O/MeOH (2:48:50) Time (2 h) Temperature (4 ºC)	Non-optimized	ACN Skin colour	HPLC/Q-ToF MS/MS CIELAB	Liang et al. (2011)
White and red cultivars	White and red skins from pomace after alcohol distillation	MAE	EtOH 50% aqueous with 0.8% (v/v) HCl LS (10 mL/g GS) Time (10 min) Power (140 W)	Non-optimized	TP	FC LC-TOF/MS	Peralbo-Molina et al. (2012)

		CTE	EtOH 50% aqueous with 0.8% (v/v) HCl LS (10 mL/g GS) Time (24 h) Temperature (40 ºC)	Non-optimized			
Garnacha	White and red skins from pomace	CTE	MeOH, EtOH, H_2O LS (1-5 mL/g GS) Time (30-90 min) Temperature (25-50 ºC)	RS-optimized	TP	FC	Pinelo et al. (2005)
Brazilian red cultivar	Red skins from pomace after sparkling winemaking	CTE	EtOH (8-92% aqueous) LS (3-17 mL/g GS) Time (60 min) Temperature (30 ºC)	Optimized (no RSM)	TP	FC	Caldas et al. (2018)
		MAE	EtOH optimal for CTE LS optimal for CTE Power density (1000 W/L) Time (30 min)	Non-optimized			
Six white and seven red cultivars	White and red skins	CTE	MeOH aqueous with 0.1% HCl LS (10 mL/g GS) Time (1h, then 24 h) Temperature (22 ºC, then 4 ºC)	Non-optimized	TP	FC	Pantelić et al. (2016)
Fourteen white and red cultivars	White and red skins	CTE	EtOH (80% aqueous) LS (10 mL/g GS) Time (60 min) Temperature (60 ºC)	Non-optimized	TP ACN	FC	Katalinić et al. (2010)
Sauvignon Blanc	White skins from veraison to harvest	CTE	EtOH (50% aqueous) LS (10 mL/g GS) Time (60 min)	Non-optimized	TP	FC	Tian et al. (2019)

			Temperature (rt)				
Five red cultivars incl. Cabernet Sauvignon Merlot Pinot Noir	Red skins from veraison to harvest	CTE	MeOH LS (10 mL/g GS) Time (15 min) Temperature (rt)	Non-optimized	TP	FC	Geană at al. (2016)
Nebbiolo	Red skins from veraison to harvest	CTE	Grape juice EtOH 10% Time (16 h) Temperature (4 ºC)	Non-optimized	TP Colour	FC Spectral A420,520,620 nm	Locatelli et al. (2016)
Shiraz	Red skins from veraison to harvest	CTE	EtOH (12% aqueous, Potassium hydrogen tartrate) Time (5 h) Temperature (27 ºC)	Non-optimized	TP ACN	FC HPLC, spectral	Fournand et al. (2006)
Cabernet Sauvignon Pinot Noir	Red skins (discs) from veraison to harvest	CTE	MeOH with 1% HCl Time (48 h)	Non-optimized	TP ACN	Colorimetrical Spectral	Dokoozlian and Kliewer (1996)
Cabernet Sauvignon Merlot Shiraz	Red skins	CTE	MeOH (60% aqueous) Temperature (rt) LS (40 mL/g GS) Time (30 s, 4 times)	Non-optimized	TP ACN	FC pH differential	Arnous and Meyer (2008)
Cabernet Sauvignon Merlot	Red skins	CTE	Acetone (80% aqueous) MeOH (60% aqueous) LS (9 mL/g GS) Time (4 h with acetone and 2.5 h with MeOH) Temperature (rt)	Non-optimized	TP	FC	Lorrain et al. (2011)
Seven white cultivars incl.	White skins from pomace	CTE	MeOH (80% aqueous) LS (4 mL/g GS)	Non-optimized	TP	FC	Sri Harsha et al. (2014)

Chardonnay Riesling			Time (2 h) Temperature (rt)				
Five red cultivars incl. Grenache Shiraz	Red skins From pomace	CTE	EtOH (10% aqueous with tartaric acid pH3.5) LS (3.5 mL/g GS) Time (1 h) Temperature (50 ºC)	Non-optimized	TP ACN	FC HPLC	Ky and Teissedre (2015)
White and red cultivars incl. Chardonnay Pinot Noir Nebbiolo	White and red skins from pomace	CTE	EtOH (60% aqueous) LS (8 mL/g GS) Time (2 h) Temperature (60 ºC)	Non-optimized	TP	FC	Moncalvo et al. (2016)

Abbreviations: A, absorbance; DW, dried waste; rt, room temperature; incl., including.

Table 2. MAE and CTE studies on other than grape skins.

Skins origin	Extraction method	Optimised by RSM	Parameter	Analytical method	Reference
Peanut skins	MAE	Optimised By RSM	TP	FC	Ballard et al. 2010
	CE	Optimised By RSM			Ballard et al. 2009
Hazelnut skins	MAE	Not optimised	TP	FC	Alexandru et al. 2014
Citrus mandarin skins	MAE	Optimised By RSM	Phenolic acids	HPLC	Hayat et al. 2009

Chapter 2

Response Surface Parallel Optimisation of Extraction of Total Phenolics from Separate White and Red Grape Skin Mixtures with Microwave-Assisted and Conventional Thermal Methods

Phenolics, along with vitamins and probiotics are called “nutraceuticals “play an important role in health and wellbeing when included in daily diet. Grape skins are the valuable source of phenolics. There has been recently an extensive research to find efficient and “clean and green” methods of phenolics and colour extracted not only from grape skins, but also from the industrial waste (e.g. post-winemaking). Although many researchers investigated the phenolic (TP) content obtained from the skins of grapes under various extraction methods, none of them presented the parallel development and response surface (RS) optimisation of a novel microwave-assisted (MAE) and conventional thermal extraction (CTE) methods of phenolic extraction from grape skins.

Mixing the skins collected at veraison with those collected at harvest allowed us to increase the pool of phenolics. Also, none of the previous studies optimised both methods in a similar way as we did, i.e. using the design of experiment (DoE) based on the same factors and sharing the same protocol in order to eliminate the method-to-method experimental errors. We were also interested in finding at which conditions the colour of the extracts, measured using CIELAB chroma (C^*), reached maximum using RS in both extraction methods within the model designed to maximise TP in white and red skin mixtures.

Considering pH dependence of anthocyanins responsible for the colour of red skins, we included that factor along with the ethanol concentration in aqueous solutions (EtOH), liquid to solid ratio (LS), temperature and time, in the preliminary screening of the MAE and CTE methods applied to the white and red skins. We found that the results for TP and *C** were higher at pH 1 than at pH 3.4 for both skin colours. In the next phase, i.e. advanced screening, we undertook the fractional factorial design for the advanced screening. It was based on five factors at two levels to find out which factors were significantly impacting TP and *C** of the skin extracts for both methods and skin colours. Although the fractional factorial design was chosen to narrow down the multi-factorial space to maximise TP and to cut the number of samples to be made and analysed for TP and *C*,* the number of samples was still large (in total 192 for both methods and skin colours in triplicate). During that phase of the study and based on the Folin-Ciocalteu assay in a cuvette for TP measurement, we developed the high throughput method for TP- first using a 96-well plate, then speeding up further that method by the use of a liquid distributing robot. As the extraction time was found not statistically significant for both methods and skin colours, we chose three factors for the MAE and CTE methods' optimisation: EtOH, LS and temperature, while keeping constant pH 1.5 and extraction time of 4 min for MAE and of 60 min for CTE. A central composite rotatable design (CCRD) along with the RS methodology were applied to find the optimal conditions at which TP reached maximum.

We also found at which conditions the colour of the extracts reached maximum, when applying the same factors and their levels as identified to maximise the TP content using the MAE and CTE methods for both skin colours. In the final phase both methods and for both skin colours were validated using optimal practical, instead of optimal, conditions after taking into account some practical constraints. Although TP was shown to be lower for the MAE than for CTE

method for both skin colours, and C^* was lower for the white skin mixture extracts using MAE and no difference was found in C^* of the red skin mixture extracts, the MAE method was found considerably faster, "cleaner and greener" than the CTE method.

To the authors' knowledge, this is the first in-depth report on comparison of a microwave-assisted extraction method with a conventional extraction method, both parallelly developed and optimised using the response surface methodology to find optimal conditions to maximise the total phenolic content (as the primary objective) and chroma (as the secondary objective), extracted separately from two skin mixtures, each of six grape *Vitis vinifera* commercial cultivars picked at veraison and harvest, and combined. The use of these mixtures has allowed us to identify general (rather than specific for a single grape variety) trade-offs between MAE and CTE methods to maximise total phenolic content and chroma. In addition, the study has shown the impact of pH on the total phenolic content and chroma of the skin extracts of white and red grapes, as well as the efficiency of both extraction methods. Finally, we have shown that using an industrial type microwave reactor allowed for precise monitoring and controlling of the extraction, in particular temperature, pressure, power, stirring and time. Presented in the following publication is the first detailed investigation of total phenolic content and colour of white and red grape skin extracts obtained using microwave-assisted and conventional thermal methods, both parallelly optimised by response surface methodology and it has been published by the *Journal of Cleaner Production,* volume 251 (online 5 Dec. 2019).

Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Mariola Kwiatkowski (note: the publishing name, Mariola, is different to the full name, Maria Jolanta)		
Contribution to the Paper	Participated in the establishment of the methods to be tested, conducted the literature review, collected biological samples and performed extraction experiments and measurements in statistically designed experimental settings. Collated the data and performed the data analysis and modelling required to answer the research questions. Prepared the comprehensive draft of the manuscript and implemented all the corrections and editions suggested by other co-authors. Mariola is the corresponding author for this manuscript.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	14/05/2019

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Dennis K. Taylor		
Contribution to the Paper	The principal supervision of Mariola, provided guidance with the concept of the experimental work and research questions, discussed the initial ideas and the relevance to the food industry, extensively commented on early and advanced version of the manuscript and the choice of the journal for the final submission.		
Signature		Date	14/05/2019

Name of Co-Author	George K. Skouroumounis		
Contribution to the Paper	Provided technical advice on designing extraction laboratory settings, revised and commented on the early version of the manuscript.		
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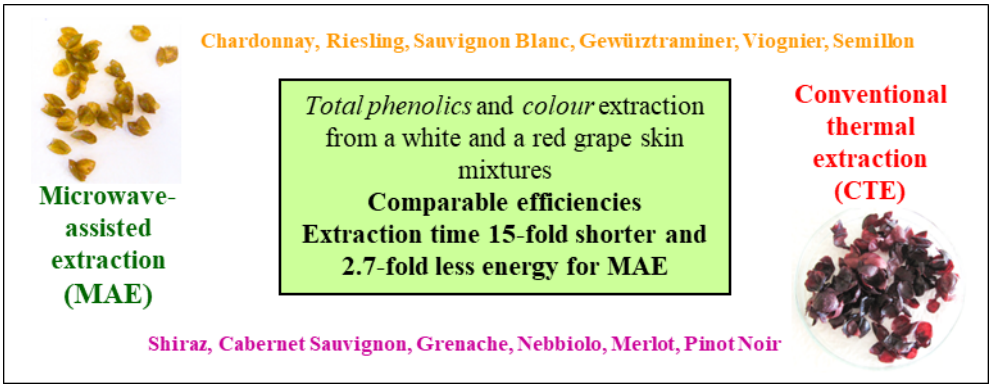
Name of Co-Author	Olena Kravchuk		
Contribution to the Paper	Discussed early concepts of the series of factorial experiments in the research and guided the first author in the analysis and interpretation of the results. Commented on the early and advanced drafts of the manuscript.		
Signature		Date	14/05/2019

Please cut and paste additional co-author panels here as required.


Chardonnay, Riesling, Sauvignon Blanc, Gewürztraminer, Viognier, Semillon
Microwave-assisted extraction (MAE)
Total phenolics and *colour* extraction from a white and a red grape skin mixtures
Comparable efficiencies
Extraction time 15-fold shorter and 2.7-fold less energy for MAE
Conventional thermal extraction (CTE)
Shiraz, Cabernet Sauvignon, Grenache, Nebbiolo, Merlot, Pinot Noir

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Response surface parallel optimization of extraction of total phenolics from separate white and red grape skin mixtures with microwave-assisted and conventional thermal methods

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ABSTRACT

Microwave-assisted extraction (MAE) was compared with conventional thermal extraction (CTE) to find optimal conditions to maximize total phenolic (TP) content and colour CIELAB C*, separately from white and red skin mixtures of six common grape cultivars each. Five factors were modelled in preliminary and advanced screenings and response surface (RS) optimization followed by the model validation. Optimized and practically acceptable levels of the factors were identified. For white skin those levels were: 60% ethanol content, 6.6 mL/g grape skins liquid to solid ratio, 70 °C temperature, pH 1.5, time of extraction 4 min and 60 min for MAE and CTE, respectively. Correspondingly, for red skin mixtures 70% ethanol content, 6.6 mL/g grape skins liquid to solid ratio, 60 °C temperature, pH 1.5, time of extraction 4 min and 60 min for MAE and CTE. The model validation experiment confirmed the reproducibility of extraction at the levels identified. Efficiency of TP extraction was comparable between MAE and CTE for both white and red skins. The colour difference between MAE and CTE was noticeable for white skins only. This research presents a novel comparison of two rival industrial methods in application to phenolics extraction from grape skins, when both methods are optimized to extract maximum TP from the same biological material and with the same solvent.

1. Introduction

The presence of phenolics in foodstuffs and their inherent antioxidant capacity aid in protecting against free radicals damaging DNA and causing lipid peroxidation, both of which have been implicated in the development of chronic diseases. Extraction of phenolics from fruit, vegetables, nuts and grains, and agricultural by-products is becoming more prevalent for the manufacture of functional foods and nutraceuticals (food additives and dietary supplements) (Gul et al., 2016; Galanakis, 2013) to assist in the prevention of such diseases.

Grapes contain numerous phenolics consisted of *flavonoids* (including anthocyanins) and *non-flavonoids* (stilbenes, hydroxybenzoic and hydroxycinnamic acids) (Iland et al., 2011; Margalit, 2004). Stilbenes, including resveratrol, are the drivers behind the health benefits. Phenolics also contribute to the colour and sensory properties impacting wine quality (Parker et al., 2007; Vidal et al., 2004).

Approximately 25% and 50% of phenolics are located in the skins of white and red cultivars, respectively (Singleton and Esau, 1969). Skins account for 10–20% of the grape berry weight (Margalit, 2004; Singleton and Esau, 1969). Skin extracts can be utilized as natural food colourants (Carocho et al., 2015) and textile dyes (Bechtold et al., 2007). Grape skins of white and red cultivars attain the highest TP content at veraison. The TP then decreases during ripening until harvest, while the anthocyanins content in red skins increases during this period (Iland et al., 2011; Kennedy, 2002). The major components of the post-winemaking waste, known as pomace, are grape skins and seeds, which can be separated if necessary. In the current commercial winemaking, pomace accounts for 10%–30% of the mass of grapes crushed (Muhlack et al., 2018).

Various methods of extraction are employed for extracting phenolics. The most typical is a conventional thermal extraction (Routray and Orsat, 2012), which requires skins to be mixed with

Abbreviations: TP, total phenolics; GS, grape skins; MAE, microwave-assisted extraction; CTE, conventional thermal extraction; RS, response surface methodology; C*, CIELAB chroma.

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solvent and heated to high temperatures for considerable time. Recently, microwave extractions, as another advanced method in extracting bioactive compounds from plant matrices (Routray and Orsat, 2012), gained popularity in processing industrial plant waste for protecting thermo-sensitive compounds while reducing the energy input required (Shahid-ul-Islam et al., 2013). Phenolics and colour can be extracted from the skins of raw grapes as well as from the skins from pomace. In a wide range of industrial applications, including Cleaner Production, it is important that methods are optimized to extract the maximum phenolics and colour from grape skins. This will reduce or better manage waste and energy consumption. Furthermore, alternative technologies can be compared, for example microwave-assisted extraction (MAE) in contrast to a conventional thermal extraction (CTE) method.

The components of grape skins from pomace were investigated for obtaining phenolic and colour compounds that are used in foodstuff and colourants (Muhlack et al., 2018: de la Cerda-Carrasco et al., 2015). Casazza et al. (2010) compared the results of phenolic extraction from the skins of Pinot Noir, isolated from white vinification pomace, using MAE and other extraction methods. However, no optimization study was done there for the MAE, which is casting doubt on the practical appeal of their maximum TP reached. The same source of skins and a response surface (RS) model from a 3^2 factorial design (for time and liquid to solid ratio factors) were utilized by Casazza et al. (2012) to find maximum TP at long extraction time of 19 h using a CTE method. Peralbo-Molina et al. (2012) compared phenolic extraction and identification from white and red skins isolated from the pomace of different cultivars employing non-optimized MAE and CTE methods, again casting doubt on whether maximum TP levels could be concluded. Pinelo et al. (2005) employed a RS-optimized CTE method with three factors in a 2^3 full factorial design for defining optimal phenolic levels from the skins of white and red Garnacha pomace. Caldas et al. (2018) compared the TP contents obtained using an optimized CTE from the skins of a Brazilian red pomace from sparkling wine production with non-optimized MAE and ultrasound-assisted (UAE) extraction methods.

Regarding colour, a study, centred only on anthocyanin content and identification from skins of a single red cultivar, was based on a 2^6 fractional factorial design for an optimized MAE method (Liazid et al., 2011). Its key findings indicated that maximum anthocyanin levels could be attained in a short extraction time (5 min) whilst temperatures above 60 °C should be avoided due to anthocyanin degradation. Similar findings, also when extracting anthocyanins from skins of a single variety, were found by Ghassempour et al. (2008) who compared the RS-optimized MAE with a non-optimized CTE method. Bechtold at al. (2007) investigated anthocyanins extraction from whole pomaces of five cultivars using a non-optimized CTE method for the purpose of textile dyeing. No MAE studies were conducted for comparison nor where skins alone utilized.

Besides the abovementioned studies, there has been no MAE and CTE studies conducted utilizing the parallel protocols to optimize all factors in order to maximize TP extraction levels. This study is designed to fill the research gap. In order to compare MAE and CTE under conditions of interest to the industry, six commercial red grape cultivars and six commercial white grape cultivars are chosen, with skins collected at harvest as well as veraison. Those cultivars (red: Shiraz, Cabernet Sauvignon, Grenache, Nebbiolo, Merlot and Pinot Noir; white: Chardonnay, Riesling, Sauvignon Blanc, Gewürztraminer, Viognier and Semillon) are typically grown in commercial vineyards in Australia and around the world (Iland et al., 2009). Consequently, the aims of this study were to: (1) evaluate the effects of five experimental factors: ethanol content, liquid to solid ratio (LS), temperature, time and pH on the TP extraction separately from white and red grape skin mixtures (6 cultivars each), then to find optimal conditions in 2^5 factorial designs for maximizing TP under the MAE or CTE methods; (2) demonstrate whether MAE or CTE should be employed as the method of choice; (3) assess the colour of extracts, represented by chroma C^*, obtained when applying the same factors and levels as identified to maximize the TP content. The experimental design (DoE) consisted of four phases: preliminary and advanced screenings, RS-optimization and model validation.

2. Materials and methods

2.1. Skin sample preparation

All grapes were *Vitis vinifera* cultivars from commercial South Australian vineyards. Preliminary screening was performed on the skins of Chardonnay grapes picked at harvest and Shiraz grapes at veraison from Longview vineyard, 2009. Advanced screening, optimization and validation studies were conducted on skin mixtures separately of white and of red grapes sourced in 2011 from C. A. Henschke & Co. The white skin mixture was prepared from Chardonnay, Riesling, Sauvignon Blanc, Gewürztraminer, Viognier and Semillon. The red skin mixture was prepared from Shiraz, Cabernet Sauvignon, Grenache, Nebbiolo, Merlot and Pinot Noir. (details Appendix A, Supplementary data).

2.2. Chemicals

For details see Appendix A, Supplementary data.

2.3. DoE and statistical analysis

The DoE used four phases: preliminary and advanced screenings, RS-optimization and model validation. Upon the clarification of factor levels in the preliminary 2^5 screening for the MAE and CTE methods, the advanced screening protocol was employed to quantify the main and two-factor interaction effects on TP and C^* and to identify factors of practical importance. A 2^3 central composite RS-optimization was then employed to find the optimal conditions for maximizing TP, and to predict the C^* at those conditions. Finally, both the MAE and CTE methods were validated as predictive models in a fully replicated validation phase.

The preliminary phase contained the following five experimental factors: EtOH, LS, temperature, time and pH. The first four factors were adopted from Li et al. (2011), which presents the MAE optimization of TP extracted from a grape seed mixture of Chardonnay, Sauvignon Blanc, Cabernet Sauvignon and Shiraz collected at veraison and harvest. The fifth factor, pH, was employed in this study due to the known correlation between pH and anthocyanin content (Mazza and Brouillard, 1987). The preliminary screening was applied to identify which factors impacted the TP extraction and evaluate the robustness of the MAE and CTE methods to extract TP and C^*, the conditions shown in Table 1. Particular focus was placed on the impact of pH (pH 1 and pH 3.4, Mazza and Brouillard, 1987) on TP and C^* for both skin colours. For both the MAE and CTE methods, three consecutive extractions were performed on the skins of Chardonnay (harvest) and Shiraz (veraison) to verify the efficiency of these extraction methods.

The advanced screening (Table 2) utilized a two-level fractional factorial resolution V design (Kuehl, 2000), to narrow down the multi-factorial space to maximize TP. The DoE procedure in Minitab 16 Statistical Software (Minitab Inc., USA) was used for the design of the experiment and the analysis of variance's (ANOVA) significance testing for the TP and C^* (at significance level $p < 0.05$).

For the optimization, a central composite rotatable design

Table 1
Experimental design for preliminary screening.

	Completely randomized design	
	MAE	CTE
Factors		
X_1: EtOH (%)	47.2	47.2
X_2: LS (mL/g GS)	45.3	45.3
X_3: Temperature (°C)	60	60
X_4: Time (min)	4.6	60
X_5: pH	1.0, 3.4	1.0, 3.4
Conditions		
Power (W)	150	N/A[a]
Pressure (psi)	200	N/A
Stirring/shaking	high	every 10 min
Cooling by compressor	on	N/A
No. of runs	1	1
No. of replicates	3	3

[a] N/A not applicable.

(CCRD) was chosen to fit second-order models and evaluate optimal conditions for maximizing TP for both the MAE and CTE methods and separately for white and red skin mixtures. The colour chroma C^* was then predicted at the TP-optimal conditions.

Several practical constraints (ethanol concentration due to safety issues and temperature due to possible degradation of phenolics; Section 3.3) were taken into account to adjust the optimal conditions for the TP from the RS-phase. In the validation phase we employed those optimized practical conditions and validated the efficiency of the MAE and CTE methods for white and red skin mixtures in an independent completely randomized experiment. We also validated the colour expected at the optimized practically acceptable conditions.

The repeatability of the TP extractions by MAE and CTE was checked by conducting three consecutive extractions with a completely randomised design. For each extraction, the same portion of solids was treated with solvent under the practical conditions (Table 2).

2.4. Extraction methods

2.4.1. MAE

A portion of skin mixture was put into a 35 mL Pyrex glass vessel and an ethanol aqueous solution added according to the values required by DoE. The pH was adjusted if required (3–5 drops of HCl). A magnetic stirring bar was added into the solution and a pressure cap placed on the vessel. The vessel was placed into a chamber of an industrial microwave reactor CEM Discover S-Class (CEM Co., USA), and MAE was performed according to the conditions presented in Tables 1 and 2. The extraction time was measured from the moment the sample reached the required temperature in the microwave reactor till the specified extraction time was completed. The temperature was controlled (±0.1 °C) using a Maxus WX801700 air compressor (Campbell Hausfeld, USA) coupled to the microwave reactor. Upon completion of extraction, the vessel was immediately placed on ice for 5 min to chill. The extract was transferred into a 10 mL tube and centrifuged using a refrigerated Hettich Universal 320R (Hettich Lab Technology, Andreas Hettich GmbH & Co. KG, Germany) at 5 °C, 10 min and 5000 rpm. The supernatant was transferred into a new 10 mL tube, and stored at −20 ± 1 °C prior to analysis. The extractions according to DoE were performed separately in a minimum of triplicates.

2.4.2. CTE

The same 35 mL Pyrex glass vessels and caps used for MAE were re-used in CTE to eliminate method-to-method errors. As in MAE, a portion of the skin mixture was placed into a vessel and an ethanol aqueous solution was added according to the DoE. Similarly, the pH was adjusted immediately as required. The vessel was capped the same way as for MAE, placed into a water bath and then heated according to the conditions presented in Tables 1 and 2. For the preliminary screening, a large glass container filled with water was heated to 60 °C on a hot plate for 1 h. For the advanced screening, optimization and validation, a Ratek WB4 water bath (Ratek Instruments Pty Ltd, Australia) with controlled temperature (±0.1 °C) was employed. The Pyrex vessel was placed in a plastic rack

Table 2
Experimental design for advanced screening, optimization and validation.

	Advanced screening		Optimization[a]		Validation	
	Half fractional factorial design 2_V^{5-1} (resolution V)		Central composite rotatable design (CCRD)		Completely randomized design	
	MAE	CTE	MAE	CTE	MAE	CTE
	White skin mixture - factors					
X_1: EtOH (%)	40, 80	40, 80	(-α)30, (−1)40, (0)55, (1)70, (α)80	(-α)30, (−1)40, (0)55, (1)70, (α)80	60	60
X_2: LS (mL/g GS)	10, 20	10, 20	(-α) 7, (−1)10, (0)15, (1)20, (α)23	(-α) 7, (−1)10, (0)15, (1)20, (α)23	7	7
X_3: Temperature (°C)	50, 70	50, 70	(-α)30, (−1)40, (0)55, (1)70, (α)80	(-α)30, (−1)40, (0)55, (1)70, (α)80	70	70
X_4: Time (min)	4, 8	60, 90	4	60	4	60
X_5: pH	1.5, 3.5	1.5, 3.5	1.5	1.5	1.5	1.5
	Red skin mixture - factors					
X_1: EtOH (%)	40, 80	40, 80	(-α)30, (−1)40, (0)55, (1)70, (α)80	(-α)30, (−1)40, (0)55, (1)70, (α)80	70	70
X_2: LS (mL/g GS)	10, 30	10, 30	(-α) 7, (−1)10, (0)15, (1)20, (α)23	(-α) 7, (−1)10, (0)15, (1)20, (α)23	7	7
X_3: Temperature (°C)	40, 60	40, 60	(-α)30, (−1)40, (0)55, (1)70, (α)80	(-α)30, (−1)40, (0)55, (1)70, (α)80	60	60
X_4: Time (min)	4, 8	60, 90	4	60	4	60
X_5: pH	1.5, 3.5	1.5, 3.5	1.5	1.5	1.5	1.5
	White and red skin mixtures – experimental conditions					
Power (W)	150	N/A[b]	150	N/A	150	N/A
Pressure (psi)	200	N/A	200	N/A	200	N/A
Stirring/shaking	high	10 min	high	10 min	high	10 min
Cooling by compressor	on	N/A	on	N/A	on	N/A
No. of runs	16	16	20	20	1	1
No. of replicates	3	3	3	3	4	4

[a] (-1) and (1) are the coded edges of the composite box design; (α) is the radius of the concentric sphere; α = 1.682.
[b] N/A not applicable.

immersed in the water bath. During extraction the samples were kept away from light by covering the water bath with a black box. The samples were vigorously shaken manually every 10 min. The same protocol as for MAE was followed upon completion of extraction. The extractions were performed in a minimum of triplicates and all replicates were extracted at the same time.

2.5. Analytical methods

2.5.1. Determination of total phenolic content

The Folin-Ciocalteu (FC) method utilizing cuvettes modified by Waterhouse (2001) was employed to measure TP of all extracts obtained using the MAE and CTE methods during preliminary and advanced screenings. A high throughput method utilizing 96-well plates and a robot was especially developed for this study during advanced screening, and further applied during the RS-optimization and validation phases (details Appendix A, Supplementary data).

2.5.2. Determination of colour

CIELAB chroma C^* was used to quantify the colour of skin extracts. The chroma is a calculated value of the squared root of $(a^*)^2 + (b^*)^2$, where a^* (redness/greenness) and b^* (yellowness/blueness) were measured using a research grade spectrophotometer (absorbance up to 5 a. u.) Cintra 40 (GBC Scientific Equipment Ltd, Australia) and employing the CIELAB tristimulus protocol (ASTM, 2000; Ohno, 2000). (details Appendix A, Supplementary data.)

2.5.3. Determination of total soluble solids and pH

(Appendix A, Supplementary data)

3. Results and discussion

3.1. Preliminary screening

The results for TP and C^* obtained over three consecutive extractions (1st, 2nd and 3rd) from the skins of Chardonnay (harvest) and Shiraz (veraison) are presented in Fig. B1 (Appendix B, Supplementary data) using the MAE and CTE methods (Table 1). No significant difference is seen between the two methods in terms of the TP extraction efficiency over all extractions (Fig. B1 (a) and (c)). For both cultivars, the first extraction accounts for approximately 80% of total TP, whilst the second and third account for approximately 15% and 5%, respectively. A pH of 3.4 compared to pH 1.0 resulted in an approximate 50% decrease in TP extraction over all three extractions. While we are not aware of any other study reporting a similar impact of pH on TP extraction for white grape skins, the results found for red skins are consistent with those previously reported (Zhao et al., 2013; Mazza and Brouillard, 1987).

In terms of C^* of the extracts, there was again no significant difference between both methods over the three extractions for both cultivars (Fig. B1 (b) and (d)). Again, there was an approximately 50% drop in colour C^* of the extracts when the extraction pH was raised from 1.0 to pH 3.4. Not surprisingly, the Shiraz skins had higher TP content and C^* than those of the Chardonnay skins, even though the Shiraz skins were picked at veraison whilst the Chardonnay skins were picked at harvest. Given the fact that the majority of the phenolics were attained from the first extraction, only a single extraction was chosen for the advanced screening and optimization with appropriate modification of the remaining factors.

3.2. Advanced screening

The fractional factorial design and the results obtained using the high throughput FC method for the advanced screening are provided in Table 3 for the white and red skin mixtures. For red skins, time (X_4) was not found significant for either TP or C^* when using MAE or CTE. This is in agreement with Ghassempour et al. (2008) and Liazid et al. (2011). For white skins, time was also not significant for TP with either method. A significant but small positive effect on C^* was noticed with increasing time for CTE, while no significant time effect on C^* was observed under MAE. Consequently, for the optimization we chose a single extraction time for MAE (4 min) and CTE (60 min) to avoid likely degradation of phenolics during a prolonged time of extraction.

The other four factors (ethanol content (X_1), liquid to solid ratio (X_2), temperature (X_3) and pH (X_5)) significantly affected TP and C^* ($p \leq 0.05$) (Table 3). Consistently for both the white and red mixtures and both methods, the lower level of ethanol, higher ratio of LS, higher temperature and lower pH increased the extraction of TP. The highest values of TP and C^* were obtained for runs 1 and 2 (Table 3). Not surprisingly, dropping the extraction temperature by 20° for both methods resulted in approximately 50% reduction in TP and C^* (compare, e.g., runs 2 and 8). For white skin mixtures, the reduction in ethanol content from 80% to 40% resulted in a small increase in TP extraction (<15% on average), while causing a large decrease in chroma C^* (of approximately 30%). Although the direction of the ethanol effect on TP and C^* was similar for the red skin mixtures (compare, e.g., runs 1 and 2), the magnitude of the effect was much smaller than for the white skin mixtures. Reduction in LS resulted in an approximately 50% reduction in TP and C^* for both methods and skin colours (compare, e.g., runs 2 and 10). A pH 1.5 was found to be superior in terms of TP and C^* (Table 3). The interaction plots for TP and C^* obtained using the MAE and CTE methods in the advanced screening are presented in Fig. B.2 and Fig. B.3 for the white and red skin mixtures, respectively (Appendix B, Supplementary data).

Both screenings allowed to fix the time and pH at their practically best values. In the optimization we focussed on the remaining three factors: ethanol content, liquid to solid ratio and temperature. As significant two-factor interactions were detected in the advanced screening, we decided to investigate the second-order response surface, described by Eq. (1):

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 \quad (1)$$

where:

X_1 = EtOH
X_2 = LS
X_3 = temperature
$b_0, \ldots, b_{33}$ = regression coefficients
Y = TP or C^* response.

3.3. Optimization

An unblocked 3-factor, 5-level central composite rotatable design (CCRD) was used to find the optimal conditions to maximize TP. For the CCRD, factor levels (coded −1 and 1), the axial points (coded $\pm \alpha = 1.682$) as well as six replications at the center point (coded 0, 0, 0), all describing a CCRD sphere, are presented in Table 4, which also records the measured values of TP and C^* for all runs. Pleasingly, the optimization further refined on the advanced screening levels of TP and C^* and resulted in further increases in

Table 3
Experimental design for the advanced screening with response of independent variables for the white and red grape skin mixtures [a, d].

Run No.	Factors[b]					Response (Y) [c]	
	X_1 (%)	X_2 (mL/g GS)	X_3 (°C)	X_4 (min)	X_5	TP (g GAE/L)	C^*
MAE (white skin mixture)							
1	40	10	70	4	1.5	1.20 ± 0.05	39.03 ± 1.22
2	80	10	70	8	1.5	1.31 ± 0.08	58.52 ± 3.11
3	40	10	50	8	1.5	0.93 ± 0.17	37.57 ± 5.40
4	80	20	70	8	3.5	0.27 ± 0.01	30.33 ± 0.64
5	40	10	70	8	3.5	0.76 ± 0.07	17.87 ± 1.83
6	40	20	50	4	1.5	0.52 ± 0.03	18.67 ± 0.99
7	40	10	50	4	3.5	0.74 ± 0.05	16.54 ± 0.75
8	80	10	50	4	1.5	1.01 ± 0.28	37.78 ± 0.55
9	40	20	70	4	3.5	0.51 ± 0.03	13.06 ± 1.01
10	80	20	70	4	1.5	0.73 ± 0.04	35.85 ± 0.61
11	40	20	70	8	1.5	0.81 ± 0.04	25.49 ± 0.64
12	80	20	50	8	1.5	0.51 ± 0.01	27.10 ± 0.07
13	80	10	50	8	3.5	0.48 ± 0.03	39.47 ± 0.84
14	80	20	50	4	3.5	0.26 ± 0.02	32.15 ± 0.91
15	80	10	70	4	3.5	0.59 ± 0.03	40.97 ± 0.56
16	40	20	50	8	3.5	0.64 ± 0.04	15.14 ± 0.06
CTE (white skin mixture)							
1	40	10	70	60	1.5	1.38 ± 0.11	46.54 ± 3.11
2	80	10	70	90	1.5	1.41 ± 0.08	76.87 ± 2.91
3	40	10	50	90	1.5	1.01 ± 0.02	37.74 ± 0.27
4	80	20	70	90	3.5	0.33 ± 0.02	26.72 ± 0.28
5	40	10	70	90	3.5	1.09 ± 0.03	22.10 ± 0.04
6	40	20	50	60	1.5	0.67 ± 0.03	22.68 ± 0.31
7	40	10	50	60	3.5	0.76 ± 0.04	17.46 ± 0.55
8	80	10	50	60	1.5	0.70 ± 0.01	41.50 ± 0.28
9	40	20	70	60	3.5	0.63 ± 0.02	14.57 ± 0.56
10	80	20	70	60	1.5	0.76 ± 0.03	43.79 ± 0.31
11	40	20	70	90	1.5	0.89 ± 0.03	31.59 ± 0.55
12	80	20	50	90	1.5	0.46 ± 0.02	32.27 ± 0.50
13	80	10	50	90	3.5	0.59 ± 0.03	42.92 ± 1.18
14	80	20	50	60	3.5	0.30 ± 0.01	30.28 ± 0.82
15	80	10	70	60	3.5	0.59 ± 0.01	39.25 ± 0.35
16	40	20	50	90	3.5	0.51 ± 0.02	12.07 ± 0.14
MAE (red skin mixture)							
1	40	10	60	4	1.5	1.16 ± 0.17	68.81 ± 1.68
2	80	10	60	8	1.5	1.10 ± 0.03	71.76 ± 0.72
3	40	10	40	8	1.5	1.03 ± 0.02	69.09 ± 2.51
4	80	30	60	8	3.5	0.32 ± 0.04	17.77 ± 1.84
5	40	10	60	8	3.5	0.88 ± 0.09	25.91 ± 1.70
6	40	30	40	4	1.5	0.43 ± 0.06	41.44 ± 3.02
7	40	10	40	4	3.5	0.91 ± 0.15	31.60 ± 1.48
8	80	10	40	4	1.5	0.93 ± 0.14	73.06 ± 0.79
9	40	30	60	4	3.5	0.54 ± 0.00	8.78 ± 0.39
10	80	30	60	4	1.5	0.49 ± 0.01	43.88 ± 2.39
11	40	30	60	8	1.5	0.57 ± 0.07	42.83 ± 3.03
12	80	30	40	8	1.5	0.32 ± 0.02	40.87 ± 3.04
13	80	10	40	8	3.5	0.65 ± 0.01	40.44 ± 4.09
14	80	30	40	4	3.5	0.32 ± 0.03	11.25 ± 1.74
15	80	10	60	4	3.5	0.71 ± 0.04	47.42 ± 1.81
16	40	30	40	8	3.5	0.42 ± 0.06	8.49 ± 1.76
CTE (red skin mixture)							
1	40	10	60	60	1.5	1.22 ± 0.04	69.81 ± 1.58
2	80	10	60	90	1.5	1.14 ± 0.07	72.81 ± 0.66
3	40	10	40	90	1.5	1.09 ± 0.01	69.45 ± 0.72
4	80	30	60	90	3.5	0.36 ± 0.02	13.44 ± 2.21
5	40	10	60	90	3.5	1.13 ± 0.06	30.61 ± 1.97
6	40	30	40	60	1.5	0.50 ± 0.01	38.93 ± 1.54
7	40	10	40	60	3.5	0.93 ± 0.05	29.21 ± 1.91
8	80	10	40	60	1.5	0.87 ± 0.02	72.40 ± 0.49
9	40	30	60	60	3.5	0.54 ± 0.01	9.29 ± 0.93
10	80	30	60	60	1.5	0.45 ± 0.02	41.31 ± 1.65
11	40	30	60	90	1.5	0.63 ± 0.01	44.33 ± 0.38
12	80	30	40	90	1.5	0.39 ± 0.01	40.89 ± 1.08
13	80	10	40	90	3.5	0.74 ± 0.03	38.26 ± 1.99
14	80	30	40	60	3.5	0.30 ± 0.01	13.13 ± 2.68
15	80	10	60	60	3.5	0.84 ± 0.05	47.95 ± 7.46
16	40	30	40	90	3.5	0.42 ± 0.00	7.11 ± 0.72

[a] Experimental conditions according to design 2_V^{5-1}.
[b] X_1 = Ethanol concentration, X_2 = Liquid: solid ratio, X_3 = Temperature, X_4 = Time, X_5 = pH.
[c] Experimental values: mean ± SD (n = 3).
[d] Completely randomised design.

Table 4
Design of experiment for RS-optimization with response of independent variables. [a, d].

Run No.	Factors [b]			Response (Y)[c]							
	X_1	X_2	X_3	MAE		CTE		MAE		CTE	
	(%)	(mL/g GS)	(°C)	TP (g GAE/L)	C^*	TP (g GAE/L)	C^*	TP (g GAE/L)	C^*	TP (g GAE/L)	C^*
				White skin mixture				Red skin mixture			
1	−1	−1	−1	0.78 ± 0.09	26.10 ± 2.19	0.96 ± 0.02	31.51 ± 0.62	0.95 ± 0.09	67.90 ± 0.33	1.33 ± 0.09	73.81 ± 0.23
2	1	−1	−1	0.62 ± 0.03	32.80 ± 1.11	0.95 ± 0.05	37.94 ± 0.55	1.14 ± 0.09	75.16 ± 0.03	1.15 ± 0.05	75.45 ± 1.25
3	−1	1	−1	0.44 ± 0.01	16.09 ± 0.84	0.65 ± 0.01	19.87 ± 0.29	0.55 ± 0.04	50.28 ± 1.14	0.84 ± 0.04	59.46 ± 0.83
4	1	1	−1	0.51 ± 0.00	21.97 ± 0.18	0.47 ± 0.02	22.28 ± 0.96	0.64 ± 0.04	59.90 ± 0.46	0.64 ± 0.02	57.13 ± 2.21
5	−1	−1	1	1.16 ± 0.02	34.01 ± 1.56	1.27 ± 0.07	43.50 ± 1.80	1.21 ± 0.04	64.47 ± 1.34	1.43 ± 0.01	66.44 ± 0.42
6	1	−1	1	1.01 ± 0.14	42.19 ± 1.13	1.28 ± 0.04	53.15 ± 1.22	1.51 ± 0.11	74.87 ± 0.23	1.51 ± 0.03	70.50 ± 1.55
7	−1	1	1	0.69 ± 0.04	20.03 ± 0.34	0.79 ± 0.02	26.29 ± 1.16	0.71 ± 0.01	50.61 ± 0.60	0.89 ± 0.01	50.75 ± 0.16
8	1	1	1	0.66 ± 0.05	27.55 ± 1.43	0.79 ± 0.02	33.90 ± 0.80	0.81 ± 0.02	60.68 ± 0.40	0.91 ± 0.02	53.87 ± 0.28
9	1.682 (α)	0	0	0.66 ± 0.01	33.03 ± 0.75	0.61 ± 0.04	35.61 ± 1.10	0.79 ± 0.05	66.55 ± 1.10	0.94 ± 0.05	66.71 ± 0.45
10	- 1.682(-α)	0	0	0.46 ± 0.02	17.01 ± 1.30	0.64 ± 0.05	21.93 ± 0.69	0.70 ± 0.06	57.23 ± 0.82	0.93 ± 0.06	58.70 ± 0.76
11	0	1.682(α)	0	0.58 ± 0.02	21.01 ± 0.94	0.59 ± 0.04	21.71 ± 0.86	0.53 ± 0.04	43.13 ± 1.35	0.63 ± 0.03	46.01 ± 1.56
12	0	- 1.682(-α)	0	1.38 ± 0.15	50.11 ± 2.86	1.35 ± 0.10	49.91 ± 0.58	1.34 ± 0.09	73.88 ± 0.15	1.64 ± 0.13	75.45 ± 0.09
13	0	0	1.682 (α)	0.99 ± 0.08	34.53 ± 1.85	1.13 ± 0.02	44.38 ± 0.52	1.09 ± 0.03	55.51 ± 0.64	1.29 ± 0.07	54.33 ± 0.23
14	0	0	- 1.682 (-α)	0.52 ± 0.01	23.23 ± 0.65	0.73 ± 0.02	25.64 ± 0.94	0.67 ± 0.04	59.81 ± 0.74	0.90 ± 0.05	63.17 ± 1.75
15	0	0	0	0.78 ± 0.11	27.80 ± 1.94	0.91 ± 0.02	31.31 ± 0.35	0.97 ± 0.01	64.70 ± 0.15	1.05 ± 0.04	64.65 ± 2.26
16	0	0	0	0.80 ± 0.15	27.05 ± 3.13	0.92 ± 0.04	31.47 ± 0.33	0.99 ± 0.10	64.60 ± 1.02	1.05 ± 0.05	63.93 ± 1.05
17	0	0	0	0.79 ± 0.12	27.79 ± 2.06	0.91 ± 0.02	31.67 ± 0.33	0.93 ± 0.05	65.43 ± 0.76	1.01 ± 0.10	63.37 ± 2.62
18	0	0	0	0.78 ± 0.11	28.58 ± 1.63	0.85 ± 0.05	31.44 ± 0.34	0.98 ± 0.08	64.65 ± 0.27	0.91 ± 0.04	60.50 ± 1.42
19	0	0	0	0.78 ± 0.04	28.32 ± 1.12	0.87 ± 0.02	30.67 ± 0.55	0.90 ± 0.07	61.15 ± 1.62	0.98 ± 0.03	59.47 ± 0.85
20	0	0	0	0.76 ± 0.10	28.06 ± 0.88	0.86 ± 0.04	30.60 ± 0.65	0.90 ± 0.08	60.12 ± 2.07	0.90 ± 0.03	58.34 ± 0.98

[a] Conditions according to central composite rotatable design (CCRD).
[b] X_1 = Ethanol content, X_2 = liquid to solid ratio, X_3 = temperature.
[c] Experimental values: mean ± SD (n = 3).
[d] Completely randomised design.

both responses. The data from Table 4 was then utilized to estimate the regression coefficients (found significant at $p \leq 0.05$) of the second-order equations (Eq. (1)) (Table 5). The optimal conditions (X_1, X_2 and X_3) were determined from the model in order to maximize TP denoted as $TP_{max\ (pred)}$. The value of C^* was estimated for those conditions, denoted $C^*_{max\ (pred)}$. The developed RS models for TP and C^* fit the experimental data well ($R^2 \geq 0.9$). The response surfaces for TP and C^* obtained using the MAE and CTE methods in the optimization are presented in Fig. B.4 and Fig. B.5 for the white and red skin mixtures, respectively (Appendix B, Supplementary data).

There were two practical constraints in relation to temperature and ethanol content. The first constraint was related to the need to decrease the temperature for MAE to operate well below the boiling point of ethanol 78.3 °C (Morrison and Boyd, 1974), as well as to avoid possible degradation of coloured phenolics in red skins above 60 °C (Liazid et al., 2011; Ghassempour et al., 2008). The second constraint was for the white skins only, for which we increased the ethanol concentration from the predicted optimal (about 47.6%) to a practical level of 60% in order to have the concentration closer to that chosen for CTE. The above changes led to only slightly lower values of TP and C^*, denoted as $TP_{max\ (pract)}$ and $C^*_{max\ (pract)}$, summarized in Table 5.

In conclusion, the following optimized practical levels of all five factors were determined by the model, to be validated: 60% ethanol content (X_1), 6.6 mL/g GS liquid to solid ratio (X_2), 70 °C temperature (X_3), 4 min and 60 min for MAE and CTE, respectively, and a pH of 1.5 for white skin mixtures; and for red skin mixtures 70% ethanol content, 6.6 mL/g GS liquid to solid ratio, 60 °C temperature, 4 min and 60 min for MAE and CTE, respectively and a pH of 1.5.

3.4. Validation

The MAE and CTE methods were validated using the practical conditions described above. We compared the values of $TP_{max\ (pract)}$ and $C^*_{max\ (pract)}$ predicted by the model equation for the practical conditions with the values obtained for a single extraction cycle (Fig. 1) at the same practical conditions. The latter values, denoted as $TP_{max\ (val)}$ and $C^*_{max\ (val)}$, are presented in Table 5 and depicted in Fig. 1. The absolute percentage error (defined as the absolute value of the difference between the validated and predicted response relative to the predicted response) were used to quantify the agreement of validation.

A little difference was found between the optimized practical and obtained validated values of TP and C^* for both methods (Table 5). For the white skin mixture, the percentage errors for the TP were 6.2% under MAE and 8.9% under CTE. For the red skin mixture, the percentage errors were 2.3% and 4.2%, correspondingly. Regarding chroma for the white skin mixture, the corresponding percentage errors were 0.6% and 8.3%, and for the red skins, 1.9% and 1.4%.

Table 5 allows for comparing the maximum values of TP and C^* obtained during the validation between MAE and CTE. In the case of red skins, $TP_{max\ (val)}$ for MAE was 11% lower than for CTE, while for white skins, this difference was 12%. Regarding the red skins, the difference in $C^*_{max\ (val)}$ between MAE and CTE was negligible, while for the white skins, $C^*_{max\ (val)}$ was 21% lower for MAE than for CTE. Although all the above results show that TP and C^* were lower (sometimes only slightly lower) for MAE than for CTE, the extraction time required for MAE was considerably shorter (15-fold).

In order to compare the efficiency of both extraction methods during validation, we assessed the TP content obtained over three consecutive extractions of the same portion of skin mixture (Fig. 1). For MAE of the white skin mixture, the TP proportions between extractions were 71%, 21% and 8%, yielding in total 2.21 g GAE/L (equivalent of 14.53 mg GAE/g GS). For CTE of the white skin mixture, these proportions were 74%, 20% and 6%, giving in total 2.37 g GAE/L (equivalent of 15.65 mg GAE/g GS), which equates to 7% more than for MAE. In the case of the red skin mixture utilizing MAE, the TP proportions were 70%, 20% and 10%, giving in total 2.26 g GAE/L (equivalent of 14.86 mg GAE/g GS), whilst for CTE,

Table 5
Estimated response surface model in the optimization, validation and practical adjustments for the MAE and CTE methods for the white and red grape skin mixtures.

Method	Response (Y)[a]	Equation	Factors			Result [b]
			EtOH (X_1) (%)	LS (X_2) (mL/g GS)	Temp. (X_3) (°C)	
White skin mixture						
MAE	TP (g GAE/L)	$Y = 0.0406X_1 - 0.0978X_2 + 0.0341X_3 - 0.0004X_1^2 + 0.0025X_2^2 - 0.0007X_2X_3$ ($p < 0.01$), $R^2 = 0.87$				
	$TP_{max\ (pred)}$		47.6	6.6	80.2	1.64 ± 0.04
	$TP_{max\ (pract)}$		60	6.6	70	1.46 ± 0.04
	$TP_{max\ (val)}$		60	6.6	70	1.55 ± 0.01
CTE	TP (g GAE/L)	$Y = 0.6211 + 0.0364X_1 - 0.0590X_2 - 0.0004X_1^2 + 0.0016X_2^2 + 0.0001X_3^2 - 0.0003X_1X_2 + 0.0001X_1X_3 - 0.0003X_2X_3$ ($p < 0.05$), $R^2 = 0.96$				
	$TP_{max\ (pred)}$		60.9	6.6	80.2	1.74 ± 0.02
	$TP_{max\ (pract)}$		60	6.6	70	1.59 ± 0.02
	$TP_{max\ (val)}$		60	6.6	70	1.76 ± 0.01
MAE	C^*	$Y = 0.9565X_1 - 3.2351X_2 - 0.0068X_1^2 + 0.0881X_2^2 - 0.0130X_2X_3$ ($p < 0.05$), $R^2 = 0.94$				
	$C^*_{max\ (pred)}$		79.7	6.6	80.2	58.42 ± 0.77
	$C^*_{max\ (pract)}$		60	6.6	70	52.37 ± 0.77
	$C^*_{max\ (val)}$		60	6.6	70	52.03 ± 0.66
CTE	C^*	$Y = 38.1365 + 0.5285X_1 - 2.2533X_2 - 0.3371X_3 - 0.0036X_1^2 + 0.0673X_2^2 + 0.0062X_3^2 - 0.0101X_1X_2 + 0.0047X_1X_3 - 0.0153X_2X_3$ ($p < 0.001$), $R^2 = 0.99$				
	$C^*_{max\ (pred)}$		80.2	6.6	80.2	75.16 ± 0.32
	$C^*_{max\ (pract)}$		60	6.6	70	60.43 ± 0.32
	$C^*_{max\ (val)}$		60	6.6	70	65.87 ± 0.45
Red skin mixture						
MAE	TP (g GAE/L)	$Y = 0.0326X_1 - 0.0002X_1^2 - 0.0005X_1X_2 - 0.0005X_2X_3$ ($p < 0.05$), $R^2 = 0.92$				
	$TP_{max\ (pred)}$		76.7	6.6	80.2	1.82 ± 0.03
	$TP_{max\ (pract)}$		70	6.6	60	1.55 ± 0.03
	$TP_{max\ (val)}$		70	6.6	60	1.59 ± 0.04
CTE	TP (g GAE/L)	$Y = 3.0391 - 0.1085X_2 - 0.0269X_3 + 0.0024X_2^2 + 0.0002X_3^2 + 0.0003X_1X_3$ ($p < 0.001$), $R^2 = 0.95$				
	$TP_{max\ (pred)}$		80.2	6.6	80.2	2.12 ± 0.03
	$TP_{max\ (pract)}$		70	6.6	60	1.70 ± 0.03
	$TP_{max\ (val)}$		70	6.6	60	1.77 ± 0.05
MAE	C^*	$Y = 72.2326 - 0.0045X_3^2$ ($p < 0.05$), $R^2 = 0.92$				
	$C^*_{max\ (pred)}$		80.2	6.6	49.1	80.66 ± 0.96
	$C^*_{max\ (pract)}$		70	6.6	60	77.56 ± 0.96
	$C^*_{max\ (val)}$		70	6.6	60	76.24 ± 0.51
CTE	C^*	$Y = 107.3000 - 0.4770X_1 - 1.5390X_2 + 0.0040X_1^2 + 0.0040X_1X_3$ ($p < 0.05$), $R^2 = 0.93$				
	$C^*_{max\ (pred)}$		80.2	6.6	34.4	84.11 ± 0.83
	$C^*_{max\ (pract)}$		70	6.6	60	77.28 ± 0.83
	$C^*_{max\ (val)}$		70	6.6	60	76.22 ± 0.31

[a] $Y_{max\ (pred)}$: values predicted by model at optimal conditions, $Y_{max\ (pract)}$: values predicted by model at practical conditions, $Y_{max\ (val)}$: values obtained at practical conditions.
[b] Predicted by model and obtained values of TP and C^*: mean ± SE (n = 3 in optimization and n = 4 in validation).

these proportions were identical at 71%, 20% and 9%, giving in total 2.49 g GAE/L (equivalent of 16.42 mg GAE/g GS), which was 10% more than for MAE. Fig. 1 also presents the values of C^* obtained for both methods over the same three consecutive extractions. No significant difference was observed between the methods for the red skin mixture (Fig. 1(d)), whilst there was a small difference for the white skin mixture (Fig. 1(b)).

3.5. Circular Economy

As an alternative to the linear economy (raw materials used-product made-waste dumped) (Ellen MacArthur Foundation, 2017) we envisage utilizing our methodology in the Circular Economy, which aims at minimising resource input, waste, emission and energy leakage by closing material and energy loops (Geissdoerfer et al., 2017).

The amounts of 879,000 tonnes of white grapes and 966,000 tonnes of red grapes were crushed in Australia in 2016 (Wine Australia, 2019). The gross production value of these crushed grapes was 1400 million AUD (FAOSTAT, 2016). By considering that pomace accounts for up to 30% of the mass of grapes crushed (Muhlack et al., 2018), approximately 263,000 tonnes of white and 289,800 tonnes of red pomace were produced in 2016. In Australia, wineries are obliged to follow guidelines to avoid fines and penalties, and hence have to record the mass of pomace, its quality and method of safe disposal (EPA, South Australia, 2016). Winemakers, together with grape growers and scientists, are already using thermochemical, biological, agricultural and environmental technologies to recover useful constituents from pomace (Muhlack et al., 2018). Red winemaking involves skin contact during wine maceration, while there is no skin contact involved in white winemaking with some exceptions such as rosé or some sparkling wines. Apart from anthocyanins, other monomeric phenolics can be recovered from pomace: stilbenes (e.g. resveratrol), flavonols (e.g. quercetin), and flavan-3-ols (e.g. catechin and epicatechin). For example, an Australian company Tarac Technologies (https://www.tarac.com.au/) operates in a closed loop system by manufacturing commercially red skin extracts from pomace for wine colour enhancement.

Red and white skin extracts can be utilized as natural food colourants as well as textile dyes. The methodology developed and the results obtained in this study may be relevant for the future

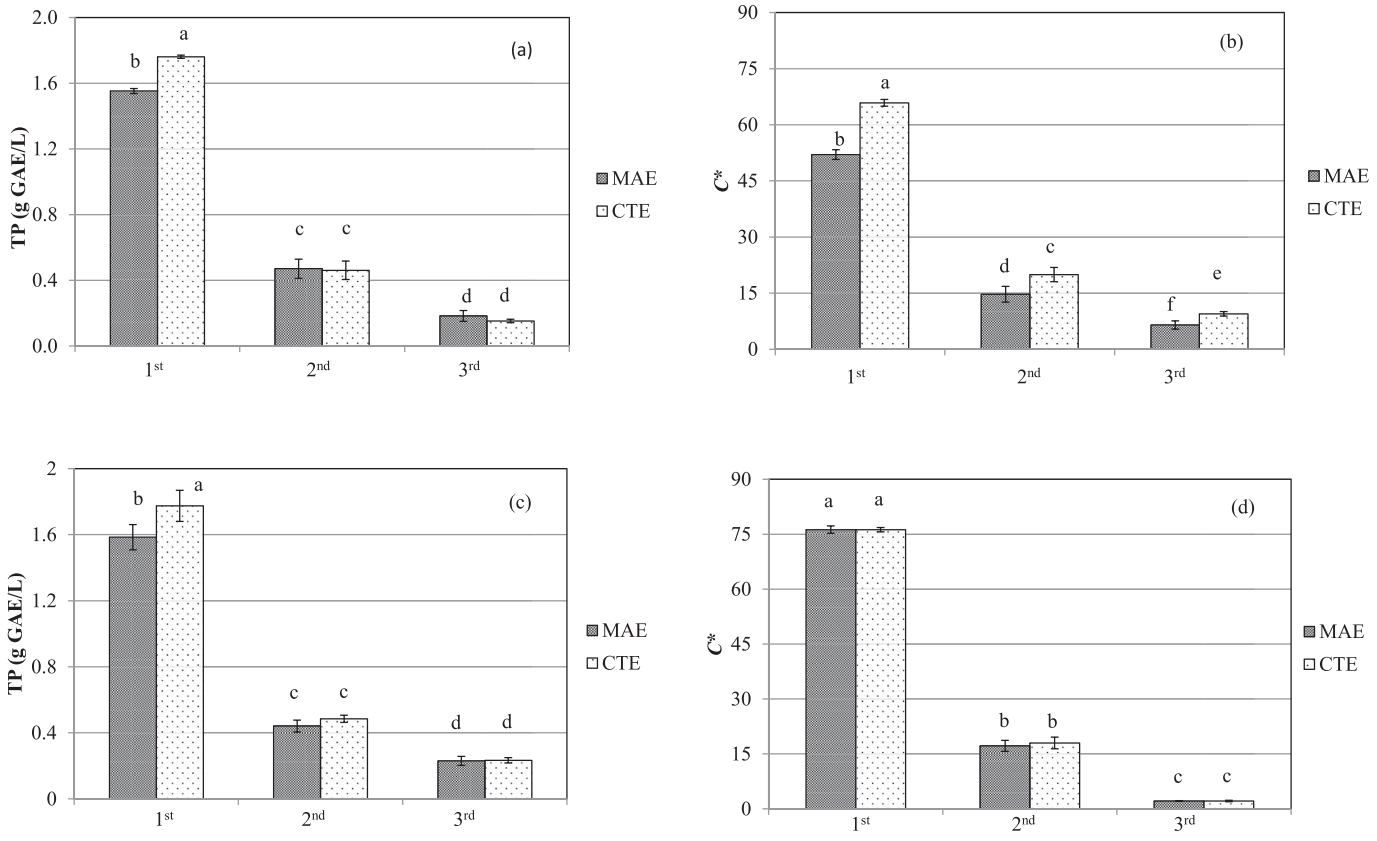

Fig. 1. Validation for total phenolics (TP) (a) and chroma (C^*) (b) of the white skin mixture, and for TP (c) and C^*(d) of the red skin mixture, obtained over three consecutive extractions (1st, 2nd and 3rd) under the conditions for MAE and CTE methods (Table 2). Experimental values: mean ± SD (n = 4). Significant differences at the 5% level.

studies on phenolics and colour extracted from skins separated from pomace. It is noted that there are machines which can separate skins from pomace at industrial scales. Both the MAE and CTE methods documented similar extraction efficiencies of phenolics and colour from white and red grape skins separately. When comparing a single extraction, the MAE method requires 15-fold less time (4 min instead of 60 min), and 2.7-fold less energy (63W instead of 167W for the equipment used in the study). This energy ratio can be further improved up to 20-fold if more efficient industrial MW equipment is used, for example CEM/MARS 6, a multimode reactor.

In order to valorise skins from grapes or pomace in future studies, TP is recalculated on the basis of kg of GAE per ton of grapes (Table 6), rather than as g of GAE/L (Table 5). (Note the colour C^* of the skin extracts is repeated from Table 5 for convenience.) In addition, the average berry weight as well as the distribution by weight of skins, pulp with juice and seeds are shown. The TP content for the red skins was 1.82 kg GAE/ton for MAE, and 2.12 kg GAE/ton for CTE, both under optimal conditions. Our CTE result was similar to the result of 1.86 kg GAE/ton reported by Singleton and Esau (1969) for red skins using a conventional method and grapes collected at harvest. For the white skins, TP was 1.64 kg GAE/ton for MAE, and 1.74 kg GAE/ton for CTE, both under optimal conditions. The latter result was higher than the one of 0.90 kg GAE/ton obtained by Singleton and Esau (1969). The difference was probably due to the fact that those authors used grapes collected at harvest, while in this study the white skin mixture also contained skins collected at veraison.

The flow chart (Fig. 2) was designed to represent the evolving holistic and systemic business model characteristic for circular ('closing the loop') rather than linear economies. We anticipate that the methodology presented in this study for phenolics and colour extractions using MAE can be applied in the valorisation of wastes: post-extraction, post-winemaking and post-non-alcoholic beverage making from white and red skins. The feasibility of our methodology transfers to extracting phenolics and colour from whole pomace has to be investigated taking into account a possibility of additionally extracted phenolics and colour from ground seeds (Appendix A, Supplementary data).

The proposed validations would be done on a laboratory scale first, followed by experiments on pilot and production scales. The use of the MAE, rather than the CTE method, on a pilot plant scale research should be performed to quantify the potential economic benefits of valorisation of the currently wasted materials. Based upon pilot plant scale studies, commercial scale implementation of such extractions should be done.

4. Conclusions

The effects of ethanol content, liquid to solid ratio, temperature, time and pH for MAE and CTE on the extraction of TP and C^* of white and red grape skin mixtures (six cultivars each) were evaluated and optimized in order to maximize TP extraction. In the DoE consisted of four phases which included preliminary and advanced screenings, RS-optimization and validation, conditions maximizing the TP extraction were identified and further refined to take into account practical constraints of safety and thermal stability of components. The optimized practically acceptable conditions identified and recommended are: 60% ethanol content, 6.6 mL/g GS liquid to solid ratio, 70 °C, 4 min and 60 min for MAE and CTE,

Table 6
Total phenolics (TP) calculated per tonne of white and red grapes obtained using MAE and CTE.

		White grapes		Red grapes	
Average berry weight [a]	g	1.08		1.09	
Distribution of berry weight:					
seeds	%	4.97		5.87	
pulp with juice	%	55.27		48.99	
skins	%	13.25		16.61	
		Predicted by model under optimal conditions [b]			
		MAE	CTE	MAE	CTE
$C^*_{max\ (pred)}$		58.42 ± 0.77	75.16 ± 0.32	80.66 ± 0.96	84.11 ± 0.83
$TP_{max\ (pred)}$	g GAE/L	1.64 ± 0.04	1.74 ± 0.02	1.82 ± 0.03	2.12 ± 0.03
$TP_{max\ (pred)}$	kg GAE/ton grapes	1.43 ± 0.03	1.52 ± 0.01	1.99 ± 0.03	2.35 ± 0.02
		Obtained under practical conditions [c]			
		MAE	CTE	MAE	CTE
$C^*_{max\ (val)}$		52.03 ± 0.66	65.87 ± 0.45	76.24 ± 0.51	76.22 ± 0.31
$TP_{max\ (val)}$	g GAE/L	1.55 ± 0.01	1.76 ± 0.01	1.59 ± 0.04	1.77 ± 0.05
$TP_{max\ (val)}$	kg GAE/ton grapes	1.36 ± 0.01	1.54 ± 0.00	1.74 ± 0.04	1.94 ± 0.05

[a] Ratio of all berries weight (6 cultivars, veraison and harvest) to number of berries used in skin mixture.
[b] Experimental values: mean ± SE (n = 3).
[c] Experimental values: mean ± SE (n = 4).

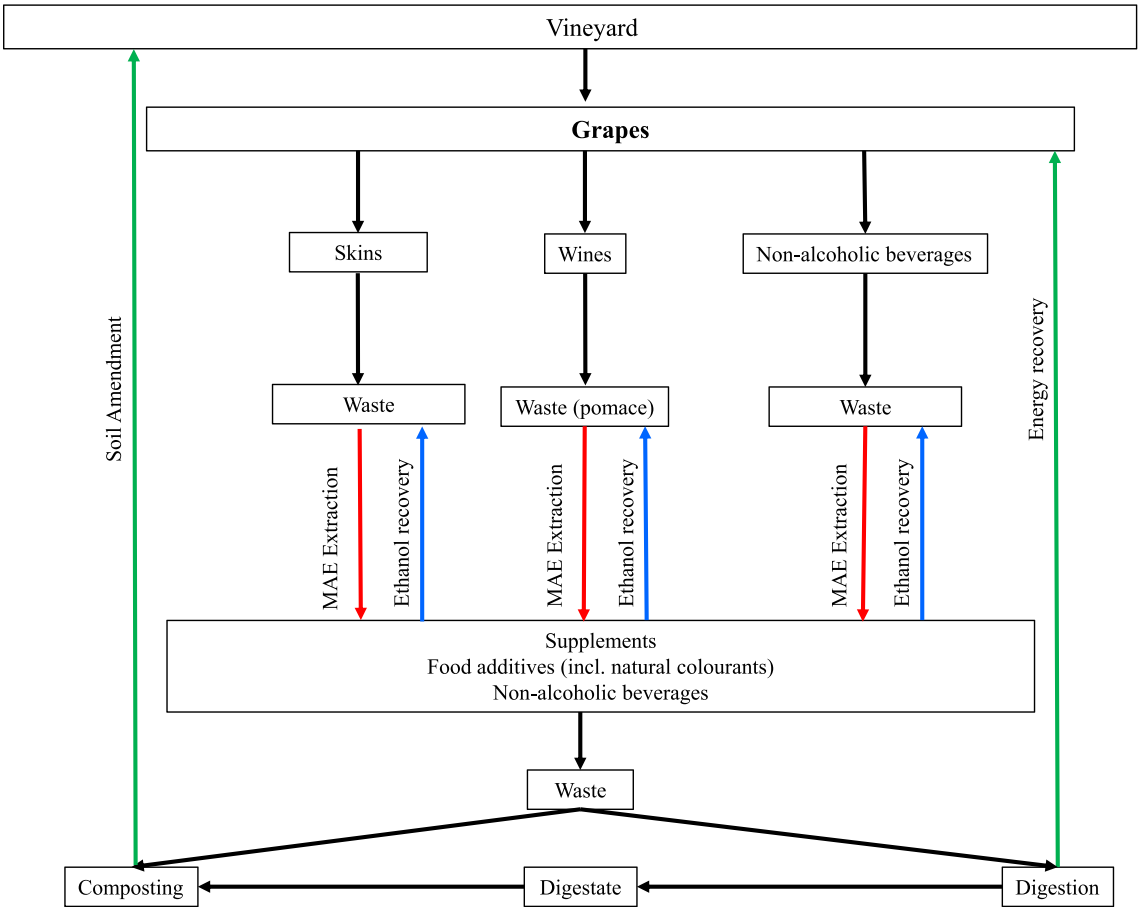

Fig. 2. Circular economy of grape processing.

respectively and a pH of 1.5 for white skin mixtures; and for red skin mixtures 70% ethanol content, 6.6 mL/g GS liquid to solid ratio, 60 °C, 4 min and 60 min for MAE and CTE, and a pH of 1.5. The validation confirmed the reproducibility of those levels. The same amount of TP was extracted under the optimal conditions by each MAE and CTE, making the methods competitive. The MAE method, however, was 2.7-fold more energy efficient and 15-fold faster for a single extraction.

The predictive models highlighted herein may be utilized by others to avoid time consuming optimization studies as they provide an indication of which factors are important in order to maximize TP extraction and C^* levels. This MAE methodology, employing industrial microwave reactor, may be relevant for the future studies on phenolics and colour extraction from red and white pomace, followed by industrial scale trials, to recover phenolics and colour of commercial values. Following Circular

Economy and to reduce landfill, it may be relevant for future studies on value-adding from red and white pomace on industrial scale to employ the MAE methodology, which is more energy and process-time efficient and can recover phenolics and colour of commercial value.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jclepro.2019.119563.

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Supplementary data

Response surface parallel optimization of extraction of total phenolics from separate white and red grape skin mixtures with microwave-assisted and conventional thermal methods

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Appendix A

A.1. Skin *sample preparation*

All grapes were *Vitis vinifera* cultivars from commercial South Australian vineyards. Preliminary screening was performed on the skins of Chardonnay grapes picked at harvest and Shiraz grapes picked at veraison from Longview vineyard, 2009. Advanced screening, optimization and validation studies were conducted on skin mixtures separately of white and red grapes sourced in 2011 from C. A. Henschke & Co. The white skin mixture was prepared from Chardonnay, Riesling, Sauvignon Blanc, Gewürztraminer, Viognier and Semillon. The red skin mixture was prepared from Shiraz, Cabernet Sauvignon, Grenache, Nebbiolo, Merlot and Pinot Noir. Approximately 20 bunches of grapes of each cultivar were picked randomly from the vines of the same row, at veraison and at harvest. All grapes were packed in freezer plastic bags and stored at -20 ± 1 ^{o}C prior to skin preparation.

For each cultivar at veraison and harvest, grape collections contained 150 to 500 frozen berries randomly selected in terms of berry size. These quantities provided approximately 50 g of skins for each time point. Each collection maintained the same ratio of berries of different colours, as observed in the grape bunches. The skins were manually removed from the frozen grape berries using a scalpel blade and frozen in liquid nitrogen as quickly as possible to minimize air and light exposure. The skins were placed into plastic freezer bags, and secured tightly with wire, and their weight was recorded to ensure that 50 g of skins were collected. The bags were then placed in a freezer (-80 ± 1 ºC) prior to extraction.

The skin mixtures from the white and red grapes were then separately prepared for extraction. An amount of 50 g of frozen skins from the contributing cultivars, each collected at veraison and harvest, were put into a plastic box, mixed thoroughly, and kept frozen (-80 ± 1 ºC) prior to grinding. This mixture was used to prepare two portions of skin powder for extraction. A portion of 5 g of frozen skins were scooped from the box using a 50 mL Falcon tube chilled in liquid nitrogen, making sure that skins of all colours were taken. The skins were ground in 1 g portions using a grinder with a chilled stainless-steel chamber. Portions were ground applying the pulse mode 6 times for 10 sec. Ground skins were transferred to a new chilled 50 mL Falcon tube. Capped tubes were put on dry ice in a Styrofoam box. This process was repeated, providing two falcon tubes of skin powder, from which each sample for extraction was prepared by taking half of the weight of skin powder required by DoE from each tube.

A.2. Chemicals

Undenatured ethanol 100% of analytical grade, sourced from Chem-Supply, South Australia, and Milli-Q-water from Synergy® Water Purification Systems (Merck Millipore, USA) were used for solvent preparation prior to extraction. Folin-Ciocalteu phenol reagent (FC reagent) was from Merck and sodium carbonate and all standards were from Sigma-Aldrich.

A.3. Determination of total phenolics utilizing cuvettes

The Folin-Ciocalteu (FC) method modified by Waterhouse (2001) was employed to measure the total phenolics (TP) of all extracts obtained using the MAE and CTE methods during preliminary and advanced screenings. A Cintra 40 UV-Visible spectrophotometer (GBC Scientific Equipment Pty Ltd, Australia) and 10 mm acrylic cuvettes were employed. To create the calibration curve for the TP calculation (expressed as gram of gallic acid equivalent per litre (g GAE/L)), the absorbance of gallic acid standard solutions (0 up to 2.5 g/L, with 10% ethanol aqueous solutions) was measured at 765 nm. A 10% ethanol aqueous solution of catechin concentration 1 g/L was analyzed to monitor the method's performance during each analysis. All measurements were performed in triplicate.

Reference: Waterhouse, A.L. (2001). Determination of total phenolics. In R.E. Wrolstad (Ed.), Current protocols in food analytics chemistry. New York: John Wiley & Sons Inc. (pp. I1.1.1-I1.1.8).

A.4. High throughput determination of total phenolics utilizing 96-well plates and a robot

To increase the throughput of the TP analyses, the FC method in a cuvette (Waterhouse, 2001) was adapted for a 96-well plate with manual liquid delivery. All FC solutions were prepared

as for the FC method in a cuvette. To ensure that the method in a 96-well plate provides the same results as the method in a cuvette, the volumes of 3 μL and 4 μL of sample/standard/blank were separately used. The chosen volume of sample/standard/blank was pipetted into the well cavity of a 96-well polystyrene plate (Costar 3596, Corning Inc., USA), and then 240 μL of Milli-Q water was added, followed by 15 μL of undiluted FC reagent. The plate covered with a lid was put into a black box (to keep away from light). The box was placed on a plate shaker and shaken for 5-8 min at 100 rpm at room temperature. Next, 45 μL of aqueous sodium carbonate solution 200 g/L was pipetted into each well and shaken for 5 min. The plate was then kept away from light for an incubation period of two hours, after which the absorbance at 765 nm was measured using a μQuant universal plate reader (Bio-Tek Instruments, USA).

The absorbances at 765 nm for the sample/standard/blank mixtures (3 μL or 4 μL) were compared to those in the cuvettes determined above. It was found that for the 4 μL of sample/standard/blank mixtures the recovery of the method was close to 100%, while for the 3 μL mixtures, it was only 79%. Therefore, a volume of 4 μL was chosen for all future analyses. Linear regression indicated a very good agreement (average $R^2 = 0.990 \pm 0.004$) between the method in a 96-well plate and that in a cuvette as determined for six different plates with white and red skin extracts and gallic acid solutions of different concentrations. Limit of Detection and Limit of Quantification were calculated as 0.007 g GAE/L and 0.024 g GAE/L, respectively. The TP calibration curve was constructed using the same standard solutions as for the method in a cuvette. All measurements were performed in duplicate.

To further increase the throughput of the TP analyses, the above method in a 96 well-plate was adapted for robotic manipulation (Corbett Robotic Systems Pty Ltd., Australia), which enabled multiple volume solution delivery through the robot's 8-channel pipette. Initially, the FC

reagent was prepared in bulk (depending on the number of analyses) by mixing 15 μL of the FC reagent and 240 μL of Milli-Q water per analysis. The volume 255 μL of the diluted FC reagent was delivered to each well, followed by 4 μL of the sample/standard/blank mixtures. After 6 min incubation, 45 μL of aqueous sodium carbonate solution (200 g/L) was added into each well and the solutions mixed utilising an 8-channel pipette of the robot. The plate was then removed from the robot, covered by a lid and kept away from light for the incubation period of two hours. Finally, the absorbance reading at 765 nm was measured using a plate reader (Tecan Infinite ™ 200 PRO Series, Switzerland). It should be noted that the full compatibility (100%) between the Tecan and μQuant plate readers was first established.

In addition, the validation method was further examined as for the method in a 96-well plate without the robot, except for employing eight different plates. Again, a very good agreement was found between the 96-well plate with the robot and the cuvette methods (average R^2 = 0.999 ± 0.000). The Limit of Detection and Limit of Quantification were calculated as 0.004 g GAE/L and 0.014 g GAE/L, respectively. The calibration curve for the TP calculation was constructed using the same solutions as for the method in a cuvette. The measurements were also performed in duplicate. This final, high throughput method was then employed for all advanced screening, optimization and validation studies.

A.5. Determination of chroma

CIELAB *L*, a*, b** were used to represent the colour of skin extracts. Chroma (*C**) was calculated as the squared root of $(a^*)^2 + (b^*)^2$, where *a** (redness/greenness) and *b** (yellowness/blueness) were measured using a research grade spectrophotometer (absorbance up to 5 a.u.) Cintra 40 (GBC Scientific Equipment Ltd, Australia) and employing the CIELAB

tristimulus protocol (ASTM, 2000; Ohno, 2000). The extracts were scanned in the range 380 - 780 nm at the daylight illuminant D65, 10-degree observer angle, and using Milli-Q water as a blank. Acrylic cuvettes (10 mm) were used for the white skin extracts, and a 1 mm quartz cuvette for the red skin extracts. A holmium filter (AZZOTA Corporation, USA) was employed for checking the spectrophotometer's performance (Allen, 2007). All measurements were performed in triplicate.

A.6. Determination of total soluble solids and pH

A digital refractometer was utilized to measure the total soluble solids (ºBrix) of the juice samples corresponding to the grape samples of which the skins were prepared for the MAE and CTE methods. The soluble solids of juice were: 9-14 ºBrix at veraison and 14-19 ºBrix at harvest for the white cultivars, and 7-14 ºBrix at veraison and 19-25 ºBrix at harvest for the red cultivars. Prior to extraction, the pH of each sample was checked and adjusted to the pH as specified by the DoE.

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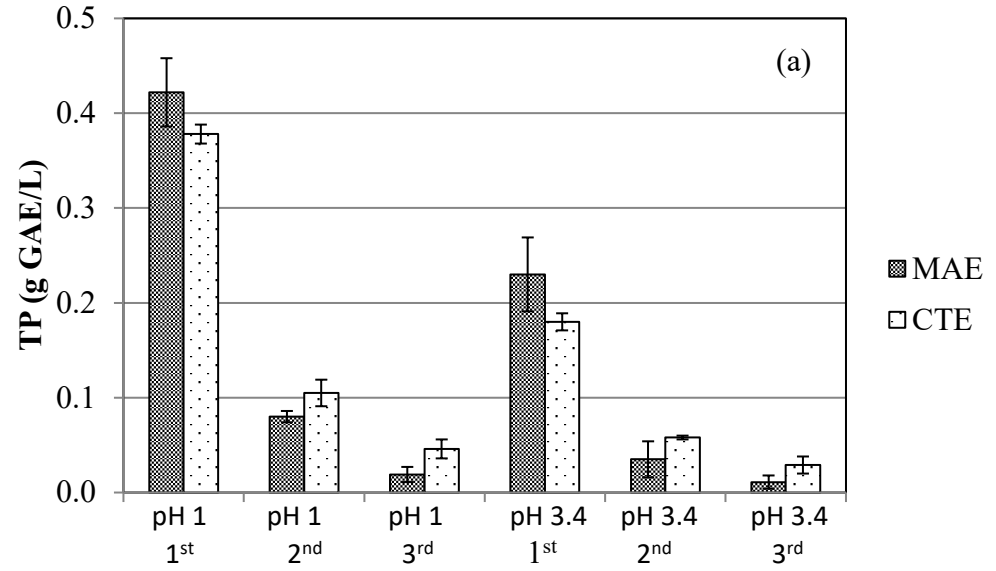
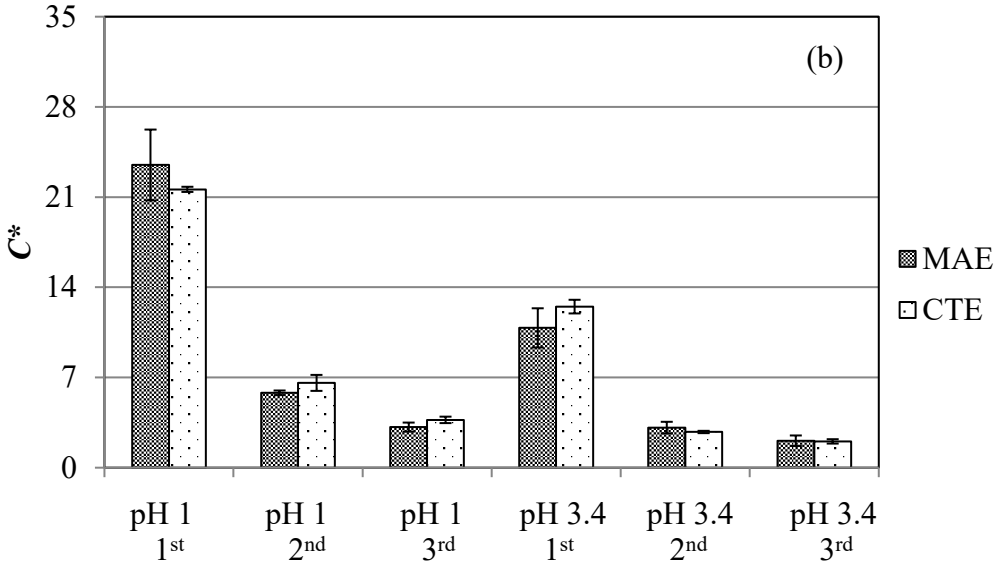
Chapter 2

Appendix B





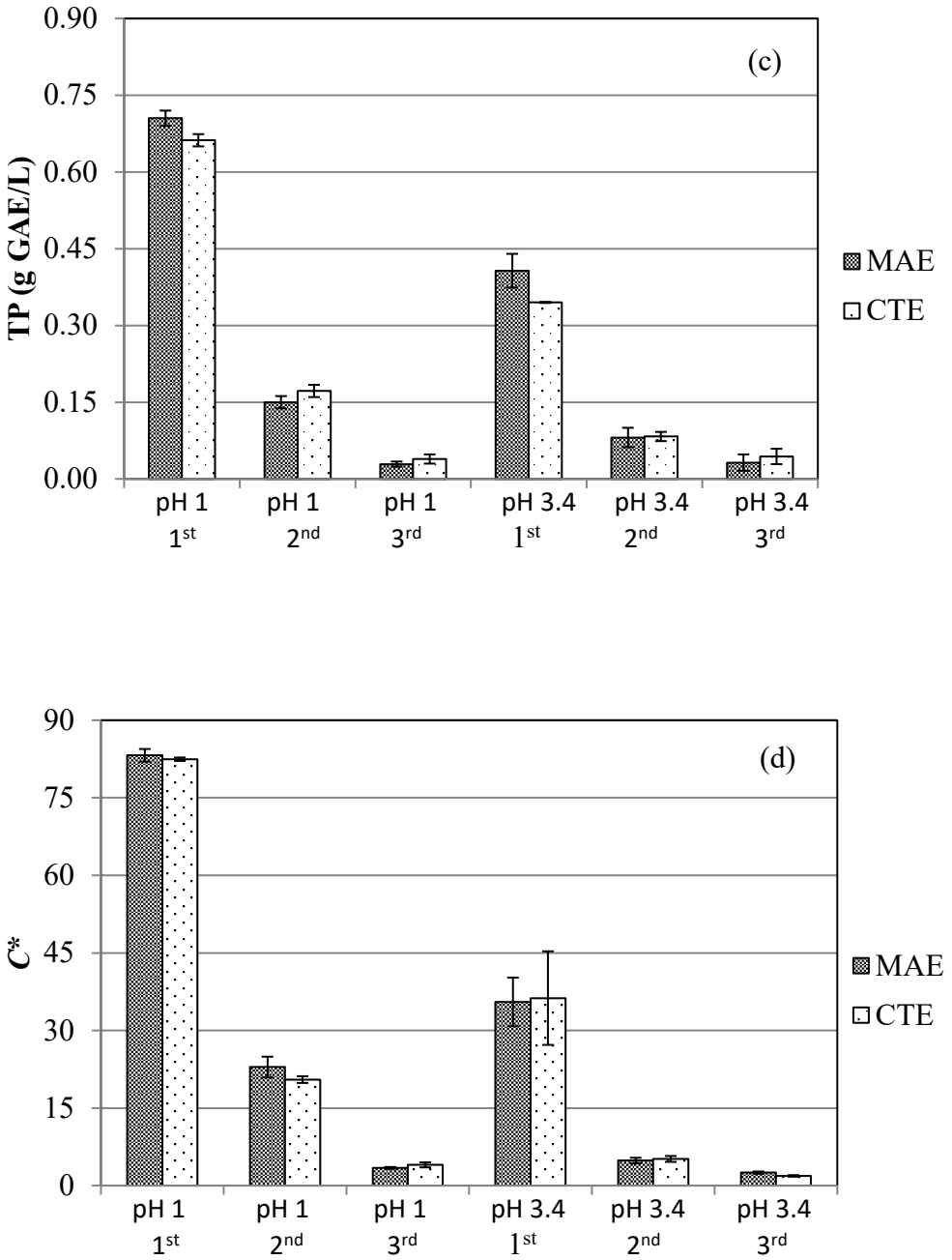


Fig. B.1. Preliminary screening for total phenolics (TP) (a) and chroma (*C**) (b) of Chardonnay skins (harvest) obtained over three consecutive extractions (1st, 2nd and 3rd). Preliminary screening for total phenolics (TP) (c) and chroma (*C**) (d) of Shiraz skins (veraison) obtained over three consecutive extractions (1st, 2nd and 3rd). All extractions employed the conditions shown in Table 1 for MAE and CTE methods. Experimental values: mean ± SD (n = 3).

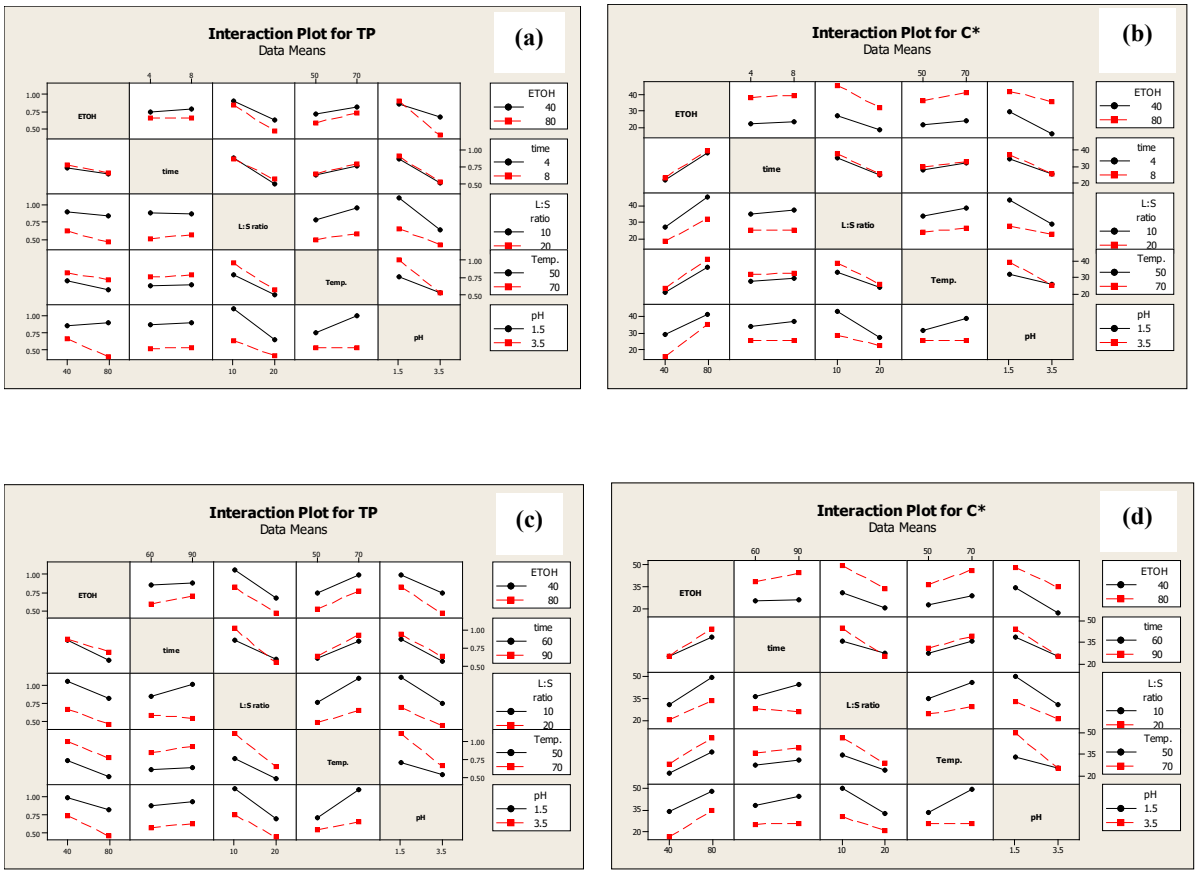


Fig. B.2. Interaction plots in advanced screening for total phenolics (TP) and chroma (*C**) of the white skin mixture obtained using MAE (a and b) and CTE (c and d) methods. All extractions employed the conditions shown in Table 2 for MAE and CTE methods. Experimental values: mean ± SD (n = 3).

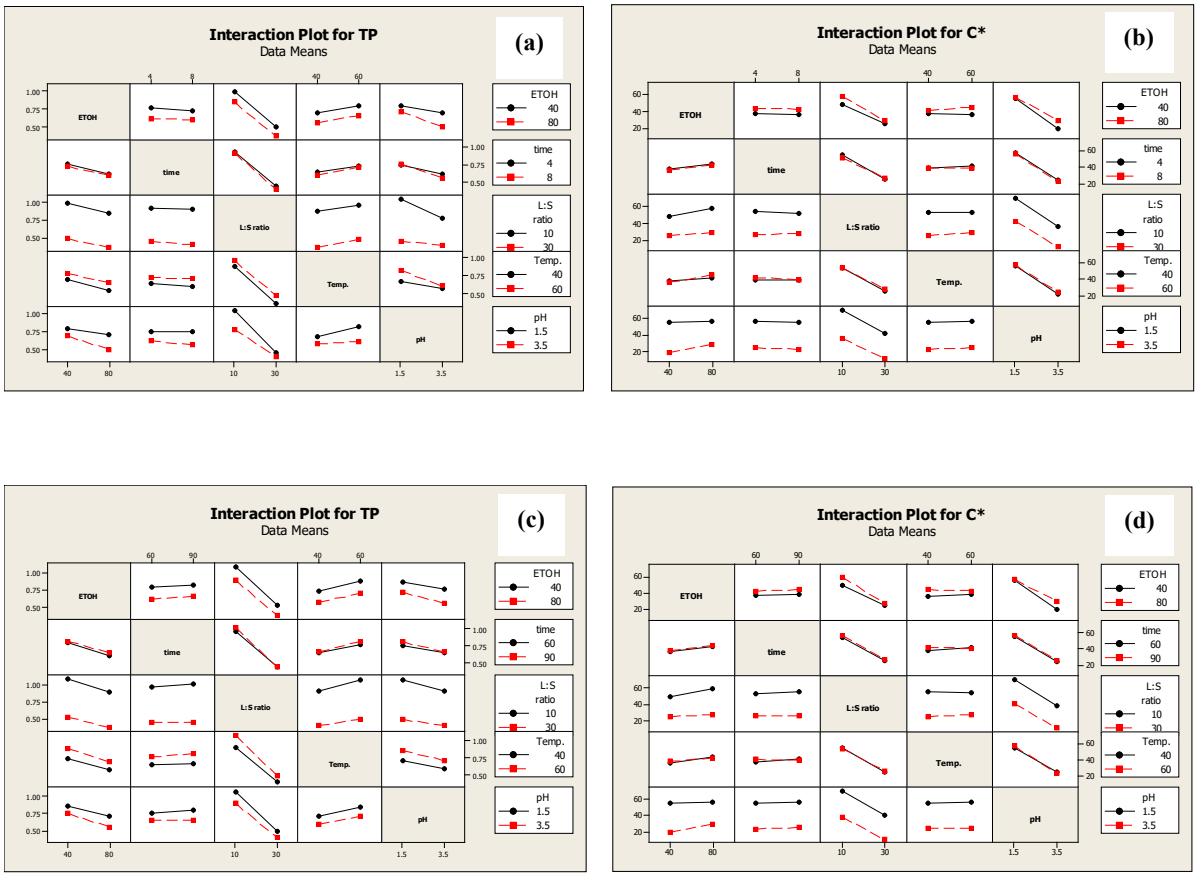
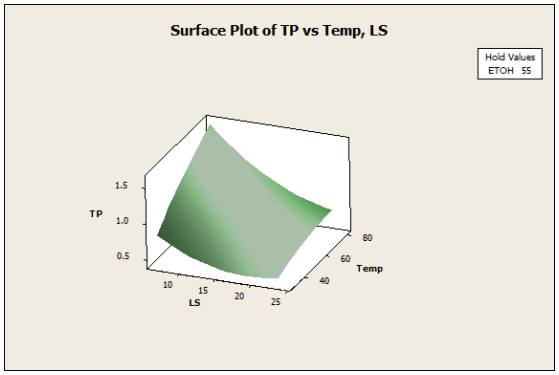
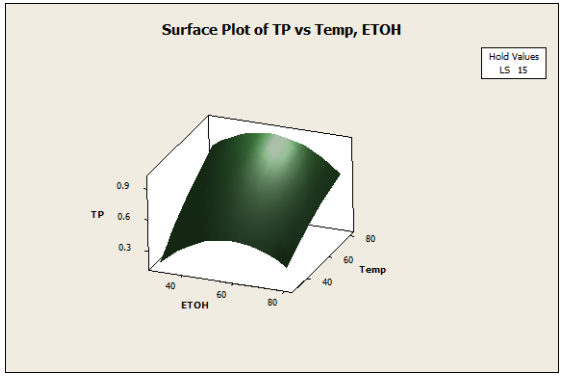
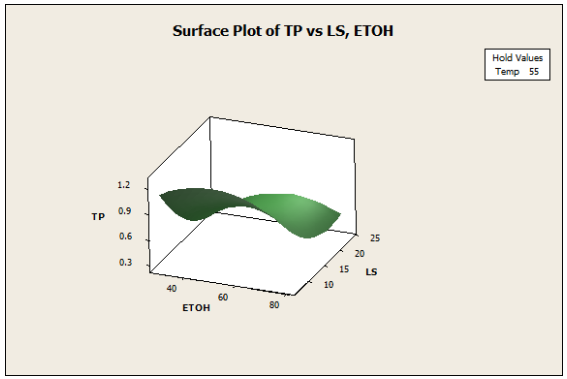


Fig. B.3. Interaction plots in advanced screening for total phenolics (TP) and chroma (*C**) of the red skin mixture obtained using MAE (a and b) and CTE (c and d) methods. All extractions employed the conditions shown in Table 2 for MAE and CTE methods. Experimental values: mean ± SD (n = 3).

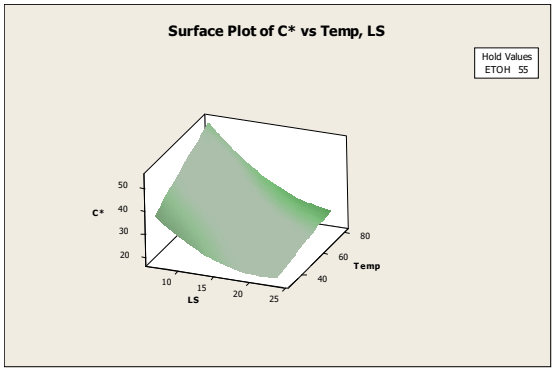
(a)

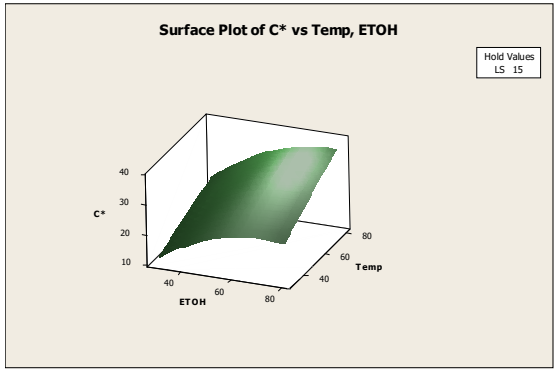

Surface Plot of TP vs Temp, LS
Hold Values
ETOH 55
TP
1.5
1.0
0.5
LS
10
15
20
25
Temp
40
60
80

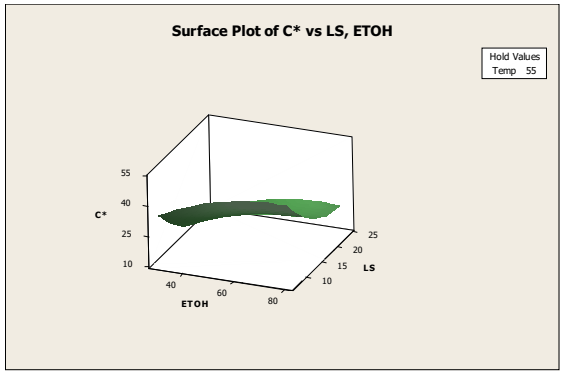

Surface Plot of TP vs Temp, ETOH
Hold Values
LS 15
TP
0.9
0.6
0.3
ETOH
40
60
80
Temp
40
60
80


Surface Plot of TP vs LS, ETOH
Hold Values
Temp 55
TP
1.2
0.9
0.6
0.3
ETOH
40
60
80
LS
10
15
20
25

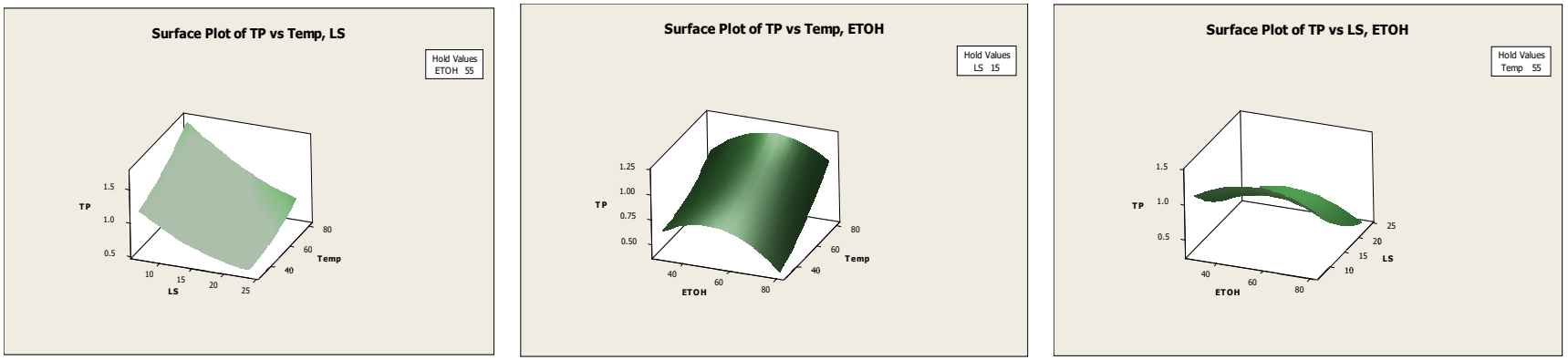
(b)


Surface Plot of C* vs Temp, LS
Hold Values
ETOH 55
C*
50
40
30
20
LS
10
15
20
25
Temp
40
60
80


Surface Plot of C* vs Temp, ETOH
Hold Values
LS 15
C*
40
30
20
10
ETOH
40
60
80
Temp
40
60
80


Surface Plot of C* vs LS, ETOH
Hold Values
Temp 55
C*
55
40
25
10
ETOH
40
60
80
LS
10
15
20
25

(c)



(d)

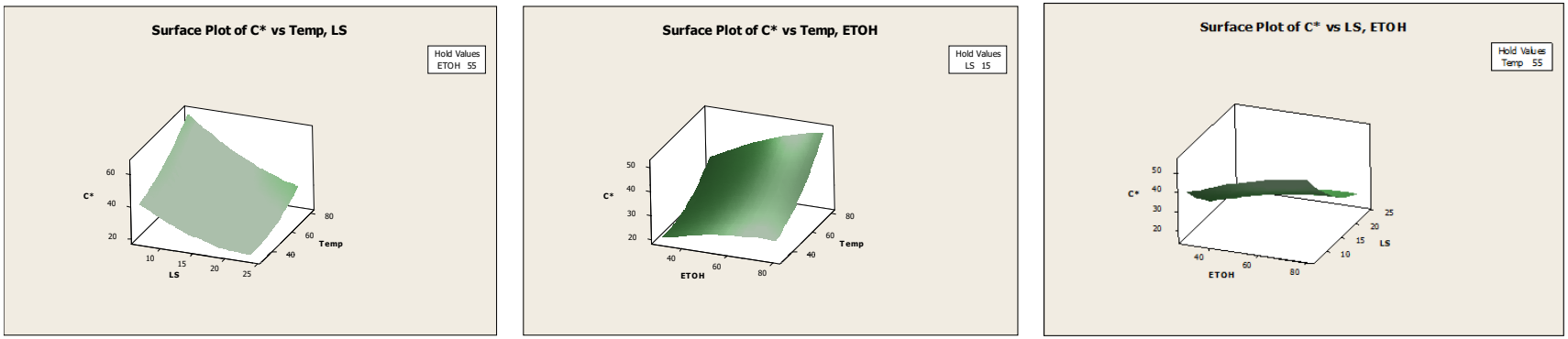
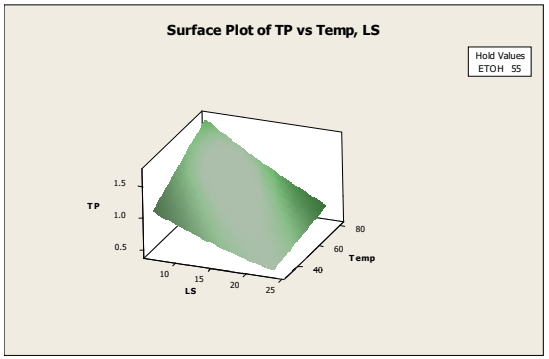
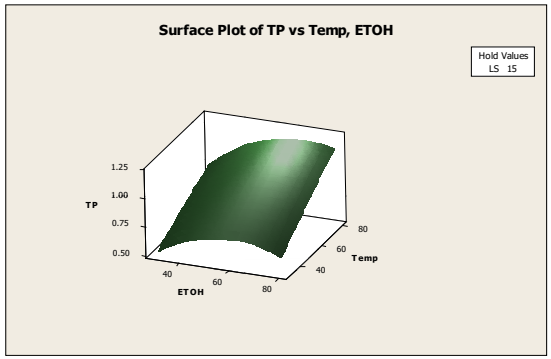


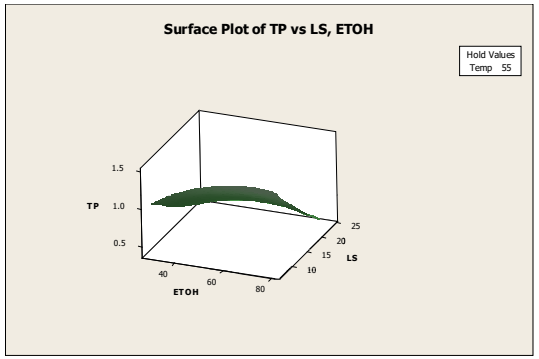
Fig. B.4. Response surface curves for: total phenolics (TP) and chroma (*C**) of the white skin mixture (six cultivars) obtained using (a and b) MAE and (c and d) CTE methods.

Chapter 2

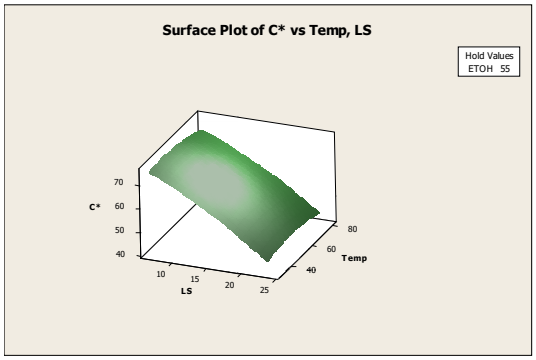
(a)

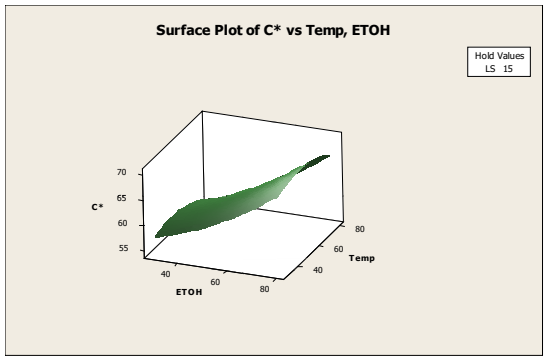

Surface Plot of TP vs Temp, LS
Hold Values
ETOH 55
TP
LS
Temp

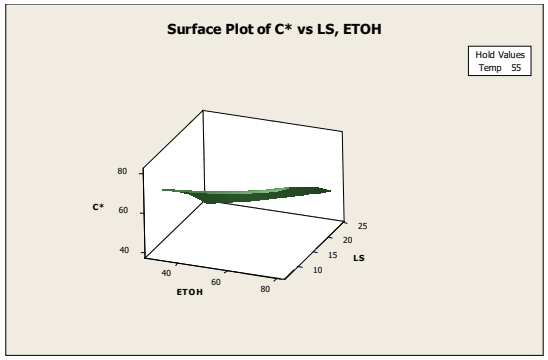

Surface Plot of TP vs Temp, ETOH
Hold Values
LS 15
TP
ETOH
Temp


Surface Plot of TP vs LS, ETOH
Hold Values
Temp 55
TP
ETOH
LS

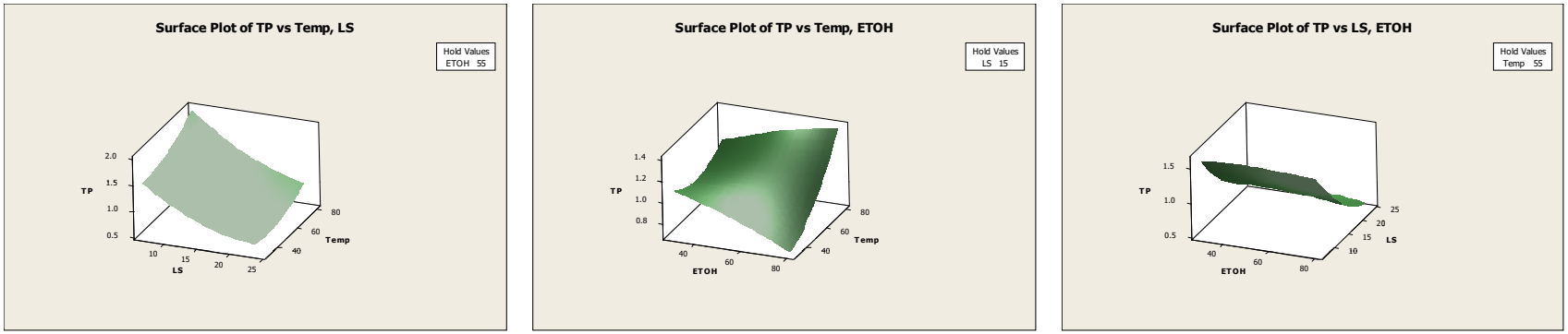
(b)


Surface Plot of C* vs Temp, LS
Hold Values
ETOH 55
C*
LS
Temp


Surface Plot of C* vs Temp, ETOH
Hold Values
LS 15
C*
ETOH
Temp


Surface Plot of C* vs LS, ETOH
Hold Values
Temp 55
C*
ETOH
LS

(c)



(d)

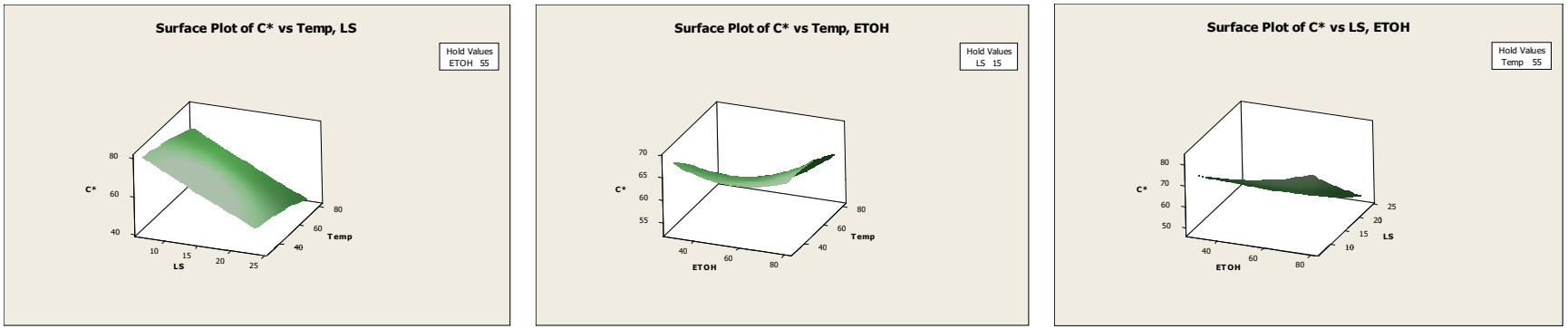


Fig. B.5. Response surface curves for: total phenolics (TP) and chroma (*C**) of the red skin mixture (six cultivars) obtained using (a and b) MAE and (c and d) CTE methods.

Chapter 3

Total Phenolics and Colour in Grape Skin Extracts of Commercial White and Red Cultivars at Veraison and Harvest Under Microwave-Assisted and Conventional Thermal Extraction Methods Optimized for White and Red Grape Skin Mixtures

Grape skin extracts are the valuable source of phenolics and can be used in manufacturing of functional foods, nutraceuticals including food colourants, skincare or textile dyes. Hence, particular attention is paid to find efficient as well as "green and clean" extraction methods. The extraction methods can be developed for extracting both phenolics and colour as we showed in the first study, in which practical conditions were found for microwave-assisted and conventional methods to extract efficiently phenolics and colour from the white and red grape skin mixtures. It was obvious for us to apply those methods in extracting phenolics and colour from skins of the individual *Vitis vinifera* L. cv. cultivars at veraison and at harvest. We were also interested which cultivar, at which time-point of berry development and which extraction method could deliver the highest TP as well as C^* for both skin colours.

In our previous study we showed how laborious was the MAE and CTE extraction methods development and their optimisation towards maximising TP and C^* separately for the white and red skin mixtures. In this study we demonstrated that utilizing the MAE and CTE methods at the practical conditions from the previous study allowed us to save time and resources. That approach was a trade-off to find the answers to our questions in this study. We were also

interested in comparing the average TP and *C** in skin extracts of the individual cultivars at veraison and harvest with the average TP and *C** in the skin mixture extracts.

A balanced unreplicated 3-factor ANOVA model was used for each skin colour separately, 2 (method)x6(cultivar)x2(time-points of berry development), of the 24 experimental treatments in performed in triplicate. Hence, the high throughput method for TP developed in the previous study was utilized herein. The TP and *C** results showed the variability among the cultivars with regard to the extraction method used as well as time-point of berry development. In general, our findings in this study were in a good agreement with the findings from previous study. For white skins, the CTE method was statistically ($p < 0.01$) more efficient than MAE in TP and *C** extraction (if veraison and harvest were combined), and for the red skins, the method effect was not significant for TP and for *C** (except for C* for Shiraz under CTE). If Grenache was excluded, all TP and C* results (at harvest) were well aligned along with TP and C* results of the red skin mixture (veraison and harvest combined); for white cultivars the alignment was more cultivar dependent. We demonstrated that MAE was the method of choice as well as we pointed out the potential value-returning cultivars to obtain maximum phenolics and colour from white and red skins.

To the best of our knowledge, our study shows the first report on comparison of the results of TP and *C** of the skin extracts of individual six white and six red grape cultivars collected separately at veraison and harvest over one vintage when utilizing the MAE and CTE RS-optimised for maximum TP and *C** methods of the white and red skin mixtures (veraison and harvest combined). Presented in the following publication is the first detailed investigation on comparison of the total phenolic content and colour of extracts obtained using response surface

optimised microwave-assisted and conventional extraction methods from skins of white and red *Vitis vinifera* L. cv. cultivars collected at veraison and harvest and has been submitted to the *Journal of Cleaner Production* (23 Feb. 2020) as a sequel to the first study (Chapter 2).

Statement of Authorship

Title of Paper	Total phenolics and colour in grape skin extracts of commercial white and red cultivars at veraison and harvest under microwave-assisted and conventional thermal extraction methods optimized for white and red grape skin mixtures
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Mariola Kwiatkowski , Olena Kravchuk, George K. Skouroumounis, Dennis K. Taylor, Total phenolics and colour in grape skin extracts of commercial white and red cultivars at veraison and harvest under microwave-assisted and conventional thermal extraction methods optimized for white and red grape skin mixtures, Journal of Cleaner Production

Principal Author

Name of Principal Author (Candidate)	Mariola Kwiatkowski (note: the publishing name, Mariola, is different to the full name, Maria Jolanta)
Contribution to the Paper	Undertook the literature review. Conducted the microwave-assisted and conventional thermal extractions of phenolics and colour from the skins of selected six white and six red commercial cultivars collected at veraison and harvest utilizing both extraction methods at the optimal practical conditions found for the white and red skin mixtures of those cultivars in the previous study by Kwiatkowski et al. published in the Journal of Cleaner Production (online 5 Dec. 2019). Collated the data and performed the multivariate data analyses to answer the research questions. Prepared the comprehensive draft of the manuscript and implemented all the corrections and editions suggested by other co-authors. Mariola is the corresponding author for this manuscript.
Overall percentage (%)	80%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 19/03/20

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Olena Kravchuk
Contribution to the Paper	Olena provided the guidance in the statistical analysis of experiments and the use of the statistical software, as well as assistance in the interpretation and presentation of results, general contribution to the manuscript preparation and editing.
Signature	Date 19/03/2020

Name of Co-Author	George K. Skouroumounis

Contribution to the Paper	George provided technical advice during the laboratory work, revised and commented on the final version of the manuscript for the final submission.		
Signature		Date	17/3/20

Name of Co-Author	Dennis K. Taylor		
Contribution to the Paper	The principal supervision of Mariola, provided the guidance with the concept of the experimental work and research questions, discussed the initial ideas and the relevance to the food and waste management industries, extensively commented on all versions of the manuscript, as well as on the choice of the journal for the final submission.		
Signature		Date	29/12/2019

Please cut and paste additional co-author panels here as required.

Microwave-assisted extraction (MAE)

Chardonnay, Riesling, Sauvignon Blanc, Gewürztraminer, Viognier, Semillon

Total phenolics and *colour* extraction from skins of six white and six red commercial grape cultivars collected at veraison and harvest, utilizing MAE and CTE optimized separately for the white and red skin mixtures

Comparable efficiencies for MAE and CTE

MAE "greener and cleaner" than CTE

Shiraz, Cabernet Sauvignon, Grenache, Nebbiolo, Merlot, Pinot Noir

Conventional thermal extraction (CTE)

Chapter 3

Total Phenolics and Colour in Grape Skin Extracts of Commercial White and Red Cultivars at Veraison and Harvest Under Microwave-Assisted and Conventional Thermal Extraction Methods Optimized for White and Red Grape Skin Mixtures

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ABSTRACT

Grape berry skins from six white and six red commercial Australian cultivars were collected at veraison and harvest over a single vintage. Phenolics and colourants were extracted from the skins of individual cultivars under a microwave-assisted (MAE) and conventional thermal extraction (CTE) methods. The settings for each method were fixed at levels maximising their total phenolic (TP) extraction from the mixture of the white skins at veraison and harvest and, unconnectedly, from the mixture of the red skins at veraison and harvest, of the cultivars used in the current study. For white and red cultivars, the current study quantifies the variation in TP and colour (CIELAB chroma *C**), which can be attributed to the joint effects of method of extraction, cultivar and time of berry development. The efficiencies of extraction methods are compared for each cultivar. For each method, the discrepancies between the average TP content achieved across corresponding individual skin collections and the TP extractions from their mixtures are investigated. The results are of interest to researchers in phenolics extraction, oenology and industrial post-winemaking waste management.

Keywords:

Chapter 3

Response surface

Pomace

By-product

Valorization

Footnote

Abbreviations: TP, total phenolics; *C**, CIELAB chroma; GS, grape skins; BW, berry weight; MAE, microwave-assisted extraction; CTE, conventional thermal extraction; RS, response surface; OIV, Organisation Internationale de la Vigne et du Vin.

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Chapter 3

1. Introduction

The interest of wine industry in phenolics and coloured compounds in grapes has sparked in application to wine production and the valorization of pomace/marc (non-fermented waste/post-fermentation waste) and waste by-products. While wine consumption has been decreasing in mature markets, a strong growth is shown in developing countries (Wine Australia, 2019). In Australia, in 2018, 861,848 tonnes of white grapes were crushed for winemaking, including Chardonnay (47%), Sauvignon Blanc (11%), Semillon (7%), Riesling (3%) and Gewürztraminer (2%). For the red cultivars, 932,334 tonnes of grapes were crushed, including Shiraz (46%), Cabernet Sauvignon (27%), Merlot (11%), Pinot Noir (6%) and Grenache (2%) (Wine Australia, 2018). Consumer choices drive the modern wine market domestically and internationally, and are taken proactively by grape growers and winemakers (Iland and Gago, 2002) who create red and white wines with adjusted flavour and mouthfeel profiles as well as blending red and white cultivars in winemaking (Iland and Gago, 2002). Continuing research in grape phenolics is considered by the industry a necessary element of the current market success (Kennedy et al., 2002).

In grapes, phenolics are mostly found in seeds and skins. The skin, constituting 10-20 % of the berry weight at maturity (Margalit, 2004; Singleton and Esau, 1969), contains approximately 25% (white) and 50% (red) phenolics (Singleton and Esau, 1969). Skins can be separated from post-winemaking waste (pomace - non-fermented waste, marc - post-fermentation waste; hence white marc is pomace), which contains skin and seeds (occasionally stems); marc accounts for 10%-30% of the mass of crushed grapes (Muhlack et al., 2018). It has been shown that pomace can retain up to 70% of phenolics for some cultivars (Ratnasooriya and Rupasinghe, 2012). Skins can be utilised for producing non-wine products rich in phenolics,

like grape skin extract; Kwiatkowski et al. (2020a) presented many examples of other skin products. As Dwyer et al. (2014) concluded, there has been a significant market potential for pomace by-products, including colourants and phenolic products.

The total phenolic (TP) content in grape skins varies depending on type of cultivar, time of berry development, region and viticultural practices for white and red cultivars (Margalit, 2004). Higher TP content per berry weight is observed at veraison (Iland et al., 2011; Kennedy, 2002). Anthocyanin content, responsible for the colour of red skins, is also cultivar and viticulture dependent, and increases from veraison to harvest for red cultivars; that change in the content of compounds responsible for the colour of white skins is not so well understood for white cultivars (Iland et al., 2011; Kennedy, 2002), although it can be seen clearly that white cultivars change from green at veraison to translucent yellow colour at harvest. The compounds responsible for red grape colour, and presumably for white grape colour, are present only in skins.

The wine and nutraceuticals industries are particularly interested in developing and optimizing novel, efficient and environmentally friendly extraction methods for bioactive compounds. A variety of methods exists for extracting phenolics from grape skins. Most traditional methods, easily accessible for the industry, are conventional thermal (CTE) and, more recent, microwave-assisted extraction (MAE) (Kwiatkowski et al., 2020a; García-Lomillo and González-SanJosé, 2017). The latter has received a substantial attention for its fast and "green and clean" performances. In Table A1 (Appendix A, Supplementary data), a selection of publications is presented to highlight a multitude of factors controlled in CTE and MAE for extracting phenolics and colour from skins harvested directly as well as separated from pomace or marc A wide range of cultivars from many countries is presented in Table A1, some papers

reporting the extractions separately at harvest and at veraison. There is a large variation in the extraction process parameters. For example, ethanol or methanol can be used at various concentrations, the temperature of extraction can vary greatly: 4-60 ºC in CTE and 50-175 ºC in MAE; the acidification part varies largely, as well as the extraction time. The TP content is generally quantified by using the Folin-Ciocalteu (FC) method and anthocyanins are quantified by the spectral or HPLC analyses.

It is difficult to compare the extraction efficiency across multiple studies, as the process settings vary greatly, only a few performed response surface (RS) optimization of their extraction process, and in many cases the space of process parameters reported is not complete. Pomace/marc phenolic extraction methods (Table A1) aim to maximise the TP extraction, although there is a concurrent interest in colour compounds, which, being predominantly phenolics, can encompass carotenoids or chlorophylls (Keller, 2010 (cited in Iland et al., 2011)). Spectral methods are employed for colour measurements. A method, developed by Ribéreau-Gayon et al. (1983) for measuring the wine colour intensity for young red wines, and recommended by Organisation Internationale de la Vigne et du Vin (OIV) to the wine industry (OIV 2018a), is laborious, requires diluting samples, and measuring the absorbance at 420 nm, 520 nm and 620 nm for calculating the colour index. As an example, Amendola et al. (2010) utilized that method for the colour measurement of the extracts obtained from the red marc, and de la Cerda-Carrasco et al. (2014) of the extracts from the whole white and red pomaces.

CIELAB is routinely used in wine industry for measuring wine colour (OIV, 2018b); it is a simple spectral method which does not require diluting samples. Liang et a. (2011) considered skin extracts from ripe berries of 57 red and 21 white cultivars, and demonstrated that the anthocyanin content (determined by HPLC-MS) in red skin extracts correlated well with the

CIELAB coordinates of skins measured in the middle part of the upper epidermis of berry; the same was not observed for white cultivars.

Amongst the white and red cultivars, colours vary from green to golden or deep red, aromatic and non-aromatic cultivars. A wide range of total soluble solids (TSS) at veraison and harvest, different number of seeds per berry and different berry sizes as well as the skin weight fractions (Tian et al., 2019; Dokoozlian and Kliewer, 1996). One can expect the efficiency of extraction methods to be cultivar specific under the same settings, or that different settings (MAE or CTE) are required for different cultivars to maximise TP and colour in skin extracts.

The extraction methods can be tuned for extracting both TP and colour. Kuck and Noreña (2016) extracted phenolics and colour (expressed by CIELAB) from the skins of Brazilian red cultivar using a CTE method, not specifically optimized. Kwiatkowski et al. (2020a) demonstrated that maximal colour (measured by CIELAB chroma C^*) was obtained for grape skin extracts from the skin mixtures of Australian commercial red or white cultivars by either MAE or CTE methods RS-optimized for maximal TP.

Employing the RS-methodology is becoming best practice in the research on plant phenolics extraction (Kwiatkowski et al., 2020a; Casazza et al. 2012; Pinelo, et al. 2005). It is not well understood how much variation would be expected for the optimal parameters for TP extraction, with the standard equipment and reagents, from grape skins of various origins, in particular, varietal and seasonal.

Herein, one-vintage individual cultivar effects were investigated on grape skin extractions conducted under the MAE and CTE RS-optimized methods for extracting maximal TP from

the skin mixtures. All six white and six red cultivars were cool-climate grown in the South Australia wine regions - five out of six cultivars listed among the Australian most processed ten cultivars (Wine Australia, 2018). The skins of the same cultivars were studied previously by the authors as mixtures (Kwiatkowski et al. 2020a). The following aims were set for this study: 1) assess TP and *C** extracted individually from the skins of the six white and six red cultivars collected separately at veraison and harvest, by utilizing the MAE and CTE methods RS-optimized for maximal TP for the skins mixtures (veraison and harvest combined) from the previous study (Kwiatkowski et al., 2020a); 2) compare the efficiency of MAE and CTE to obtain maximal TP for the commercial cultivars chosen; 3) compare the average TP and *C** in the skin extracts across individual cultivars at veraison and harvest separately with the average TP and *C** for the inclusive white and red skin mixture extracts.

2. Materials and methods

Full details about sample preparation, chemicals, extraction, the RS-optimization of extraction settings and analytical methods can be found in Kwiatkowski et al. (2020a); a brief description is presented below for convenience.

2.1. Skin sample preparation

Six white (Chardonnay, Riesling, Sauvignon Blanc, Semillon, Gewürztraminer and Viognier) and six red (Shiraz, Cabernet Sauvignon, Merlot, Pinot Noir, Grenache and Nebbiolo) *Vitis vinifera L.*cv. grape cultivars were chosen from the South Australian cool climate commercial vineyards (C.A. Henschke & Co.) and collected at veraison and harvest in 2011. Berries were kept for two years at -20 ^{o}C in sealed plastic bags and closed plastic boxes in a freezer for the

purposes of the project reported by Kwiatkowski et al. (2020a) and this study. As required, skins were peeled off, frozen in liquid nitrogen and kept at -80 ºC. Kwiatkowski et al. (2020a) used part of the berries collected to prepare separately the white and red skin mixtures in June, 2012, using 50g of the skins of each cultivar at veraison and 50g at harvest. The remaining berries were peeled for this study in October, 2013; about 4.5g of skins at veraison and 4.5g of skins at harvest prepared separately for each cultivar for one extraction method. A summary of the grapes used for this study is presented in Table A2 (Appendix A, Supplementary data).

2.2. Extraction methods

The extraction of phenolics and colour from the white and red skins of individual cultivars collected at veraison and harvest was done under the MAE and CTE methods, and RS-optimized by Kwiatkowski et al. (2020a) to achieve maximal TP separately for the white and red skin mixtures. Herein, the protocols of extraction were streamlined with the protocols at the validation phase in Kwiatkowski et al. (2020a) to minimize the method-to-method experimental error. In brief, for each extract a portion of ground skins (1.5 g) was placed into a 35 ml Pyrex glass vessel and 10 mL aqueous ethanol was added: 60% v/v for the white cultivars or 70% v/v for the reds. The pH was adjusted to pH 1.5 with 3-5 drops of HCl. This provided a liquid to solid (LS) ratio of 6.59 mL/g (GS). The vessel was tightly capped.

For MAE, a single vessel was placed into a chamber of an industrial microwave reactor CEM Discover S-Class (CEM Co., USA), coupled with a Maxus WX801700 air compressor (Campbell Hausfeld, USA) to control temperature (± 0.1 ºC) of 70 ºC (whites) or 60 ºC (reds) over 4 minutes. The extractions were performed in triplicates.

For CTE, up to 9 vessels with samples were placed at once in a plastic rack immersed into a water bath (Ratek Instruments Pty Ltd, Australia) with controlled temperature (± 0.1 ºC), and heated at 70 ºC (whites) or 60 ºC (reds) over 60 minutes. During extraction the water bath was covered with a black box. Three replicates of each cultivar-by-time of development were extracted at once.

2.3. *Analytical methods for TP and colour*

The TP content in skin extracts was measured in duplicates using a high throughput Folin-Ciocalteu method developed by Kwiatkowski et al. (2020a). TP is expressed as gram of gallic acid equivalent per litre (g GAE/L).

As in Kwiatkowski et al. (2020a), the CIELAB chroma $C^* = [(a^*)^2 + (b^*)^2]^{1/2}$, was calculated from a^* (redness/greenness) and b^* (yellowness/blueness) colour coordinates (-100 to +100 range for both) returned by a research grade Cintra 4040 spectrophotometer (absorbance measurement up to 5 a.u.) (GBC Scientific Equipment Ltd, Australia) and the CIELAB tristimulus method (OIV, 2018b).

2.4. Statistical design and analysis of experiment

Days of skin preparation were logistically confounded with the effects of cultivar and time of development. Care was taken to ensure the laboratory conditions and techniques were standardised and kept consistent to resolve that confounding. The white and red skins were extracted a week apart. All combinations of cultivars and time of development were randomized within each extraction method; sample triplicates of each extraction were

performed in a non-randomised way. A balanced unreplicated 3-factor ANOVA model was used for each skin colour separately, 2(method)x6(cultivar)x2(time of development), of the 24 experimental treatments with 3 subsamples each. The three-factor interaction was used as the error term with 5 degrees of freedom. The ANOVA was applied to the TP and *C** data with Minitab 19 Statistical Software (Minitab Inc., USA). The average values and variations in sub-samples were analysed. The Anderson-Darling test for normality was applied to check for outlining cultivars at each time of development in each method of extraction. A two-sample t-test with separate estimation of variances was used to compare the average TP and *C** of skin mixtures to the corresponding averages of the individual skin extractions. A similar analysis of CIELAB coordinates is reported in the Appendix A, Supplementary data.

3. Results and discussion

Table A2 (Appendix A. Supplementary data) summarises the physiological data of the berries collected at veraison and harvest. Among the white cultivars, Semillon was characterised by the largest berry weight at veraison (1.15 g/berry) and at harvest (1.35 g/berry), and the highest harvest maturity (21.1 degrees Brix). The second smallest berry at veraison was Sauvignon Blanc (0.71 g/berry). There were uncharacteristically small berries of Gewürztraminer at veraison (0.32 g/berry) in comparison to the average berry weight of 1.03 g/berry at veraison in Kwiatkowski et al. (2020a). The discrepancy is explained by the small number of berries sourced in 2011 and shared between the two studies. The small berries left from previous study, however, were suitable for the aims of this study. At harvest, Riesling berries had the second large weight (1.25 g/berry), while the other four white cultivars ranged 0.91 – 1.09 g/berry. Amongst the red cultivars, Grenache berries were the largest at veraison (1.42 g/berry) and

harvest (1.94 g/berry), and also had the highest total soluble solids. The smallest berries were in Cabernet Sauvignon at veraison (0.81 g/berry) and harvest (1.13 g/berry).

The weight fraction of skin amongst the white cultivars ranged 10-13% w/w at veraison and 13-17% w/w at harvest (the unusually high percentage, 24%, at veraison for Gewürztraminer is explained by its small berry size). The corresponding weight fraction range amongst the red cultivars was 14-20% w/w at veraison and 16-25% w/w at harvest. These ranges agree with the estimation of skin weight between 10-20% of mature berry weight reported by Singleton and Esau (1969) and Margalit (2004). The skin weight percentages (Table A2) were used for expressing the TP in individual extracts as kg GAE/ton grapes (Table A4).

3.1. Phenolics in white cultivars

The comparison of the efficiency of extraction from individual white and red cultivars was performed under a fixed set of extraction parameters established in Kwiatkowski et al. (2020a) for maximising TP separately in the mixtures of the white and red skins under MAE and CTE. Table 1 shows the average TP content and the standard deviations of three replicated extracts for each combination of cultivar, time of development and method of extraction. The ANOVA model of unreplicated 6x2x2 factorial design was applied to study the main effects of cultivar, time of development and method of extraction, and their two-factor interactions. The three-factor interaction effect with 5 degrees of freedom was used for the estimation of uncontrolled random error.

Significant effects were observed of cultivar ($p = 0.01$), time of development ($p < 0.01$) and extraction method ($p < 0.01$); the cultivar-by time interaction effect was significant ($p = 0.02$).

The average TP decrease from veraison to harvest was 0.47 g GAE/L across the cultivars, but the change was significantly smaller ($p < 0.01$) for Chardonnay (an insignificantly small increase of 0.08 g GAE/L) and significantly larger ($p = 0.03$) for Sauvignon Blanc (a decrease of 0.78 g GAE/L) and noticeably larger ($p = 0.07$) for Riesling (a decrease of 0.71 g GAE/L). The TP across both methods and times of development ranged from the overall average of 1.7 g GAE/L for Gewürztraminer and Viognier to 1.2 g GAE/L for Riesling. The average difference between CTE and MAE was 0.26 g GAE/L.

The percentage error can be used for comparing extraction methods as defined by Eq. (1):

$$\text{TP Percentage error} = (|TP_{CTE} - TP_{MAE}| / TP_{CTE}) * 100 \quad (1)$$

The average percentage error was 15% (consistently with the 12% for the white skin mixture in Kwiatkowski et al., 2020a). Across cultivars, TP extracted by CTE was higher by 5-25% when compared to MAE at veraison, whilst at harvest the CTE TP values were higher by 9-21%.

The decrease in TP for Sauvignon Blanc from veraison (1.48 mg GAE/g BW under MAE and 1.56 mg GAE/g BW under CTE) to harvest (1.31 mg GAE/g BW under MAE and 1.47 mg GAE/g BW under CTE) agreed with the TP decrease from ~ 2.7 mg GAE/ g BW at veraison to ~ 1.5 mg GAE/g BW at harvest reported by Tian et al. (2019) for skin extracts of Sauvignon Blanc under a non-optimized CTE method.

Sri Harsha et al. (2014) extracted phenolics in three consecutive extractions with a non-optimized CTE method (Table A1) from skins separated from Chardonnay and Riesling

pomaces from two wineries. TP for the combined extracts was found respectively: 4.6 g GAE/kg and 6.7 g GAE/kg of dry product for Chardonnay, and 11.1 g GAE/kg and 10.9 g GAE/kg of dry product for Riesling. Herein, the average TP in a single extraction from the skins collected at harvest was 9.1 g GAE/kg GS (MAE) and 11.5 g GAE/kg GS (CTE) for Chardonnay, and 5.6 g GAE/kg GS (MAE) and 6.1 g GAE/kg GS (CTE) for Riesling.

The discrepancy in the results in this study and the comparisons in the literature may be explained by viticultural and environmental effects. The authors looked separately at the hypothesis of outliers in the selection of cultivars sourced from the same producer. The average TP for each cultivar at veraison and harvest are plotted in Fig. 1(A) on the normal probability scale separately for CTE and MAE. The displays put the cultivars' TP contents ordered from smallest to largest on the x-scale against their cumulative distribution percentage (from 1/6 to 5/6) on the y-scale. In each panel, the extracts are separated into harvest and veraison collections. The best-fit normal trends are shown with the thin solid straight lines of the corresponding colour and described with the mean and standard deviations shown in the legend in each panel. If a cultivar TP is in agreement with this normal expectation, it lies between the corresponding 95%-confidence curves shown on the plot. If all the cultivars are in agreement with the overarching normal probability model, their TPs align along the straight line of the best fit on the corresponding normality plot. As can be seen in Fig. 1(A), the normal probability plots do not show any abnormalities of cultivars TPs in either CTE or MAE. The Anderson-Darling normality test results are shown in the legends. The hypotheses that there is an overarching normal probability model for "whites" at harvest and veraison are not rejected (the p-values are far above the common significance level of 0.05). One may notice that the third small percentile on the MAE plot is slightly off-guard (this corresponds to Semillon both at

harvest and veraison), but this is not sufficient to disturb the overall normality of the selection of cultivars.

The overall average TP across all cultivars at veraison and harvest herein agrees with TP from the white skin mixture under MAE and CTE in Kwiatkowski et al. (2020a). The average TPs herein are 1.70 g GAE/L under CTE and 1.44 g GAE/L under MAE in comparison to the mixture TPs of 1.76 g GAE/L under CTE and 1.55 g GAE/L under MAE, which are shown in Table 1 and depicted with the bold vertical reference lines in Fig.1(A). The two-sample t-tests (with separate variance estimations) comparing the results of the 12 extractions in this study against the four validation replicates in Kwiatkowski et al. (2020a) do not show a significant difference ($p > 0.40$). There are no abnormalities amongst the six white cultivars (Fig. 1(A)), meaning that their TP results agree with the expectations from the normal probability model with the average TP, and the standard deviation capturing the natural variation amongst those *Vitis vinifera* grape species. Under each extraction method, the TP for the white skin mixture is placed between the harvest and veraison individual extracts (Fig. 1(A)), suggesting that the level of TP extracted from the white skin mixture expressed the phenolics contributed both by harvest and veraison skins of each cultivar.

3.2. Colour in white cultivars

CIELAB average *L*, a*, b** of the white skin extracts under MAE and CTE at veraison and harvest are shown in Table A3, followed by their statistical analysis (Appendix A, Supplementary data). The chroma C^* values are shown in Table 1. The ANOVA model of unreplicated 6x2x2 factorial design was applied to the chroma data. The model fitted the main effects of method, cultivar and time of development, and their two-way interactions.

All effects but the cultivar-by-method interaction effects were significant ($p < 0.01$ each). The time-by-method effect, although significant, was small and can be explained by the fact that more chroma was extracted with CTE at veraison (73.5) in comparison to harvest (67.2), while no difference in chroma was observed between harvest and veraison for MAE. The method effect was consistent for all cultivars, with CTE resulting in larger chroma (70.3) in comparison to MAE (60.0). On average, the veraison extracts had higher chroma (67.2) in comparison to harvest (63.1). However, a strong cultivar-by-time effect was present, with the interaction effects (i.e. an additional effect to the sum of cultivar and main effects) significant for all cultivars but Viognier. For Semillon, the chroma was significantly lower at veraison (49.1) than at harvest (64.5). An insignificant difference was observed between veraison and harvest for Chardonnay. The other four cultivars had the chroma significantly higher at veraison, although the magnitude of the difference was cultivar specific (Table 1).

The values of percentage error were calculated according to Eq. (2) for comparing the extraction methods:

$$C^* \text{ percentage error} = (|C^*_{CTE} - C^*_{MAE}| / C^*_{CTE}) * 100. \quad (2)$$

The overall percentage error for chroma was 15% (21% for the white skin mixture in Kwiatkowski et al., 2020a); the C^* values obtained using CTE were higher by 13-26% at veraison and 10-15% at harvest for all cultivars.

There appeared to be no reports in the research literature of the CIELAB chroma C^* for skin extracts of individual white cultivars we could use for reference herein. Liang et al. (2011)

reported the chroma results in the middle portion of the upper epidermis of the ripe Semillon (25.83) and Chardonnay (29.41) berries. Those results were significantly lower than the *C** results of extracts herein for Semillon (59.32 under MAE, 69.67 under CTE) and Chardonnay (65.76 under MAE, 73.42 under CTE) skins at harvest.

Fig. 2(A) presents the normal probability plots of MAE and CTE harvest and veraison extractions. The bold vertical reference lines show the chroma of the white skins mixture under the optimal practical conditions in Kwiatkowski et al. (2020a). The tests for normality are insignificant for CTE extractions both at harvest and veraison. For MAE, the test of normality is insignificant for harvest, while Semillon at veraison behaves significantly different to the distribution of the other five cultivars. The average *C** for CTE across the six cultivars (70.31) was significantly higher ($p < 0.01$, two-sample t-test with separate variance estimations) than the *C** value for the white skin mixture (65.87) reported by Kwiatkowski et al. (2020a). The *C** average for MAE across the six cultivars (59.97) was also significantly higher ($p<0.01$, two-sample t-test with separate variance estimations) than the *C** achieved for the mixture (52.03) by Kwiatkowski et al. (2020a). It can be suggested that the following two groups of cultivars deviated from the average *C** (Fig. 2(A)): (I) at veraison Chardonnay and Semillon, and at harvest Riesling and Sauvignon Blanc; (II) the remaining cultivars. Referring to CIELAB coordinates (Table A3), group I is characterised by a lower *a**, meaning that group II were more of golden tones, presumably from the combination of red and yellow pigments, with Gewürztraminer dominating due to its varietal characteristics (some pink/red pigments were present in the skins at harvest). Both methods extracted more colour from Riesling at veraison, Chardonnay at harvest, and Gewürztraminer at veraison and harvest under their optimal practical conditions for the white skin mixture.

Table 1 shows the average TP of skin extracts for the six red cultivars at veraison and harvest, with MAE and CTE at the optimal practical conditions in Kwiatkowski et al. (2020a). The ANOVA of unreplicated 6x2x2 factorial design was applied, and only detected a significant effect of cultivar ($p < 0.01$). There was a weak cultivar-by-time effect ($p = 0.09$), mostly noticeable for Merlot, for which TP at veraison was significantly lower than at harvest ($p = 0.015$), opposite to the change in Nebbiolo ($p = 0.05$), and no significant changes for the other four cultivars. Neither the overall effect of method of extraction nor cultivar-by-method effects were found significant.

Referring to Table 1, and applying Eq. (1), the percentage error between average TP of combined data at veraison and harvest between CTE and MAE was 4%, in comparison to 11% for the red skin mixture in Kwiatkowski et al. (2020a). The percentage errors between CTE and MAE for cultivars ranged from 26% for Pinot Noir (veraison) to -13% for Grenache (veraison) and Cabernet Sauvignon (harvest), to essentially no difference for Merlot. The relative changes herein can all be allocated to the random uncertainty in the experiment.

For Merlot, the TP increase from veraison (MAE 9.83 mg GAE/g GS, CTE 9.71 mg GAE/g GS) to harvest (MAE 12.30 mg GAE/g GS, CTE 12.00 mg GAE/g GS) was consistent with the findings of Ivanova et al. (2011), who reported an increase in TP from ~10.2 mg GAE/g GS at veraison to 33.3 mg GAE/g GS at harvest in Merlot skins under an ultrasonic extraction non-optimized for maximum TP. In contrast to the increase in the Merlot results herein, Geană et al. (2016) reported a slight decrease from 9.7 mg GAE/g GS at veraison to ~8 mg GAE/g

GS at harvest under CTE non-optimized for maximum TP. Their values of TP in skin extracts were similar to the TP in this study at veraison, while noticeably lower at harvest.

Fournand et al. (2006) reported a TP increase in Shiraz skin extracts from veraison (~ 1.5 g GAE/L at 17.4 ºBrix) to harvest (~ 2.2 g GAE/L at 27.2 ºBrix) under a non-optimized CTE method over 5 hours (TP remaining constant after 3 hours of extraction). Their TP at veraison was slightly lower than the TP obtained herein under CTE (1.78 g GAE/L). Pantelić et al. (2016) extracted TP of 10.13 mg GAE/g GS from Shiraz skins at harvest with a non-optimized CTE, which agrees with the TP in the Shiraz skin extracts at harvest herein (10.83 mg GAE/g GS).

Pinelo et al. (2005) obtained the TP of 0.5 g GAE/L and 0. 4 g GAE/L for the extracts of Grenache skins separated from the pressed and distilled pomace, respectively, under RS-optimized CTE using an ethanolic solvent. Herein TP in the Grenache skins at harvest was of a comparable order: 0.87 g GAE/L under MAE and 0.82 g GAE/L under CTE (Table 1).

The average TP for Cabernet Sauvignon and Pinot Noir did not significantly differ at veraison and harvest. For Cabernet Sauvignon under MAE, there was no difference in TP between veraison and harvest, while a small decrease was noticed under CTE. That insignificant decrease from veraison (CTE 9.13 mg GAE/g GS) to harvest (CTE 7.84 mg GAE/g GS) is consistent with the small decrease reported by Geană et al. (2016) for Cabernet Sauvignon, from veraison (20 mg GAE/g GS) to harvest (~15 mg GAE/g GS), under their non-optimized CTE method. A small increase was observed in Pinot Noir from 9.81 mg GAE/g GS at veraison to 11.73 mg GAE/g GS at harvest under MAE, and a small decrease from 13.25 mg GAE/g GS to 10.76 mg GAE/g GS under CTE. Neither change was statistically significant. Dokoozlian

and Kliewer (1996) reported the increase in TP from veraison to harvest for the skins of Cabernet Sauvignon and Pinot Noir extracted using a non-optimized CTE. The TP content for Pinot Noir skins did not change from veraison to harvest in Geană et al. (2016).

The normal probability plots (Fig. 1(B)) do not demonstrate any abnormalities, and the harvest and veraison selections of six cultivars are adequately described by the corresponding overarching normal distributions. The average TP across the 12 cultivar-by time of development extracts by each extraction method was significantly lower than the TP of those skins' mixture under the same conditions in Kwiatkowski et al. (2020a). This difference was stronger for CTE ($p < 0.01$), between the average TP of 1.52 g GAE/L for the 12 individual extracts and the mixture TP of 1.77 g GAE/L in Kwiatkowski et al. (2020a). For MAE, the corresponding difference was between 1.43 g GAE/L and 1.59 g GAE/L ($p = 0.02$). The comparisons were done with two-sample t-tests with separate variance estimations. No abnormalities were detected amongst the six red cultivars with the normality test. The TP of a few individual cultivars were above or close to the mixture TP (Fig. 1(B)) suggesting that the mixture-optimized conditions by Kwiatkowski et al. (2020a) extracted more from Merlot, Pinot Noir, Shiraz and Nebbiolo by both methods.

3.4. Colour in red cultivars

CIELAB average *L*, a*, b** of the red skin extracts under MAE and CTE at veraison and harvest are shown in Table A3, followed by their statistical analysis (Appendix A, Supplementary data). The chroma *C** for individual skin extracts, collected at veraison and harvest, under MAE and CTE at the optimal practical conditions (Kwiatkowski et al., 2020a)

are presented in Table 1. The ANOVA of unreplicated 6x2x2 factorial design was applied to test for the main and two-factor interaction effects. The effects of cultivar, time and cultivar-by-time of development were significant ($p < 0.01$ for each). The effects of methods of extraction, and method-by-time were not significant ($p > 0.10$). Both extraction methods returned statistically similar ($p > 0.10$) results for the individual cultivars except for Shiraz, for which CTE returned 20% higher C^* than MAE (64 against 58, $p = 0.025$). The average chroma C^* at harvest (63) was significantly higher ($p < 0.01$) than at veraison (17); such a substantial increase would be expected due to the increase in the anthocyanin content within the skins during berry ripening (Iland et al., 2011). The magnitude of the increase in chroma between veraison and harvest, however, was significantly cultivar dependent, and the largest for Merlot (from 9 to 77, $p < 0.01$). Grenache extracts exhibited lower colour at harvest in comparison with the other extracts of the other five cultivars. The veraison to harvest change was also the smallest for Grenache (from 9 to 24, $p < 0.01$).

The normal probability plots for harvest and veraison extractions by each method are presented in Fig 2(B). There is a significant lack of normality, more so for CTE. Both for CTE and MAE, Grenache at harvest does not confirm to the distribution of the other five cultivars ($p = 0.05$ and $p = 0.02$ correspondingly). At veraison, there is a noticeable curvature in the normality plot with CTE, caused by Pinot Noir returning slightly smaller than expected chroma while Shiraz slightly higher ($p = 0.07$).

Interestingly, the chroma value of 76 of the skin mixtures was still significantly higher ($p < 0.01$ for each extraction method) than the averages of the six cultivars at harvest (62.5 for CTE and 63.5 for MAE – in comparison, 18.3 for CTE and 16.2 for MAE at veraison). However, if Grenache at harvest is excluded, the chroma of the mixture agrees with the average chroma of

the five remaining cultivars at harvest (70.2 for CTE and 71.4 for MAE, $p > 0.1$ for both). The finding by Kwiatkowski et al. (2020a) that the same colour was extracted from the red skin mixture under both methods has been confirmed herein (Fig. 2(B)), if Grenache was excluded. The average percentage error was 3% herein in comparison with 0% in the previous study (Table 1). The remaining five cultivars at harvest aligned well with the average C^* for the red skins mixture, indicating that the optimal practical conditions in Kwiatkowski et al. (2020a) achieved a comparable extraction efficiency of the coloured compounds from the skins of individual cultivars. It can be conjectured that anthocyanins dictated the values of C^* extracted from individual skins and their mixtures. In comparison to the colour of white cultivars Fig. 2(A), different development was observed for the colour of the red skins driven by anthocyanins (Fig. 2(B), high values of a^* Table A3).

3.5. Future research and industrial applications

Extracting valuable phenolic and coloured compounds from grape skins is relevant to the current research, benchtop and commercial trends in grape science and value-adding technologies in winemaking, and in wine and grape industry waste management. The skins can be sourced from primary fruit, or separated from pomace/marc (up to 30% of the mass of grapes crushed as reported for Australian conditions by Muhlack et al. 2018). As Sette et al. (2020) have recently demonstrated, it is possible to achieve almost zero-waste valorization in grape industries by properly implementing the principles of Circular Economy (Ellen MacArthur Foundation, 2017) and extracting valuable elements up to the stage of bio-charcoal. This current study demonstrates that the valorization of skins would further add to the processes in Sette et al. (2020) by enabling the extraction of cultivar-specific colour compounds and phenolics. Those compounds would have high market value, as they are sought after by the

manufacturers of functional foods and nutraceuticals including food natural colourants (García-Lomillo and González-SanJosé, 2017; Gul el al. 2016; Carocho et al., 2014; Galanakis, 2013), skincare (Taylor, 2016) or textile dyes (Bechtold et al., 2007).

Sette et al. (2020) draw attention to the importance of valorization for regional industries, which naturally brings the necessity of optimizing extraction processes to meet specific local viticultural conditions and varietal selection in wine production. Herein, the authors strongly advocate that for the step of extracting bioactive compounds to be efficient, the extraction processes must be RS-optimized, and the preference given to more energy efficient methods. In their RS-optimized benchtop extractions from skins harvested directly from grapes, Kwiatkowski et al. (2020a) achieved the TP extraction under MAE comparable to that under CTE, while MAE required a 15-fold shorter extraction time and 2.7-fold less energy usage. The result was obtained by Kwiatkowski at al. (2020a) with a small industrial microwave reactor, and it is undoubtful, that the energy ratio can be further improved if a more efficient industrial microwave reactor is used. The extraction methodology developed by Kwiatkowski at al. (2020a) and utilized herein for twelve individual Australian commercial grape cultivars, can be applied wider in valorization of not only pomace/marc, but also the waste from non-alcoholic beverage making. The high amount of phenolics in grape waste makes it difficult for bacteria to digest the waste if it discharged directly as landfill (Paisio et al. 2014). On the other hand, the anaerobic digestate from properly processed waste with a substantial reduction in bioactive compounds, can be used directly or mixed with other organic materials as a soil amendment (Almeida et al., 2013) to improve overall soil and plant health, and to reduce fertilizer costs.

Chapter 3

This study reports the results for phenolics and colour extractions from grape skins of individual cultivars sourced in the same region in South Australia in 2011 and under fixed extraction conditions, RS-optimized separately for the white and red mixtures of those skins. This approach is closing the gap in the literature by allowing one to compare individual cultivars under controlled extraction conditions. This approach can be extended by including more cultivars or a wider range of regional conditions. The authors point that the skins were sourced directly from berries, and the results can be different if skins are taken from pomace or marc. White winemaking involves pressing the juice to be fermented and no skin contact in general, therefore a minimal loss of TP and C^* in skins in pomace would be expected. However, a substantial loss could be expected in red marc, due to the skin contact during fermentation. After fermentation ethanol concentration does not exceed 14% in table wine (Margalit, 2004), hence, there is a potential for extracting phenolics and colour under MAE or CTE from marc due to high ethanol concentration (60% for white or 70% for red skins) used in both methods. Referring to Table A4, and choosing MAE as a "green and clean", efficient and energy-and resources saving method, we would nominate the following potential value-returning winners for extracting TP for pomace skins: among the white cultivars Gewürztraminer at harvest (1.52 kg GAE/ton grapes) and Sauvignon Blanc at veraison (1.51 kg GAE/ton grapes), and among the red cultivars, Merlot (2.62 kg GAE/ton grapes), followed by Cabernet Sauvignon (2.23 kg GAE/ton grapes) and Nebbiolo (2.11 kg GAE/ton grapes) at harvest. The best candidates for maximum colour under MAE at harvest are: among the whites, Chardonnay (C^* = 66) and Gewürztraminer (C^* = 66), and among the reds Merlot (C^* = 78), Shiraz (C^* = 76) and Cabernet Sauvignon (C^* = 73) (Table 1).

Future research should not only concentrate on extraction methods for recovering the compounds from grapes, but also on the "green and clean", energy-saving and efficient

methods of phenolics and colour analyses. In Kwiatkowski et al. (2020a) a high throughput FC method was developed to speed up the TP analyses workload, and employed herein. This method can be used as a reference method in the process optimization in the industry, however more robust method is needed on the production scale. The attenuated total reflectance mid-infrared (ATR-MIR) spectroscopy of the skin extracts was studied by Kwiatkowski et al. (2020b), who built predictive models between the spectra and the TP and *C** data of the skin mixture extracts obtained by Kwiatkowski et al. (2020a). Kwiatkowski et al. (2020b) concluded that the spectral method is a good candidate for industrial applications, though still under development.

4. Conclusions

Continuing the research by Kwiatkowski et al. (2020a), total phenolics and chroma were assessed for individual grape skin extracts of six white and six red commercial cultivars collected at veraison and harvest with conventional thermal and microwave-assisted extraction methods, both under their RS-optimized practical conditions found separately for the white and red skin mixtures of the same cultivars (veraison and harvest combined).

For white skins, CTE was statistically ($p < 0.01$) more efficient (15%) than MAE in phenolics and colour extraction (if veraison and harvest were combined), which was in agreement with Kwiatkowski et al. (2020a). However, the method effect was not significant for TP in red skins, and TP under CTE was only 4% larger (in comparison to 11% in Kwiatkowski et al. (2020a)); consistently with Kwiatkowski et al. (2020a), no statistical difference was found in colour extraction, (except for Shiraz, *C** under CTE exceeded by 19% *C** under MAE). For red cultivars except Grenache, the average results for TP and *C** (only at harvest) were aligned

along the TP and *C** of the red skin mixture (veraison and harvest combined). For the white cultivars the average results for TP for Chardonnay (veraison and harvest), Gewürztraminer (harvest) and for Riesling, and Semillon (veraison), were well aligned with the average TP for skin mixture; and for *C** at harvest: under CTE Viognier and Semillon, and under MAE Sauvignon Blanc and Riesling, well aligned with average *C** for skin mixture.

Comparing two methods, MAE, as more energy and resources efficient, should be the method of choice to obtain maximum phenolics and colour from the white and red skins from primary fruit or separated from pomace/marc, or whole pomace, in manufacturing dietary supplements, food additives including natural colourants, skincare and natural dyes for textiles in efficient and environmentally green way. Under MAE, the potential value-returning winners for extracting maximum TP from white skins were Gewürztraminer at harvest and Sauvignon Blanc at veraison, and from red skins Merlot, Cabernet Sauvignon and Nebbiolo at harvest; for extracting colour, Chardonnay and Gewürztraminer, and Merlot, Shiraz and Cabernet Sauvignon, all at harvest.

The efficiency of MAE can be further improved by employing more efficient industrial microwave reactors. The findings can be also valuable in the economic decision making when (regarding the time-point of berry development) and which cultivar to choose for the future processing of skins and whole grapes, particularly when grapes are in over-supply. Using spectral assessment of phenolics would enable a quick process of optimization of extraction conditions.

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Appendix A, Supplementary data

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Table 1
Total phenolics (TP) and colour (*C**) extracted using the MAE and CTE methods from the skins of the white and red grape cultivars, collected at veraison and harvest [a].

Cultivar	TP (g GAE/L)				*C**			
	MAE		CTE		MAE		CTE	
	Veraison	Harvest	Veraison	Harvest	Veraison	Harvest	Veraison	Harvest
White cultivars								
Chardonnay	1.36 ± 0.02	1.39 ± 0.04	1.58 ± 0.06	1.75 ± 0.08	60.39 ± 0.11	65.76 ± 1.07	71.37 ± 2.24	73.42 ± 1.03
Riesling	1.43 ± 0.03	0.85 ± 0.01	1.76 ± 0.08	0.93 ± 0.04	64.40 ± 0.68	53.42 ± 0.24	78.74 ± 0.73	59.07 ± 1.33
Sauvignon Blanc	1.99 ± 0.14	1.18 ± 0.03	2.09 ± 0.01	1.33 ± 0.02	62.40 ± 1.27	49.77 ± 1.03	74.56 ± 2.46	58.01 ± 0.97
Semillon	1.45 ± 0.08	1.29 ± 0.04	1.92 ± 0.02	1.51 ± 0.04	41.92 ± 2.87	59.32 ± 1.22	56.38 ± 1.60	69.67 ± 0.49
Viognier	1.79 ± 0.07	1.31 ± 0.04	2.40 ± 0.03	1.45 ± 0.01	64.09 ± 0.91	60.77 ± 0.83	76.57 ± 1.60	68.18 ± 0.70
Gewürztraminer	1.81 ± 0.16 [b]	1.46 ± 0.03	2.09 ± 0.08	1.60 ± 0.10	72.34 ± 1.83	65.63 ± 0.74	83.55 ± 3.23	74.58 ± 0.92
White skin mixture [c]	**1.55 ± 0.01**	**1.55 ± 0.01**	**1.76 ± 0.01**	**1.76 ± 0.01**	**52.03 ± 0.66**	**52.03 ± 0.66**	**65.87 ± 0.45**	**65.87 ± 0.45**
Red cultivars								
Shiraz	1.47 ± 0.09	1.29 ± 0.07	1.78 ± 0.05	1.64 ± 0.05	39.90 ± 3.18	75.57 ± 0.71	49.03 ± 0.59	78.75 ± 0.40
Cabernet Sauvignon	1.25 ± 0.04	1.34 ± 0.04	1.39 ± 0.13	1.19 ± 0.02	23.45 ± 2.45	72.91 ± 1.13	26.06 ± 2.91	67.36 ± 0.53
Merlot	1.49 ± 0.12	1.87 ± 0.04	1.47 ± 0.03	1.82 ± 0.02	9.16 ± 0.13	77.65 ± 0.22	9.58 ± 0.24	77.12 ± 0.41
Pinot Noir	1.49 ± 0.10	1.78 ± 0.00	2.01 ± 0.04	1.63 ± 0.09	12.33 ± 0.32	67.45 ± 0.57	11.83 ± 0.45	61.75 ± 1.68
Grenache	1.20 ± 0.29	0.87 ± 0.02	1.06 ± 0.01	0.82 ± 0.02	9.40 ± 0.38	23.85 ± 0.29	9.27 ± 0.11	24.46 ± 0.52
Nebbiolo	1.72 ± 0.03	1.36 ± 0.18	1.96 ± 0.07	1.46 ± 0.04	3.20 ± 0.42	63.79 ± 5.55	4.05 ± 0.21	65.85 ± 0.99
Red skin mixture [c]	**1.59 ± 0.04**	**1.59 ± 0.04**	**1.77 ± 0.05**	**1.77 ± 0.05**	**76.24 ± 0.51**	**76.24 ± 0.51**	**76.22 ± 0.31**	**76.22 ± 0.31**

[a] Experimental values: mean ± SD (n = 3).
[b] Grape berries smaller in comparison with the rest of cultivars.
[c] Kwiatkowski et al. (2020a); skin mixture combines skins collected at veraison and at harvest); experimental values: mean ± SD (n = 4).
Note: The results can be re-calculated by using the conversion rule: TP [g GAE/g GS] = (10 mL/1000 mL) * TP [g GAE/L] * (1 g GS/1.5175 g GS).

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Figure captions

Fig. 1: Normal probability plots of TP extractions from individual white (A) and red (B) skins at harvest and veraison. Bold reference lines are the TP from the red and white skin mixtures in Kwiatkowski et al. (2020a). CH, Chardonnay; R, Riesling; SB, Sauvignon Blanc; S, Semillon; G, Gewürztraminer; V, Viognier; SH, Shiraz; CS, Cabernet Sauvignon; M, Merlot; PN, Pinot Noir; GR, Grenache; N, Nebbiolo.

Fig. 2: Normal probability plots of CIELAB chroma in the extractions from individual white (A) and red (B) skins at harvest and veraison. Bold reference lines are the average chroma from the red and white skin mixtures in Kwiatkowski et al. (2020a). CH, Chardonnay; R, Riesling; SB, Sauvignon Blanc; S, Semillon; G, Gewürztraminer; V, Viognier; SH, Shiraz; CS, Cabernet Sauvignon; M, Merlot; PN, Pinot Noir; GR, Grenache; N, Nebbiolo.

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Fig. 1(A)

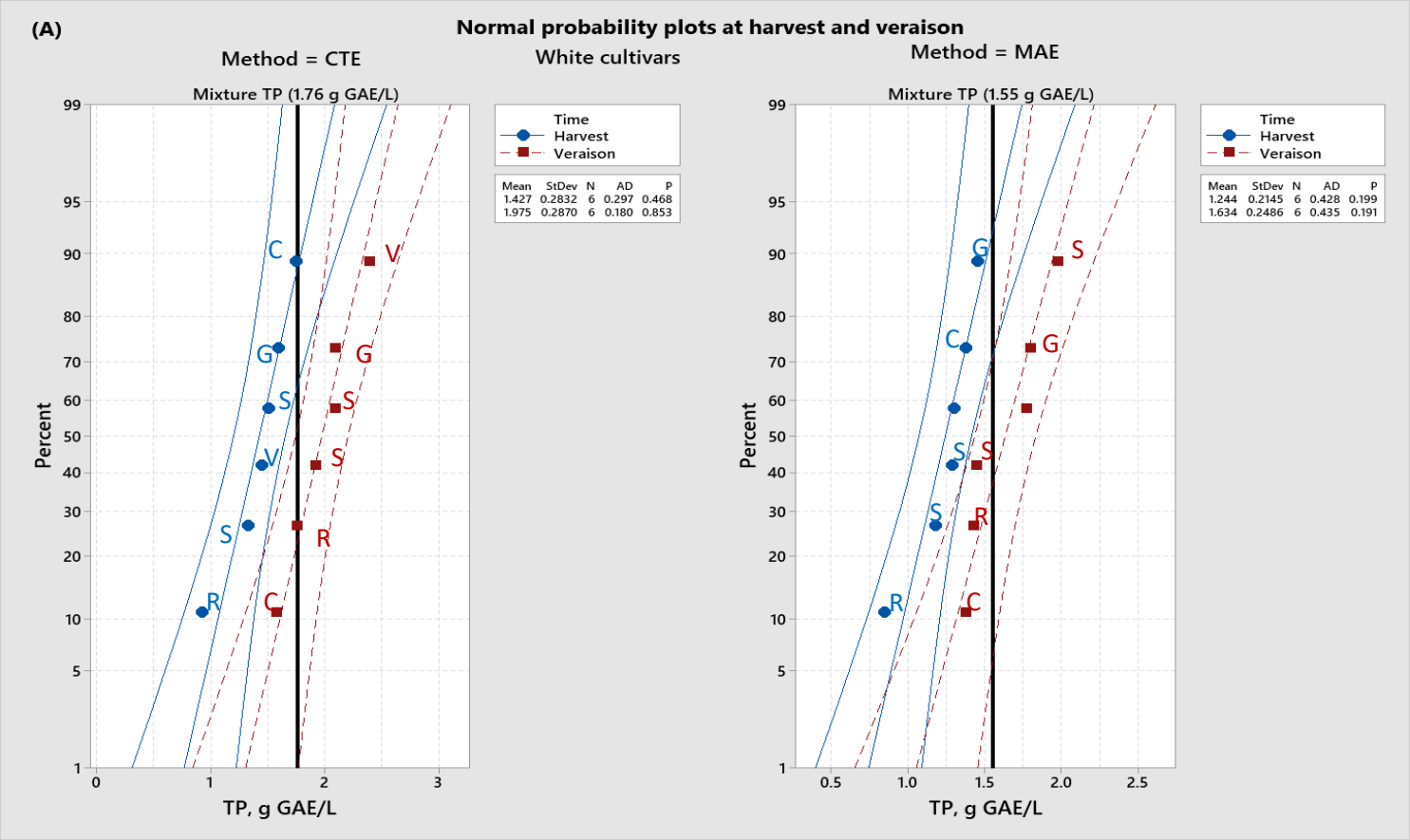

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Fig. 1 (B)

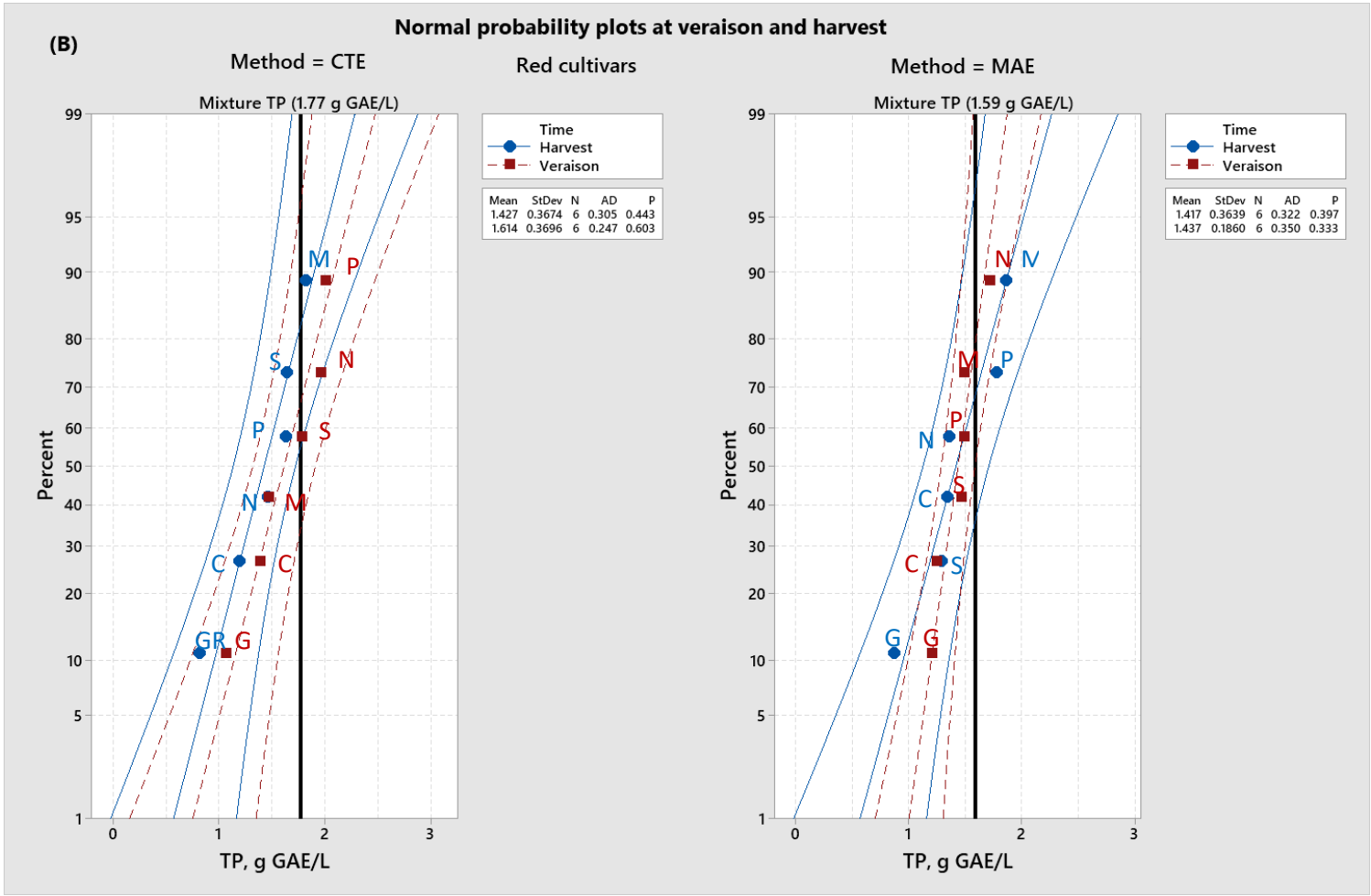

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Fig. 2(A)

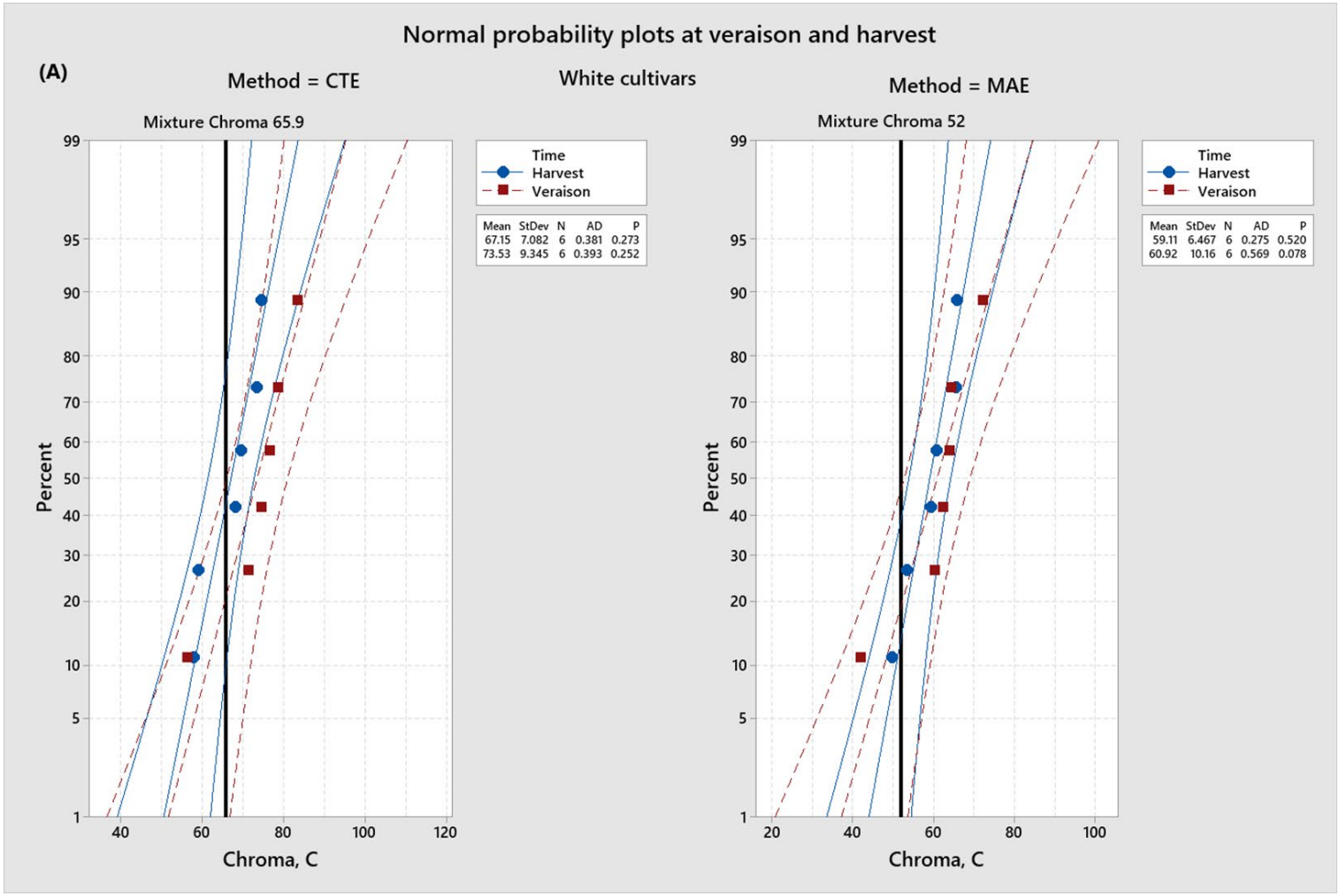
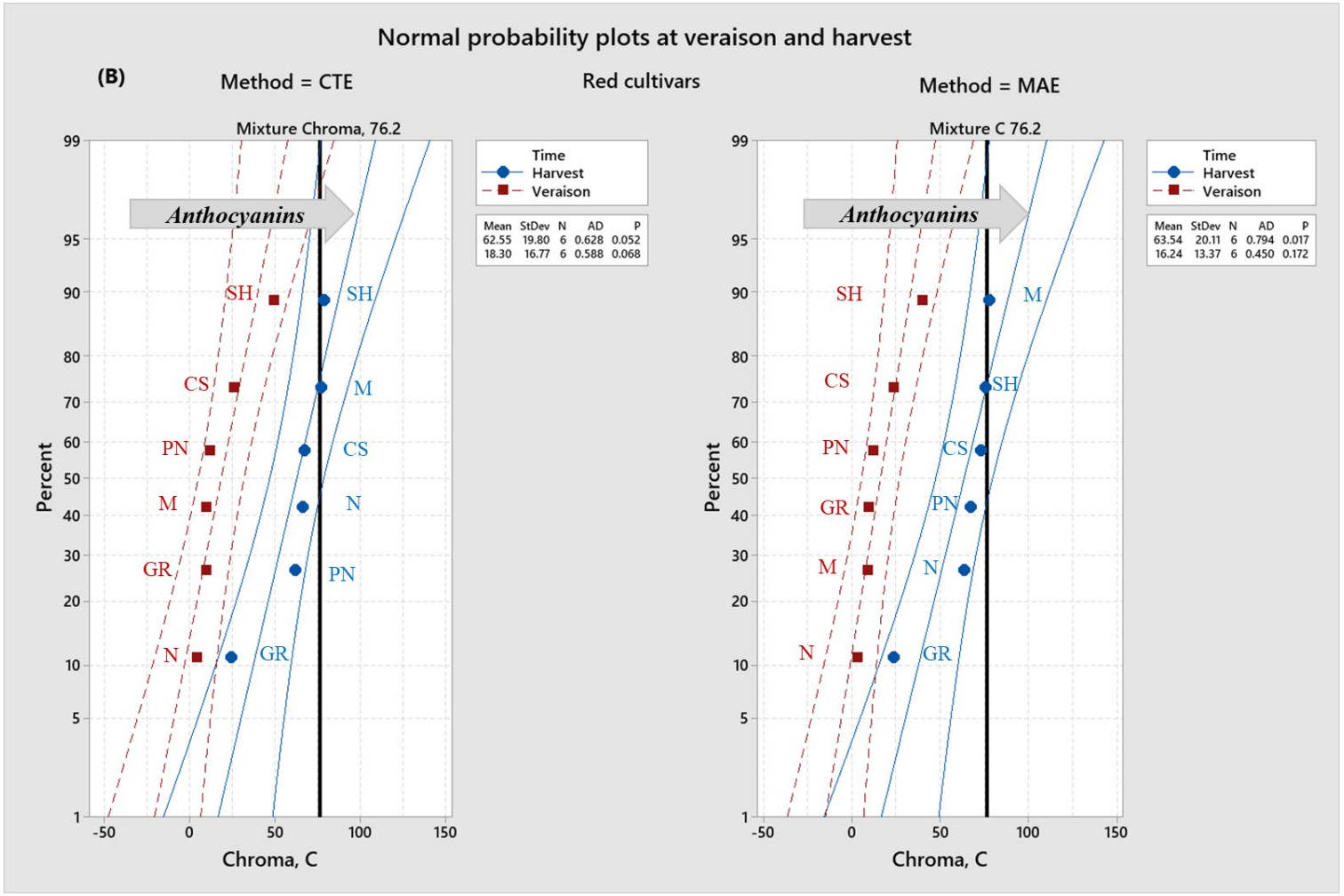


Fig. 2(B)


Normal probability plots at veraison and harvest
(B)
Method = CTE
Red cultivars
Method = MAE
Mixture Chroma, 76.2
Mixture C 76.2
Anthocyanins
Time
Harvest
Veraison
Mean StDev N AD P
62.55 19.80 6 0.628 0.052
18.30 16.77 6 0.588 0.068
Mean StDev N AD P
63.54 20.11 6 0.794 0.017
16.24 13.37 6 0.450 0.172
Percent
Chroma, C
SH
CS
PN
M
GR
N

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Supplementary data

Total phenolics and colour in grape skin extracts of commercial white and red cultivars at veraison and harvest under microwave-assisted and conventional thermal extraction methods optimized for white and red grape skin mixtures

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Appendix A

Table A1
A review of MAE and CTE research studies on phenolics extraction from grape skins peeled from berries or separated from pomace.

Cultivar	Skins origin	Extraction method	Investigated factors	DoE Optimisation	Target extract	Analytical method	Reference
Iranian cultivar Shahani	Red skins	MAE	MeOH, EtOH (0.1% 12N HCl) LS (2.5-10 mL/g GS) Temperature (60-80 ºC) Time (2-10 min)	RS-optimized	ACN	HPLC & LC-MS/MS	Ghassempour et al. (2008)
		CTE	MeOH (0.1% 12N HCl) LS (25 mL/g GS) Time (48 h) Temperature (rt)	Non-optimized			
Pinot Noir	Red skins from marc from white-vinification	MAE	MeOH under nitrogen LS (5 mL/g DW) Temperature (110 ºC)	Non-optimized	TP	FC	Casazza et al. (2010)
		CTE	EtOH or MeOH Temperature (rt) Time (19 h)	Non-optimized			
		CTE	EtOH LS (3.3-10 mL/g DW) Time (9-29 h) Temperature (rt)	RS-optimized	TP	FC	Casazza et al. (2012)
Tintilla de Rota	Red skins	MAE	MeOH (50-80%) Stirring (on/off) Temperature (50-100 ºC) Time (5-20 min) Power (100-500 W)	RS-optimized	ACN	HPLC	Liazid et al. (2011)

			LS (12.5-25 mL/g GS)				
Red Globe (table cultivar) Napoleon	Red skins	MAE	MeOH (50% aqueous) 1 mL of stock solution in 20 ML MeOH Temperature (50-175 ºC)	Non-optimized	ACN	HPLC	Liazid et al. (2007)
Moscatel	White skins		Power (500 W) Time (20 min)				
Chinese Kyoho cultivar	Red skins	MAE	EtOH 95% with 1.5 M HCl (85:15) Energy density (20-40 W/mL) Citric acid (0.2-0.6 mol/L) LS (15-20 mL/g GS) Power (400-800 W) Time (20-60 s)	RS-optimized	ACN	HPLC-MS	Li et al. (2012)
Bordo	Red skins	CTE	Aqueous citric acid 2%, (w/v) LS 1:3 (w/v) Time (20 h) Temperature (22 ºC)	Non-optimized	TP Colour of powders ACN	FC CIELAB Spectral A520,700nm	Kuck and Noreña (2016)
57 red and 21 white cultivars	White and red skins	CTE	Formic acid/ H_2O/MeOH (2:48:50) Time (2 h) Temperature (4 ºC)	Non-optimized	ACN Skin colour	HPLC/Q-ToF MS/MS CIELAB	Liang et al. (2011)
White and red cultivars	White and red skins from pomace	MAE	EtOH 50% aqueous with 0.8% (v/v) HCl LS (10 mL/g GS) Time (10 min) Power (140 W)	Non-optimized	TP	FC LC-TOF/MS	Peralbo-Molina et al. (2012)

		CTE	EtOH 50% aqueous with 0.8% (v/v) HCl LS (10 mL/g GS) Time (24 h) Temperature (40 ºC)	Non-optimized			
Garnacha	White and red skins from pomace	CTE	MeOH, EtOH, H_2O LS (1-5 mL/g GS) Time (30-90 min) Temperature (25-50 ºC)	RS-optimized	TP	FC	Pinelo et al. (2005)
Brazilian red cultivar	Red skins from pomace from sparkling winemaking	CTE	EtOH (8-92% aqueous) LS (3-17 mL/g GS) Time (60 min) Temperature (30 ºC)	Optimized (no RSM)	TP	FC	Caldas et al. (2018)
		MAE	EtOH optimal for CTE LS optimal for CTE Power density (1000 W/L) Time (30 min)	Non-optimized			
Six white and seven red cultivars	White and red skins	CTE	MeOH aqueous with 0.1% HCl LS (10 mL/g GS) Time (1h, then 24 h) Temperature (22 ºC, then 4 ºC)	Non-optimized	TP	FC	Pantelić et al. (2016)
Fourteen white and red cultivars	White and red skins	CTE	EtOH (80% aqueous) LS (10 mL/g GS) Time (60 min) Temperature (60 ºC)	Non-optimized	TP ACN	FC	Katalinić et al. (2010)
Sauvignon Blanc	White skins from veraison to harvest	CTE	EtOH (50% aqueous) LS (10 mL/g GS) Time (60 min)	Non-optimized	TP	FC	Tian et al. (2019)

			Temperature (rt)				
Five red cultivars incl. Cabernet Sauvignon Merlot Pinot Noir	Red skins from veraison to harvest	CTE	MeOH LS (10 mL/g GS) Time (15 min) Temperature (rt)	Non-optimized	TP	FC	Geană at al. (2016)
Nebbiolo	Red skins from veraison to harvest	CTE	Grape juice EtOH 10% Time (16 h) Temperature (4 ºC)	Non-optimized	TP Colour	FC Spectral A420,520,620 nm	Locatelli et al. (2016)
Shiraz	Red skins from veraison to harvest	CTE	EtOH (12% aqueous, Potassium hydrogen tartrate) Time (5 h) Temperature (27 ºC)	Non-optimized	TP ACN	FC HPLC, spectral	Fournand et al. (2006)
Cabernet Sauvignon Pinot Noir	Red skins (discs) from veraison to harvest	CTE	MeOH with 1% HCl Time (48 h)	Non-optimized	TP ACN	Colorimetrical Spectral	Dokoozlian and Kliewer (1996)
Cabernet Sauvignon Merlot Shiraz	Red skins	CTE	MeOH (60% aqueous) Temperature (rt) LS (40 mL/g GS) Time (30 s, 4 times)	Non-optimized	TP ACN	FC pH differential	Arnous and Meyer (2008)
Cabernet Sauvignon Merlot	Red skins	CTE	Acetone (80% aqueous) MeOH (60% aqueous) LS (9 mL/g GS) Time (4 h with acetone and 2.5 h with MeOH) Temperature (rt)	Non-optimized	TP	FC	Lorrain et al. (2011)
Seven white cultivars incl.	White skins from pomace	CTE	MeOH (80% aqueous) LS (4 mL/g GS)	Non-optimized	TP	FC	Sri Harsha et al. (2014)

Chardonnay Riesling			Time (2 h) Temperature (rt)				
Five red cultivars incl. Grenache Shiraz	Red skins From pomace	CTE	EtOH (10% aqueous with tartaric acid pH3.5) LS (3.5 mL/g GS) Time (1 h) Temperature (50 ºC)	Non-optimized	TP ACN	FC HPLC	Ky and Teissedre (2015)
White and red cultivars incl. Chardonnay Pinot Noir Nebbiolo	White and red skins from marc	CTE	EtOH (60% aqueous) LS (8 mL/g GS) Time (2 h) Temperature (60 ºC)	Non-optimized	TP	FC	Moncalvo et al. (2016)

Abbreviations: A, absorbance; DW, dried waste; rt, room temperature; incl., including.

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Table A2

Viticultural data of the white and red grape cultivars collected at veraison and harvest [a].

Cultivar	Number of 50-berry lots		Average total soluble solids (degrees Brix)		Average berry weight (g)		Average % skins of the grape berry weight (% w/w)	
	Veraison	Harvest	Veraison	Harvest	Veraison	Harvest	Veraison	Harvest
White cultivars								
Chardonnay	4	3	11.7 ± 0.4	17.2 ± 0.9	0.90 ± 0.11	0.91 ± 0.07	11.3 ± 2.1	12.9 ± 0.5
Riesling	4	3	8.6 ± 0.4	16.6 ± 0.8	0.82 ± 0.05	1.25 ± 0.18	12.1 ± 1.2	16.5 ± 0.8
Sauvignon Blanc	6	3	9.4 ± 0.6	15.7 ± 0.4	0.71 ± 0.06	1.09 ± 0.08	11.5 ± 3.0	16.9 ± 1.3
Semillon	3	3	11.4 ± 0.9	21.1 ± 0.2	1.15 ± 0.11	1.35 ± 0.07	13.4 ± 2.3	13.2 ± 0.9
Viognier	4	3	9.6 ± 0.9	13.6 ± 0.3	0.92 ± 0.07	0.91 ± 0.10	10.1 ± 2.0	14.8 ± 0.5
Gewürztraminer	2	3	15.7 ± 0.0	17.8 ± 0.5	0.32 ± 0.02	1.09 ± 0.02	23.9 ± 3.1	15.8 ± 1.3
Red cultivars								
Shiraz	3	2	11.3 ± 0.4	21.5 ± 0.4	1.04 ± 0.01	1.53 ± 0.06	16.1 ± 1.0	20.5 ± 0.8
Cabernet Sauvignon	3	2	10.5 ± 0.6	21.9 ± 0.2	0.81 ± 0.04	1.13 ± 0.03	19.9 ± 1.0	25.3 ± 1.0
Merlot	3	2	11.0 ± 0.9	19.8 ± 0.4	1.04 ± 0.08	1.46 ± 0.11	13.5 ± 0.3	21.3 ± 1.3
Pinot Noir	3	2	12.0 ± 0.8	19.0 ± 0.0	1.12 ± 0.13	1.49 ± 0.02	14.6 ± 1.4	16.0 ± 1.7
Grenache	2	2	13.5 ± 0.1	24.7 ± 0.4	1.42 ± 0.04	1.94 ± 0.05	19.1 ± 0.5	25.4 ± 1.9
Nebbiolo	3	2	7.4 ± 0.2	24.2 ± 0.2	1.02 ± 0.01	1.35 ± 0.03	17.1 ± 0.5	23.5 ± 5.3

[a] Experimental values: mean ± SD.

Table. A3

CIELAB coordinates [a] for grape extracts obtained from skins collected at veraison and harvest for the white (a) and red (b) cultivars using the MAE and CTE methods.

(a)

		*a**		*b**		*L**	
Method	Cultivar	Veraison	Harvest	Veraison	Harvest	Veraison	Harvest
MAE	Chardonnay	10.57 ± 0.85	14.86 ± 0.58	59.45 ± 0.26	64.06 ± 0.97	80.22 ± 0.44	77.05 ± 0.51
	Riesling	12.64 ± 0.32	9.40 ± 0.24	63.15 ± 0.69	52.59 ± 0.26	80.04 ± 0.21	82.84 ± 0.20
	Sauvignon Blanc	12.58 ± 1.00	8.74 ± 1.01	61.11 ± 1.11	48.99 ± 0.88	77.97 ± 0.81	81.80 ± 0.83
	Semillon	4.83 ± 1.33	12.85 ± 1.04	41.63 ± 2.73	57.91 ± 1.06	86.47 ± 1.37	78.39 ± 0.80
	Viognier	13.31 ± 0.62	13.21 ± 0.49	62.69 ± 0.80	59.32 ± 0.76	76.56 ± 0.72	75.29 ± 0.48
	Gewürztraminer	17.61± 1.13	29.06 ± 0.75	70.16 ± 1.61	58.84 ± 0.49	75.59 ± 0.92	69.12 ± 0.59
CTE	Chardonnay	20.99 ± 1.76	22.44 ± 0.53	68.21 ± 1.80	69.90 ± 1.13	72.58 ± 1.32	71.77 ± 0.45
	Riesling	28.18 ± 1.01	18.32 ± 0.75	73.52 ± 0.40	56.16 ± 1.17	68.91 ± 0.71	76.58 ± 0.59
	Sauvignon Blanc	25.17 ± 1.83	15.84 ± 0.78	70.18 ± 1.95	55.80 ± 0.82	68.73 ± 1.56	76.24 ± 0.63
	Semillon	15.78 ± 0.63	23.61 ± 0.40	54.13 ± 1.49	65.54 ± 0.39	77.75 ± 0.61	70.57 ± 0.31
	Viognier	27.69 ± 1.42	24.05 ± 1.11	71.39 ± 1.20	63.79 ± 0.33	65.96 ± 1.14	67.62 ± 0.80
	Gewürztraminer	30.53 ± 2.85	36.84 ± 0.73	77.76 ± 2.36	64.85 ± 0.69	66.98 ± 2.49	63.07 ± 0.61

(b)

		*a**		*b**		*L**	
Method	Cultivar	Veraison	Harvest	Veraison	Harvest	Veraison	Harvest
MAE	Shiraz	38.17 ± 3.10	74.87 ± 0.84	-11.62 ± 0.76	-10.24 ± 0.88	79.12 ± 1.63	55.86 ± 0.80
	Cabernet Sauvignon	22.55 ± 2.31	71.63 ± 1.32	-6.42 ± 0.84	-13.56 ± 0.93	87.12 ± 1.09	58.20 ± 1.45
	Merlot	9.15 ± 0.12	77.47 ± 0.22	0.40 ± 0.10	-5.38 ± 0.07	93.75 ± 0.34	54.86 ± 0.09
	Pinot Noir	12.27 ± 0.32	66.35 ± 0.56	-1.23 ± 0.35	-12.17 ± 0.34	93.08 ± 0.31	62.59 ± 1.28
	Grenache	9.33 ± 0.27	23.22 ± 0.27	0.45 ± 1.33	-5.45 ± 0.14	93.55 ± 0.53	85.25 ± 0.19
	Nebbiolo	2.05 ± 0.21	63.57 ± 5.75	2.46 ± 0.37	-4.92 ± 2.05	97.88 ± 0.71	67.48 ± 3.70
CTE	Shiraz	47.35 ± 0.55	78.39 ± 0.44	-12.73 ± 0.25	-7.47 ± 0.43	77.97 ± 0.57	56.35 ± 0.11
	Cabernet Sauvignon	25.20 ± 2.84	65.58 ± 0.57	-6.63 ± 0.67	-15.37 ± 0.13	86.49 ± 0.66	62.66 ± 0.48
	Merlot	9.54 ± 0.23	76.95 ± 0.44	0.88 ± 0.12	-5.22 ± 0.48	93.57 ± 0.44	55.06 ± 0.34
	Pinot Noir	11.73 ± 0.45	60.51 ± 1.76	1.56 ± 0.16	-12.29 ± 0.26	91.85 ± 0.29	65.22 ± 1.35
	Grenache	9.27 ± 0.11	23.84 ± 0.51	0.17± 0.08	-5.44 ± 0.13	96.88 ± 0.14	89.09 ± 0.65
	Nebbiolo	1.70 ± 0.17	65.73 ± 1.04	3.67 ± 0.26	-3.83 ± 0.89	98.15 ± 0.06	66.17 ± 0.76

[a] Experimental values: mean ± SD (n = 3).

Chapter 3

Table A4

Total phenolics (TP) recalculated as kilogram of gallic acid (GAE) equivalent per tonne of grapes, extracted using the MAE and CTE methods from the skins of the white and red grape cultivars, collected at veraison and harvest [a].

Cultivar	TP (kg GAE/ton grapes)			
	MAE		CTE	
	Veraison	Harvest	Veraison	Harvest
White cultivars				
Chardonnay	1.01 ± 0.01	1.18 ± 0.03	1.18 ± 0.04	1.49 ± 0.07
Riesling	1.14 ± 0.02	0.92 ± 0.01	1.40 ± 0.06	1.01 ± 0.04
Sauvignon Blanc	1.51 ± 0.11	1.31 ± 0.03	1.58 ± 0.01	1.48 ± 0.02
Semillon	1.28 ± 0.07	1.12 ± 0.03	1.70 ± 0.02	1.31 ± 0.03
Viognier	1.19 ± 0.05	1.28 ± 0.04	1.60 ± 0.02	1.41 ± 0.01
Gewürztraminer	2.85 ± 0.25 [b]	1.52 ± 0.03	3.29 ± 0.13	1.67 ± 0.10
White skin mixture [c]	**1.35 ± 0.01**	**1.35 ± 0.01**	**1.54 ± 0.01**	**1.54 ± 0.01**
Red cultivars				
Shiraz	1.56 ± 0.10	1.74 ± 0.09	1.89 ± 0.05	2.22 ± 0.07
Cabernet Sauvignon	1.64 ± 0.05	2.23 ± 0.07	1.82 ± 0.17	1.98 ± 0.03
Merlot	1.33 ± 0.11	2.62 ± 0.06	1.31 ± 0.03	2.55 ± 0.03
Pinot Noir	1.43 ± 0.10	1.88 ± 0.00	1.93 ± 0.04	1.72 ± 0.09
Grenache	1.51 ± 0.37	1.46 ± 0.03	1.33 ± 0.01	1.37 ± 0.03
Nebbiolo	1.94 ± 0.03	2.11 ± 0.28	2.21 ± 0.08	2.26 ± 0.06
Red skin mixture [c]	**1.74 ± 0.04**	**1.74 ± 0.04**	**1.94 ± 0.05**	**1.94 ± 0.05**

[a] Experimental values: mean ± SD (n = 3).

[b] Grape berries smaller in comparison with the rest of cultivars.

[c] Kwiatkowski et al. (2020a); skin mixture combines skins collected at veraison and at harvest; experimental values: mean ± SD (n = 4).

A.1. CIELAB colour coordinates analysis

The colour coordinates of each individual extraction were obtained with a research grade Cintra 4040 spectrophotometer (absorbance measurement up to 5 a.u.). The triplet of colour coordinates was returned to each sample as presented in Table A4. The ranges of coordinates are 0 (black) to 100 (white) for L^* (luminance), -100 (green) to 100 (red) for a^*, and -100 (yellow) to 100 (blue) for b^*. The analysis of chroma C^* (the length of the vector on the (a^*, b^*) plane) is presented in the main paper.

The ANOVA model of unreplicated 6x2x2 factorial experiment was applied to the average values of triplicates of each cultivar-by-time of development-by-extraction method for red and white cultivars separately.

A.1.1. Colour analysis of white cultivars

The main effects of cultivar and methods were significant ($p < 0.01$ for each) for the a^* coordinate. The interactions cultivar-by-time and method-by-time were also significant ($p < 0.01$ for each). The latter effect, although significant, was small and not practically interesting. It is reasonable to say that the a^* coordinate did not change noticeably over time for both methods of extraction. The cultivar-by-time interaction is more complicated. The a^* coordinate changed differently between veraison and harvest across cultivars. There was a very small difference for Chardonnay (about 17) and Viognier (about 20), the values were significantly higher at harvest for Gewürztraminer (24 at veraison and 33 at harvest) and Semillon (18 and 10), and significantly smaller for Riesling (20 and 14) and Sauvignon Blanc (19 and 12). The largest positive difference from the average a^*-colour was observed for Gewürztraminer (the average $a^* = 28$) and the largest negative difference was for Semillon (the average $a^* = 14$).

The Anderson-Darling normality test detected the MAE extraction at harvest from Gewürztraminer skin as an outlier in comparison to the rest of the cultivars. Otherwise the cultivars look like a random collection from a normal super-population of white cultivars both at veraison and harvest whether with MAE or CTE.

All three main effects and the cultivar-by-time and method-by time were significant for b^* ($p < 0.01$ for each). The cultivar-by-time and cultivar-by-method interaction effects were significant ($p < 0.01$ for each). Slightly but significantly higher b^* values were observed at veraison (64) in comparison to harvest (60). On average, CTE's b^* was higher (66) than MAE's b^* (58). The method-by-time effect, although significant, was not noticeable, with CTE's harvest value being slightly lower (by 1 unit) than one would expect from the sum of the main effects. The cultivar effect was significant and explained the bulk of differences in the colour. Semillon at veraison was an outlier in comparison to the other veraison MAE extractions. This effect was also the major contributor to the cultivar-by-time effect, with Semillon being the only cultivar, whose colour b^* was significantly ($p < 0.01$) lower at veraison (48) than at harvest (62); a small negative difference observed for Chardonnay was not significant. The other four cultivars exhibited a higher value of b^* at veraison.

The main effects of cultivar and method of extractions were significant for L^* ($p < 0.01$ for each), as well as the interaction effects of cultivar-by-time and method-by-time ($p < 0.01$ for each). L^* values were higher for MAE (78) in comparison to CTE (71), the interaction effect, although significant, did not affect noticeably this difference. No abnormalities amongst the six cultivars were detected by the normality tests on L^* in their extracts at harvest or veraison by CTE or MAE. The cultivar main effect was driven by the natural variation in cultivars from the smallest for Gewürztraminer (69) and the largest for Semillon (78). This main trend broke

at harvest, when the value for Semillon was much lower than one would expect in the absence of cultivar-by-time interaction, and Semillon was not a cultivar with the highest L^*.

The two-way relationship between the coordinates are illustrated in the matrix plot in Fig. A1(A). On the (b^*, a^*) scatter, the linear trends between the coordinates are clearly noticeable, separately, for harvest and veraison. The outlining behaviour of Gewürztraminer at harvest, previously tested with the normality test, is also clearly observed here as an off-trend in comparison to the colour vector of the other five varieties.

A.1.2. Colour analysis of red cultivars

For a^*, the main effects of cultivar and time, and the cultivar-by-time interactions were significant ($p < 0.01$ for each). For all cultivars, a^* increased from veraison (16) to harvest (68), but the change for Grenache was minimal (from 9 at veraison to 23 at harvest) in comparison to the other cultivars. By the Anderson-Darling normality test, Grenache at harvest was detected an outlier in comparison to the other cultivars by CTE ($p = 0.05$) as well as MAE ($p < 0.01$). For CTE, Shiraz at harvest was also noticeably off-trend with the other cultivars ($a^* = 42$ in comparison to the average of 12 for the other five varieties).

For b^*, similarly, significant effects were the main effects of cultivar and time and their interaction. On average, b^* decreased from -2 to -8 from veraison to harvest. That trend was consistent for all cultivars, with the exception of Shiraz, whose b^* value was slightly negative at veraison, and did not change much at harvest. For all other cultivars, the values at veraison were positive. No abnormalities were detected amongst the six cultivars by each method.

For L^*, the main effects of cultivar and time and their interactions were significant ($p < 0.01$ for each). The method and method-by-time effects were noticeable, but much smaller and only border-line significant ($p = 0.05$ for each). At veraison, L^* was higher (91) than at harvest (65), which was consistent to all cultivars, with the exception of Grenache, for which the difference was much smaller (95 at veraison and 87 at harvest, averaged over the two methods of extractions). The Anderson-Darling test detected Grenache an outlier at harvest in the CTE extractions amongst other cultivars.

The CIELAB coordinates are plotted on the matrix plot in Fig. A1(B). One can see the Grenache at harvest behaves along the veraison trend for the rest of cultivars in all three scatters. There is a clear correlation between a^* and b^* at veraison, and the two coordinates are not correlated at harvest, bringing a richer range of colours in the skin extracts.

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Chapter 4

ATR-MIR Spectroscopy Predicts Total Phenolics and Colour for Extracts Produced by Microwave-Assisted or Conventional Thermal Extraction Methods Applied Separately to Mixtures of Grape Skins from White or Red Commercial Cultivars

As presented in Chapters 2 and 3, we found the TP high throughput analyses still too laborious and time consuming. To achieve additional improvements in the future research on phenolics and colour extraction from grape skins as well as in manufacturing nutraceuticals, supplements, skincare or textile dies, we decided to investigate the efficacy of infrared spectroscopy to quickly and efficiently analyse a large number of samples.

Due to its simplicity, robustness, cost effectiveness, and being environmentally friendly the vibrational spectroscopy was considered. That choice was made taking into consideration the benefits of using the mid-infrared (MIR, 4000-400 cm^{-1}) spectroscopy over NIR (NIR, 13400-4000 cm^{-1}), the latter as successfully used in the analysis of compositional parameters in wine grape homogenate extracts and juice. They included sharper spectral peaks and no limitations regarding low concentrations of the analyte. MIR is based on bending and stretching of the sample molecules. It is noted that in the last decade there has been a substantial improvement in the quality of analysis using attenuated total reflectance mid-infrared (ATR-MIR) instruments.

Chapter 4

This paper shows the investigation of the four models (i.e., TP and *C** for white skins, and TP and *C** for red skins) built between the raw spectra and their second derivatives (Savitzky-Golay, 20 points) of the ATR-MIR spectra and the chemical data for TP and *C** of the white and red grape skin mixture extracts collected during the advanced screening, RS-optimisation and validation of the MAE and CTE methods (total number of samples 240 per one skin colour) to predict TP and *C** using partial least square (PLS1) regression. We examined the full range of the spectra of 4000-400 cm^{-1}, and the sub-ranges of 4000-1100 cm^{-1}, 1500-400 cm^{-1}, 1500-1100 cm^{-1} and 1457-1168 cm^{-1}. We have found out that the PLS1 models built between the TP and *C** reference data and the second derivative of the spectra range of 4000-1100 cm^{-1} provided the best results for both TP and C*for each skin colour, offering fair classification, hence suitable for screening purpose. We have also identified some spectral ranges and peaks to flag differences between the white and red skins.

To the authors' knowledge this research represents the first report on investigating the efficacy and benefits of the ATR-MIR spectroscopy to measure the total phenolics content (TP) and colour (chroma *C**) of the white and red grape skin mixture extracts. The findings have allowed us to propose the robust, efficient and "green and clean" analytical methods of measuring TP and *C** by the ATR-MIR spectra of the white and red grape skin extracts. Presented in the following publication is the first detailed investigation of the attenuated total reflectance mid-infrared (ATR-MIR) spectroscopy to predict total phenolics content and colour of white and red grape skin mixture extracts obtained using microwave-assisted and conventional extraction methods and it has been accepted for publication by *Food Analytical Methods* (6 Jan. 2020).

Statement of Authorship

Title of Paper	Attenuated total reflectance mid-infrared (ATR-MIR) spectroscopy predicts total phenolics and colour for extracts produced by microwave-assisted or conventional thermal extraction methods applied separately to mixtures of grape skins from white or red commercial cultivars
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Mariola Kwiatkowski, Daniel Cozzolino and Dennis K. Taylor. Attenuated total reflectance mid-infrared (ATR-MIR) spectroscopy predicts total phenolics and colour for extracts produced by microwave-assisted or conventional thermal extraction methods applied separately to mixtures of grape skins from white or red commercial cultivars, Food Research International

Principal Author

Name of Principal Author (Candidate)	Mariola Kwiatkowski (note: the publishing name, Mariola, is different to the full name, Maria Jolanta)		
Contribution to the Paper	Participated in the establishment of the mathematical models to be tested, conducted the literature review, collected the skin extracts obtained in the first part of the PhD project (revised version of the manuscript submitted to the Journal of Cleaner Production on 29th Oct.2019) and performed the ATR-MIR scanning. Collated the data and performed the multivariate data analyses and modelling to answer the research questions. Prepared the comprehensive draft of the manuscript and implemented all the corrections and editions suggested by other co-authors.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	11/11/2019

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Daniel Cozzolino		
Contribution to the Paper	Daniel provided guidance with the concept of the experimental work and research questions related to the use of spectroscopy, extensively commented on early and advanced versions of the manuscript and the choice of the journal for the final submission. Daniel is the corresponding author for this manuscript. The contribution % is 10%		
Signature		Date	19/12/2019

Name of Co-Author	Dennis K. Taylor		
Contribution to the Paper	Dennis is the principal supervisor of Mariola, provided guidance with the concept of the experimental work and research questions, discussed the initial ideas and the relevance to the food industry, and the research novelty and impact, extensively commented on early and advanced versions of the manuscript and the choice of the journal for the final submission. The contribution % is 10%		
Signature		Date	19/12/2019

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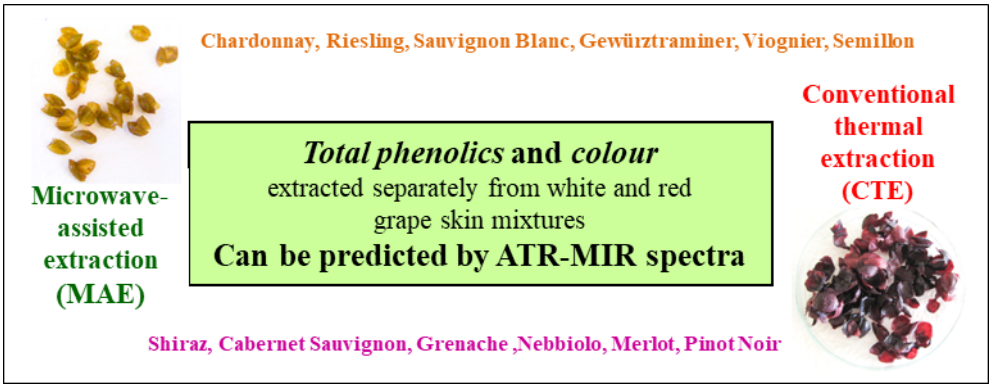
Chardonnay, Riesling, Sauvignon Blanc, Gewürztraminer, Viognier, Semillon
Microwave-assisted extraction (MAE)
Total phenolics and *colour*
extracted separately from white and red grape skin mixtures
Can be predicted by ATR-MIR spectra
Conventional thermal extraction (CTE)
Shiraz, Cabernet Sauvignon, Grenache ,Nebbiolo, Merlot, Pinot Noir

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ATR-MIR Spectroscopy Predicts Total Phenolics and Colour for Extracts Produced by Microwave-Assisted or Conventional Thermal Extraction Methods Applied Separately to Mixtures of Grape Skins from White or Red Commercial Cultivars

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Abstract
Attenuated total reflectance mid-infrared (ATR-MIR) spectra were collected from 480 grape skin extracts produced in a wide range of conditions. The conditions were created at different phases in parallel response surface RS-optimisation experiments aiming to maximise the total phenolics in red and white grape skin extractions by microwave-assisted (MAE) or conventional thermal (CTE) methods. Skins mixtures for six white and six red Australian commercial cultivars were prepared from grapes collected at veraison and harvest. The total phenolics were measured for individual extracts with the Folin-Ciocalteu method. Partial least squares (PLS1) regression was employed on the spectra to predict the total phenolics (TP) and colour (CIELAB chroma C^*) of individual extracts. Models based on the raw spectra and their second derivative (Savitzky-Golay, 20 points) were examined using the full range of spectra 4000–400 cm^{-1}, and sub-ranges: 4000–1100 cm^{-1}, 1500–400 cm^{-1}, 1500–1100 cm^{-1} and 1457–1168 cm^{-1}. The PLS1 models based on the second derivative of the range of 4000–1100 cm^{-1} provided the best results for both TP and C^*, for each skin colour. Overall, the residual predictive deviation (RPD) exceeded 3.0, indicating the PLS1 models' ability for fair classification, suitable for screening. A comparative analysis was performed for the range of 4000–1100 cm^{-1} between the spectra of white and red skin mixture extracts: the peaks at 3840 cm^{-1}, 2940 cm^{-1}, 2358 cm^{-1}, 2197 cm^{-1}, 2136 cm^{-1}, 1725 cm^{-1}, 1100 cm^{-1} and 1148 cm^{-1} are flagged to be investigated in further research. ATR-MIR spectroscopy can reduce the time and costs of TP and C^* analyses in grape skin extracts.

Keywords Chroma CIELAB · Green chemistry · Food chemistry · Nutraceuticals · Natural colourants

Introduction

Fruits are a rich source of phenolics, and when included in daily diet, bring benefits to health due to phenolics' antioxidant capacity (Gul et al. 2016; Galanakis 2013). Phenolics contribute to the colour, aroma, flavour, taste and mouth-feel of grape-based products, including wines (Iland et al. 2011; Parker et al. 2007; Vidal et al. 2004). Phenolics extracted from the grape plant material directly or post-winemaking waste (pomace) can be utilised in the production of nutraceuticals (food additives, including natural colourants, and dietary supplement) and functional foods (foods with added bioactive compounds) (Gul et al. 2016; Galanakis 2013; Shahidi 2009). In industry, grape skin extracts are used as food additives (Carocho et al. 2014; Jackman et al. 1987) and textile (Shahid-ul-Islam et al., 2013; Bechtold et al., 2007) natural colourants.

Skin accounts for 10–20% of the grape berry weight (Margalit 2004; Singleton and Esau 1969), and approximately 25% and 50% of phenolics are located in the skins of white and red cultivars, respectively (Singleton and Esau 1969). During winemaking, approximately 10–30% of the mass of grapes crushed for the process constitutes a waste (pomace) (Muhlack et al. 2018). Winemakers, grape growers and

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scientists have been using various methods to recover useful constituents from pomace, including obtaining phenolic and colour compounds that could be used in foodstuff and as colourants (Muhlack et al. 2018, de la Cerda-Carrasco et al. 2015). A decade ago, Bechtold et al. (2007) reported a thermal extraction method for extracting anthocyanins (known to drive the colour of red skins) from whole pomace with the aim of using the extract as alternative to the synthetic colourants in dyeing textiles.

Recently there has been growing interest in developing alternative extraction methods more environmentally friendly in terms of chemical and energy usage in comparison with the conventional thermal extraction (CTE) methods, which can be used in valorisation of winery bi-products (Kalli et al. 2018). Microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE) are two examples of the alternative extraction methods (Routray and Orsat, 2012), which have been investigated under different settings by many oenology and wine chemistry researchers in the last decade. A multi-phase response surface (RS) (Kuehl, 2000), experiment has been recently reported by Kwiatkowski et al. (2020) demonstrating the way to optimise extraction conditions for grape skins for MAE and CTE from a complex biological material. Kwiatkowski et al. (2020) used FC method for quantifying phenolics and CIELAB for colour. Other chemical methods have been employed by other researchers for phenolic assessment (Aleixandre-Tudo et al. 2019). All methods are time consuming and require toxic and expensive compounds.

There are several spectroscopy methods of phenolic assessment reported in the literature. Teixeira dos Santos et al. (2017) and Machado and Domínguez-Perles (2017) reported on the applicability and potential of the vibrational spectroscopy for estimating phenolics in the wine industry, including by-products.

The attractiveness of the near-infrared spectroscopy (NIR, 13400–4000 cm^{-1}) is due to its simplicity, robustness, cost effectiveness, and being environmentally friendly (Cozzolino 2014). It was applied for efficient in-cuvette analysis of compositional parameters in wine and grape homogenate extracts (Cozzolino et al. 2011; Cozzolino et al. 2008a; Cozzolino et al. 2008b; Janik et al. 2007; Cozzolino et al. 2004). Cozzolino et al. (2007) analysed wine compositional parameters using a non-destructive in-bottle NIR measurements. By using the NIR hyperspectral imaging, Jara-Palacios et al. (2016) successfully determined 27 individual phenolics in the seeds, skins and stems isolated from the Spanish white grape pomace. The total phenolics and colour density were efficiently predicted by the spectra of the red grapes employing a contactless Fourier transform (FT) NIR on-line instrument (Aleixandre-Tudo et al. 2019).

Cozzolino (2014) and Cozzolino (2011) discuss the benefits when using the mid-infrared (MIR, 4000–400 cm^{-1}) spectroscopy over NIR: sharper spectral peaks and no limitations regarding low concentrations of the analyte. In the last decade, there has been a substantial improvement in the quality of analysis using MIR techniques (Lu and Rasco 2012). Fragoso et al. (2011) predicted efficiently total phenolics (RPD = 4.5), total anthocyanins (RPD = 3.5) and condensed tannins (RPD = 3.8) in red grape homogenate extracts at various stages of berry development by the FT-MIR spectra in the range of 1457–1168 cm^{-1}. The second derivative transformation (Savitzky-Golay, 5 point) to the FT-IR spectra was efficiently used to measure TP in the range of 1560–1177 cm^{-1}, and for condensed tannins in the ranges of 1670–950 cm^{-1} and 3750–2125 cm^{-1} in seeds of two Greek native grape cultivars (Kyraleou et al. 2015). The attenuated total reflectance MIR (ATR-MIR) was employed to investigate a number of compositional parameters, except phenolics, of the Sauvignon Blanc grape homogenate at various stages of berry development (Musingarabwi et al. 2016). Cozzolino et al. (2011) presented various compositional parameters, excluding phenolics, of Australian commercial white and red wines using ATR-MIR. ATR-MIR was applied in to analyse phenolics and other compositional parameters in Shiraz wines (Ristic et al. 2016) and in white grape juices (Shah et al. 2010).

To the best of our knowledge, no studies have reported the use of MIR to evaluate the TP content and CIELAB chroma C^* of skin mixture extracts, obtained under various methods from the high quality white and red grape cultivars.

Using the RS methodology, Kwiatkowski et al. (2020) optimised MAE and CTE methods for maximizing the TP content extracted separately from white and red grape skin mixtures of typical commercial cultivars. Each mixture was composed from the skins of six white or red cultivars, correspondingly, collected at two time points of berry development—veraison and harvest. In addition to preliminary screening of parameters of extraction, the design of experiment (DoE) consisted of three phases: advanced screenings, RS-optimisation and the validation of the MAE and CTE methods, in which 480 samples of skin extracts were collected. The TP was measured with a high-throughput Folin-Ciocalteu assay (Kwiatkowski et al. 2020).

In this study, we are investigating the ATR-MIR spectral data additionally collected on individual grape skin extracts in the experiment reported by Kwiatkowski et al. (2020) to build predictive models between the spectra and the chemical data of TP and C^*. Our aims in this study are to (1) investigate the feasibility of the ATR-MIR spectroscopy to measure TP and C^* of white and red grape skin mixture extracts, obtained under the MAE and CTE methods optimised for maximal TP content; (2) quantify differences in the ATR-MIR spectra between the white and red skin mixture extracts; (3) investigate the feasibility of infrared spectroscopy to speed up further the TP analyses of skin extracts in the future similar projects, enabling researchers to enjoy the simplicity, robustness, cost effectiveness and environmental consciousness of spectroscopy methods (Cozzolino 2014).

Materials and Methods

The full details of the grape skin sample preparation, extraction, design of experiment and analytical methods can be found in Kwiatkowski et al. (2020), a brief description is presented below for convenience. The spectral data collection is described thoroughly below.

Skin Sample Preparation

The *Vitis vinifera* six white (Chardonnay, Riesling, Sauvignon Blanc, Semillon, Viognier and Gewürztraminer) and six red (Shiraz, Cabernet Sauvignon, Grenache, Nebbiolo, Merlot and Pinot Noir) grape cultivars were collected at veraison and harvest, sourced from South Australian cool climate commercial vineyards (C. A. Henschke & Co.).

Design of Experiment

In Kwiatkowski et al. (2020), the design of experiment (DoE) consisted of four phases: the preliminary and advanced screenings, RS-optimisation and validation of the MAE and CTE methods. The chosen levels of five experimental factors: ethanol concentration (EtOH), liquid to solid ratio (LS), temperature, time and pH are shown in Table 1. The optimal conditions were found using the RS methodology for maximizing TP extracted from the white and red skin mixtures using the MAE and CTE methods. In addition, using the same factors and their levels as identified for maximising TP, the optimal conditions for C^* of the extracts were found using both methods. The MAE and CTE methods' validations were conducted under the optimal practical conditions chosen due to practical constrains explained in Kwiatkowski et al. (2020).

The white and red skin mixture extracts collected during the advanced screening, RS-optimisation and the MAE and CTE methods' validation phases were used in this study.

Samples Collected in Extraction Methods

The MAE and CTE methods were parallelly RS-optimised and both shared the same protocol to minimise the method-to-method experimental error (Kwiatkowski et al. 2020). Briefly, each sample was placed in a 35-mL Pyrex vessel and capped. For MAE, a single sample was placed in a chamber of an industrial microwave reactor CEM Discover S-Class (CEM Co., USA), for which temperature was controlled (± 1 °C) by a Maxus WX801700 air compressor (Campbell Hausfeld, USA) coupled with the microwave reactor. For CTE, multiple samples were put in a plastic rack immersed in the Ratek WB4 water bath (Ratek Instruments Pty Ltd.,

Table 1 Experimental design for advanced screening, RS-optimisation and validation studies of the MAE and CTE methods

	Advanced screening		RS-optimisation		Methods' validation	
	MAE	CTE	MAE	CTE	MAE	CTE
White skin mixture—factors						
EtOH (%)	40, 80	40, 80	30, 40, 55, 70, 80	30, 40, 55, 70, 80	60	60
LS (mL/g GS)	10, 20	10, 20	7, 10, 15, 20, 23	7, 10, 15, 20, 23	7	7
Temperature (°C)	50, 70	50, 70	30, 40, 55,70, 80	30, 40, 55,70, 80	70	70
Time (min)	4, 8	60, 90	4	60	4	60
pH	1.5, 3.5	1.5, 3.5	1.5	1.5	1.5	1.5
Red skin mixture—factors						
EtOH (%)	40, 80	40, 80	30, 40, 55, 70, 80	30, 40, 55, 70, 80	70	70
LS (mL/g GS)	10, 30	10, 30	7, 10, 15, 20, 23	7, 10, 15, 20, 23	7	7
Temperature (°C)	40, 60	40, 60	30, 40, 55, 70, 80	30, 40, 55, 70, 80	60	60
Time (min)	4, 8	60, 90	4	60	4	60
pH	1.5, 3.5	1.5, 3.5	1.5	1.5	1.5	1.5
White and red skin mixtures settings						
Power (W)	150	N/A	150	N/A	150	N/A
Pressure (psi)	200	N/A	200	N/A	200	N/A
Stirring/shaking	High	10 min	High	10 min	High	10 min
Cooling by compressor	On	N/A	On	N/A	On	N/A
No. of treatments	16	16	20	20	1[a]	1[a]
No. of replicates	3	3	3	3	4	4

N/A denotes not applicable

[a] Consecutive first, second and third extractions of phenolics from the same portion of solids were used for the validation of the MAE and CTE methods

Australia) with controlled (± 1 °C) temperature, covered by a black box to eliminate light exposure.

In the advanced screening and RS-optimisation, extracts were obtained in a single extraction. Three consecutive extractions (1st, 2nd and 3rd) were applied during the MAE and CTE methods' validation.

Analytical Methods for TP and Colour

A high-throughput Folin-Ciocalteu assay, utilizing a 96-well plate and a liquid dispensing robot, was developed by Kwiatkowski et al. (2020) to measure the TP content in the skin mixture extracts. TP is expressed as gram of gallic acid equivalent per litre (g GAE/L).

The colour of the skin extracts, expressed as CIELAB chroma C^* (ASTM 2000; Ohno 2000), was calculated from a^* (redness/greenness) and b^* (yellowness/blueness) measured by the Cintra 40 spectrophotometer (GBC Scientific Equipment Ltd., Australia).

ATR-MIR Spectroscopy

The ATR-MIR spectra of the skin extracts were collected during the advanced screening, RS-optimisation and validation of the MAE and CTE methods in Kwiatkowski et al. (2020). For each skin mixture, the total number of extracts was 240, obtained by both methods (96 extracts from advanced screening, 120 extracts from RS-optimisation and 24 extracts from the methods' validation, Table 1). The chemical data of TP and C^* are used as the reference data in this study for the qualitative and quantitative analyses by the ATR-MIR spectra.

To record the ATR-MIR spectra, a few drops of an extract were poured on a platinum diamond of an ATR single reflection sampling module cell mounted on a Bruker Alpha instrument (Bruker Optics GmbH, Ettlingen, Germany), allowing to acquire spectra between 4000 and 375 cm^{-1}.The spectra were collected using the OPUS software (version 6.5, Bruker Optics). The spectrum of each sample was obtained by taking the average of 64 scans at a resolution of 8 cm^{-1}, with the scanner velocity of 7.5 kHz and a background of 64 scans. The Milli-Q-water was used to obtain the background reference spectra. After each sample, the crystal was washed with Milli-Q-water and dried with a soft Kimwipes tissue. Spectra were exported from the OPUS software in GRAMS format (*.spc) into The Unscrambler software (version 7.6, CAMO ASA, Norway). To improve the signal-to-noise ratio, the second derivative transformation was applied to the spectra using a twenty-point Savitzky-Golay smoothing (Dambergs et al. 2015).

Theory and Calculations for the Analysis of Spectral Data

Principal component analysis (PCA) is a main tool of unsupervised methods in pattern recognition (multivariable analysis) methods, and it has been used in the last two decades when investigating food quality or authenticity. It is a dimension-reduction technique helping to reduce the number of variables, detecting outliers, finding relationships between the treatments and variables as well as trends (Cozzolino et al. 2019; Jiménez-Carvelo et al. 2019). In this study, the PCA with full cross-validation (leave-one-out) was performed on the averaged ATR-MIR of triplicates of spectral data of the white and red skin mixture extracts for the qualitative evaluation (there were four replicates for the multiple extractions in the validation phase); for more details on samples refer to Table 1.

Partial least squares (PLS1) regression is a main tool of supervised methods in chemometrics pattern recognition methods for quantitative evaluation. The model building consists of the calibration set (or training set) and the validation set (or test set). For the assessment of the quality of quantification rate in the model, the cross-validation is applied to the calibration set as an internal validation, followed by the model external validation applied to another set of samples (Jiménez-Carvelo et al. 2019). Herein, the models were built to predict TP and C^* using the individual (not averaged) ATR-MIR spectra. The ATR-MIR scans were procured for the white and red skin mixture extracts, the total number of 240 scans for each skin mixture: a half of the extracts was used for the PLS1 calibration (training set, number of samples $n = 120$) and the other half for the PLS1 validation (test set, $n = 120$). The PLS1 calibration models were created between the TP and C^* data (both standardised, 1/SD) and ATR-MIR spectra (raw or second derivative), separately for the white and red skin extracts. The lowest number of PLS1 components was used to avoid overfitting (Cozzolino et al. 2011; Naes et al. 2002). The statistics calculated for calibration data sets were as follows: the mean and standard deviation (SD), coefficient of determination in cross-validation (R^2), bias and slope. The full cross-validation (leave-one-out, with standard error of cross-validation SECV) and the residual predictive deviation (RPD = SD/SECV; Cozzolino et al. 2011; Fearn 2002; Naes et al. 2002; Williams 2001) were used to classify the PLS1 calibration models. The PLS1 validation was performed using the spectra of the training set of the skin extracts, separately for the white and red extracts. The PLS1 validation statistics were as follows: the coefficient of determination (R^2), standard error of prediction (SEP), bias and slope, as well as the relative standard deviation (RSD = (SEP/mean) × 100; Cozzolino et al. 2011).

The authors used R^2, RPD and RSD to classify the efficacy of the models. They followed the approach presented in Fearn

(2002) and Williams (2001) which recommends that: (a) if $R^2 > 0.98$, then the model is usable in any applications; (b) if $R^2 = 0.92–0.96$ then the model is usable in most applications, including quality assurance; and (c) if $R^2 = 0.83–0.90$, then model is usable with caution for most applications, including research. When RPD was ≥ 3, the model is considered suitable for quantitative determination of chemical parameters (Fearn 2002; Williams 2001). Finally, when RSD is < 20%, the model is classified as adequate to predict chemical parameters (Cozzolino et al. 2011).

Results and Discussion

ATR-MIR Spectra

The ATR-MIR spectra in the whole range of 4000–400 cm^{-1} of the solvents used for phenolic extractions (Fig. A.1, Appendix A, Supplementary data) and of the gallic acid ethanol aqueous solutions used for the TP calibration curve (Fig. A.2. Appendix A, Supplementary data) were investigated in order to find the regions in which ethanol, water and gallic acid absorbed. The following five concentrations of ethanol in aqueous solutions: 30%, 40%, 55%, 70% and 80% (Table 1) were used during the RS-optimisation of the MAE and CTE methods, and their raw spectra and second derivative (Savitzky-Golay transformation, 20 points), as well as the spectra of water and ethanol 100%, are presented respectively in Fig.A.1(a) and Fig.A.1(b). In Fig.A.1. (a), two groups of the spectra ranges could be identified: (I) 4000–3000 cm^{-1}, 2600–1500 cm^{-1} and 800–400 cm^{-1} representing the stronger water than ethanol absorption regions; and (II) 3000–2600 cm^{-1} and 1500–800 cm^{-1} representing the stronger ethanol than water absorption regions. In those groups, the following prominent peaks could be identified respectively at: (I) 3283 cm^{-1}, 2100 cm^{-1}, 1635 cm^{-1} and 409 cm^{-1}; (II) 3316 cm^{-1}, 2971 cm^{-1}, 2925 cm^{-1}, 2877 cm^{-1}, 2727 cm^{-1}, 1642 cm^{-1}, 1485 cm^{-1}, 1452 cm^{-1}, 1420 cm^{-1}, 1379 cm^{-1}, 1325 cm^{-1}, 1268 cm^{-1}, 1087 cm^{-1}, 1044 cm^{-1}, 879 cm^{-1}, 804 cm^{-1}, 635 cm^{-1} and 431 cm^{-1}. The corresponding peaks and troughs are presented in Fig. A.1. (b). The peaks in Fig. A.1. are in the agreement with Stuart et al. (1996) who reported that alcohols (and phenols) absorb in the fingerprint region of 1300–1000 cm^{-1}, as well as in the region of 3650–2500 cm^{-1}: the peaks in the range of 2962–2890 cm^{-1} represent asymmetrical and symmetrical C–H stretching in CH_3 and CH_2 groups, as well as the peaks at 1380 cm^{-1}, 1465 $cm^{-1}$and 1450 cm^{-1} represent C–H deformation in CH_3 and CH_2 groups. As reported in Stuart et al. (1996), the frequencies of bonds vibrations depend on the masses of the atoms in the bond and the bond stiffness, hence the importance of hydrogen bonding in infrared spectroscopy. In Fig. A.1. (b), the peak at 3687 cm^{-1} with the troughs at 3372 cm^{-1} and 3207 cm^{-1} (corresponding with the peak at 3283 cm^{-1} in Fig. A.1. (a)), as well as the trough at 1635 cm^{-1} (corresponding with the peak at 1635 cm^{-1} in Fig. A.1(a)) represent the hydrogen bonding regions of stronger absorption by the O–H group in water than in ethanol.

The raw spectra (Fig. A.2(a)) and their second derivative (Fig. A.2. (b and c)) of the 10% ethanol aqueous solutions of gallic acid at different concentrations (0.05–2.5 g GAE/L) were examined to identify the spectral regions in which phenolics absorbed. The whole range of the spectra 4000–400 cm^{-1} included the main phenolic region of 1500–1100 cm^{-1} reported by Cozzolino et al. (2011) and the region of 1457–1168 cm^{-1} chosen by Fragoso et al. (2011); the latter denoted further as the Fragoso range. For reference, the spectra of water and 100% ethanol are also included in Fig. A.2. The same two groups (I and II) of peaks related to the absorption regions of water and ethanol as discussed in Fig. A.1. can be identified in Fig. A.2. Additionally, the peaks' height is proportional to the gallic acid concentration. Due to a low concentration of ethanol in water (10%), gallic acid absorption is more prominent in the stronger water than ethanol absorption regions (I). Figure A.2. (c) presents the enlarged main phenolic region of the second derivative of the spectra. In general, the absorption peaks of gallic acid followed the absorption peaks of ethanol and water.

The identification of the phenolic absorption regions in the white and red skin extracts was performed by utilising the information obtained from Fig. A.1 and Fig. A.2. Figure 1 presents the second derivative of the spectra in the range of 4000–400 cm^{-1} for the white (Fig. 1a) and red (Fig. 1b) skin extracts obtained when applying the factors and their levels during the RS-optimisation phase (Table 1). The peaks and their height correspond with different concentrations of phenolics and ethanol in the solvent. The five groups of the spectra observed in Fig. 1 represent the five concentrations of ethanol in aqueous solutions. The spectra of the white and red skin extracts are very similar, and they contain similar peaks to those discussed in Fig. A.1. and Fig. A.2. The peak at 2358 cm^{-1}, which is only noticeable in the white skin extracts (Fig. 1a) in comparison with the spectra of the red skin extracts (Fig. 1b), is associated with the absorption of phenolics similar to the weak absorption of gallic acid around this region—too small to be prominent in Fig. A.2.

The change in the height of peaks in the spectra related to differences in the phenolic content is shown in Fig. 2. As an example, the CTE method was chosen when extracting phenolics at the practical optimal conditions over three consecutive extractions from the same portion of skins during the methods' validation phase (Table 1) to test the efficiency of both extraction methods for the white and red skin mixtures. The three consecutive extractions provided 70%, 20% and 10% of the total phenolic content, respectively, regardless of the extraction method and skin colour (Kwiatkowski et al. 2020). The difference in the peaks' height in Fig. 2 presents a similar relationship between the corresponding spectra of the

extracts obtained in the first, second and third (1st, 2nd and 3rd) extractions due to the differences in TP, confirming the regions in which phenolics absorb. The difference was particularly notable in the region of stronger water than ethanol absorption (I), because the ethanol concentration was only 10% of the aqueous solutions.

Qualitative Analysis (PCA)

Based on the discussion above, the following spectra ranges were chosen for further qualitative principal component analyses (PCA): (a) full range of 4000–400 cm^{-1}; (b) main phenolic (fingerprint) range of 1500–1100 cm^{-1}; (c) range of 1457–1168 cm^{-1} selected by Fragoso et al. (2011); and (d) two additional ranges of 4000–1100 cm^{-1} and 1500–400 cm^{-1} extending the main phenolic region towards the upper and lower bands of the full range of spectrum.

The principal component analyses were applied to the raw spectra and their second derivative (Savitzky-Golay transformation, 20 points) to find the trends and similarities between the spectra for different treatments (i.e., the five factors at different levels according to DoE), as well to identify outliers. These analyses were separately conducted for the white and red skin extracts during all the phases of DoE (data not shown). This section presents a general discussion of the results for all PCA models. A detailed discussion of the PCA for the region chosen as most suitable to predict TP and *C** is provided in a separate "PCA and PLS1 for the Best Spectral Region 4000–1100 cm^{-1}"section below.

The first two principal components (PCs) explained close to 100% of the variation among the treatments, regardless of the spectral range, skin colour and, whether the raw spectra or their second derivative were used. The ethanol-water concentration in aqueous solutions was the main factor separating the treatments (explained by PC1), followed by pH and LS ratio (both explained by PC2); these findings were in a good agreement with those presented in Kwiatkowski et al. (2020). For all spectra ranges, the PCA scores plots were characterised by six clusters for the white skin extracts and five clusters for the red skin extracts. The number of clusters was related to the number of the different concentrations of ethanol in aqueous solutions used in all phases of DoE (Table 1). The height of the PCA loadings (particularly explained by PC2 and PC3) around the peak 2358 cm^{-1} and the associated with it trough at 2390 cm^{-1} were larger for the white than for the red skin extracts, supporting our conjecture that this region was related to phenolics in larger abundance in the white than in the red skins (Fig. 1).

Quantitative Analysis (PLS1)

The partial least squares (PLS1) regression models were built between the TP and *C** data of the skin mixture extracts collected during all phases of DoE, to predict TP and *C**using the ATR-MIR spectra (raw and second derivative) at the same ranges as chosen for the PCA analyses. The PLS1 models were created separately for the white and red skin mixture extracts due to matrix differences. The number of extracts was 240 for each skin mixture (i.e. 96 extracts from advanced screening, 120 extracts from RS-optimisation and 24 extracts from the validation for both methods, Table 1): a half of the extracts was used for the PLS1 calibration (i.e. a training set, number of samples $n = 120$) and the other half for the PLS1validation (i.e. a test set, $n = 120$).The calibration and validation statistics for those models, as well as the values of the standard error of the laboratory methods (average of the MAE and CTE errors), are presented in Table 2.

For a given spectra range, only the model performing better (i.e. using either raw spectra or their second derivative) is presented herein. In most cases, the TP and *C** models based on the second derivative provided better results in terms of RPD, SECV, R^2, SEP and RSD. The models based on the range of 4000–1100 cm^{-1} provided the best predictions for TP and *C** for both the white and red skin extracts, except for TP for the red skin extracts, for which RPD, SECV and RSD were very close to the corresponding values for the best models based on the ranges of 1500–1100 cm^{-1} and 1457–1168 cm^{-1}. Hence, we chose the models based on the second derivative of the spectra in the range of 4000–1100 cm^{-1} as best predicting both TP and *C**. Since RPD for this range was ≥ 3.0, but less than 4.9 for the four models (i.e., TP and *C** for white skins, and TP and *C** for red skins), the models can be used for fair classification, and hence screening (Williams 2001). Those models for which RSD < 20% were adequate to predict TP and *C** (Cozzolino et al. 2011).

PCA and PLS1 for the Best Spectral Region 4000–1100 cm^{-1}

The PCA score plots obtained when using the ATR-MIR spectra at the range 4000–1100 cm^{-1} chosen as the best to predict TP and *C** for the white (Fig. 3a) and red (Fig. 3b) skin extracts presented a good discrimination among the treatments. The first two components explained 99% of the variation among the treatments, regardless of the skin colour. The ethanol concentration in aqueous solutions was the main factor affecting the separation of the treatments, followed by the LS ratio, temperature, pH and extraction time. It was in a good agreement with the findings in Kwiatkowski et al. (2020) in which the four factors except the extraction time were found significantly ($p \leq 0.05$) affecting TP and *C**. The PCA scores for MAE and CTE methods with the corresponding treatments (i.e. at the same levels of factors except time) are close to each other within each cluster in Fig. 3, confirming the similarities in the TP and *C** results obtained under both extraction methods. The PC1 and PC2 loadings (not shown) indicated the strongest ethanol absorption in the ranges of 3000–2700 cm^{-1} and

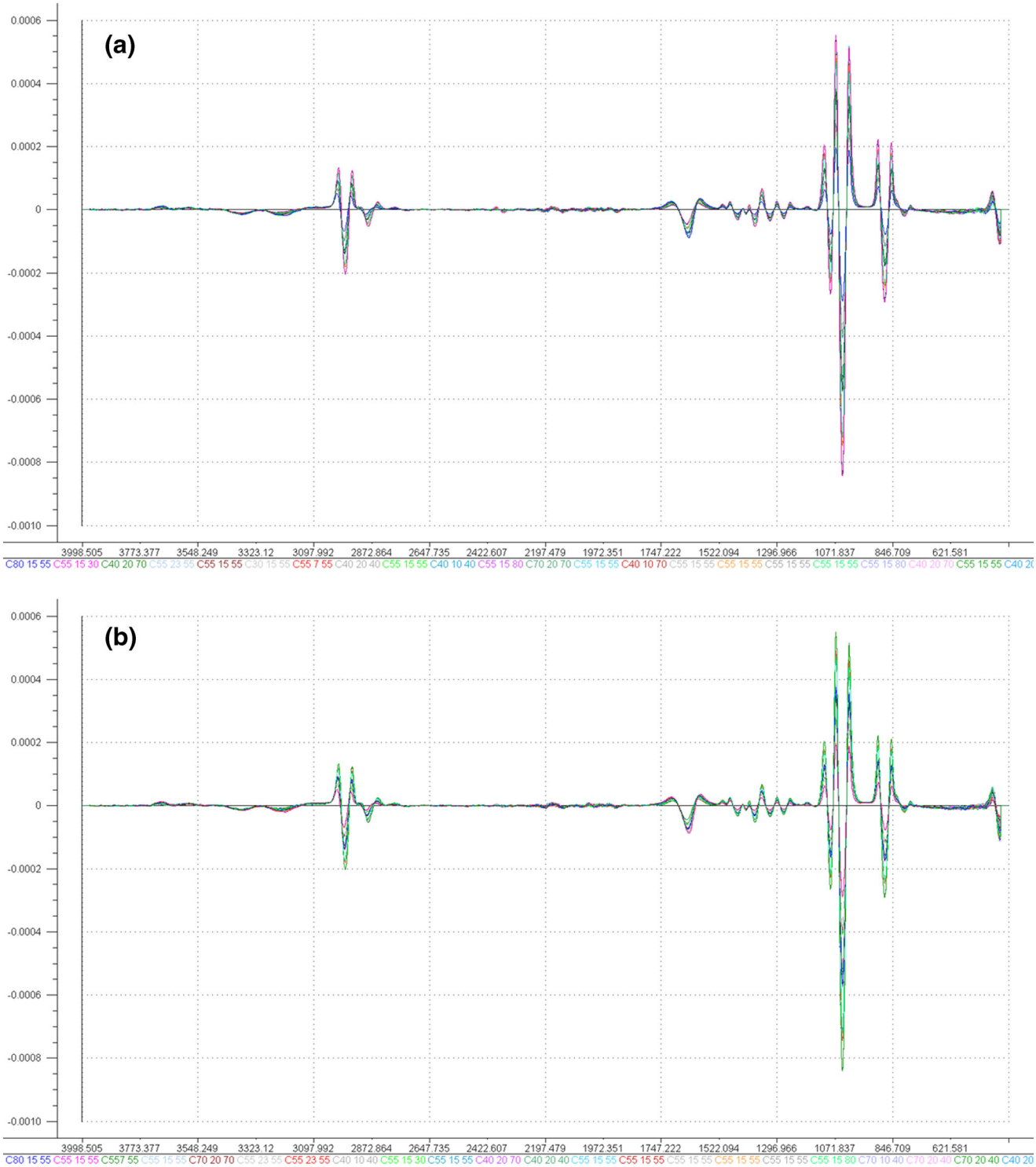

Fig. 1 Second derivative of the ATR-MIR spectra at the range of 4000–400 cm^{-1} of the white (**a**) and the red (**b**) grape skin mixture extracts, (number of samples $n = 120$, each), obtained under MAE and CTE methods during the RS-optimisation phase. Sample code 'C80 15 55' means that the extract was obtained using CTE method, 80% ethanol aqueous solution, liquid:solid ratio LS 15 mL/g GS and temperature 55 °C

1500–1100 cm^{-1}. The PC3 loadings (not shown) for the white skin extracts indicated prominent signals at 2358 cm^{-1} and the associated with it at 2390 cm^{-1} i.e. the region we conjecture represented the phenolic absorption specific for the white skins, as presented in Fig. 1a, in which phenolics absorbed stronger for the white than for the red skin extracts.

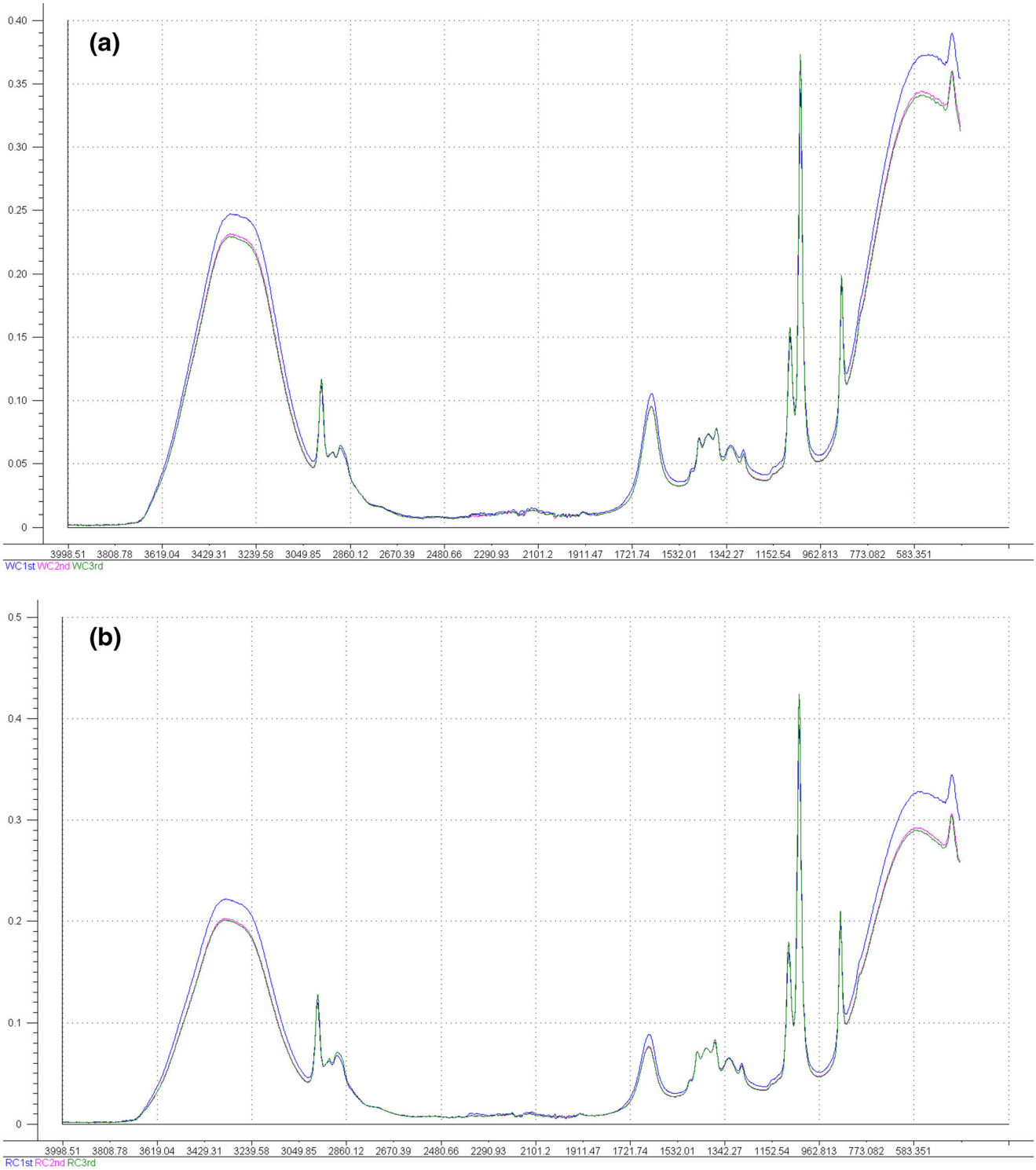

Fig. 2 Averaged (number of replicates $n = 4$) ATR-MIR raw spectra at the range of 4000–400 cm^{-1} of the white (**a**) and of the red (**b**) grape skin mixture extracts, obtained over three consecutive extractions (1^{st} (in blue), 2^{nd} (in pink) and 3^{rd} (in green)) during the validation phase of the CTE method

The PLS1 regression coefficients (five PLS terms) for TP and *C** obtained from the calibration sets for the white skin extracts are presented respectively in Fig. 4a and b, while for the red skin extracts in Fig. 4c and d. The regions of the phenolic absorption overlapped with the regions of water and ethanol absorptions identified in Fig. A.1. and Fig. A.2 (Appendix A, Supplementary data). The prominent peaks, discussed below, were identified at similar regions for both TP and *C** for the white and red skins.

Table 2 PLS1 model statistics (number of samples for calibration and validation $n = 120$, and latent variables $T = 5$, each) for total phenolics (TP) and chroma (C^*) for the white skin mixture and the red grape skin mixture extracts, respectively, by the ATR-MIR spectra in chosen ranges using second derivative in most cases (raw spectra were used instead of their second derivative). The models were based on the combined data for the extracts collected during advanced screening, RS-optimisation and validation studies of the MAE and CE methods

Variable	Spectra range	PLS1 calibration (training set)								PLS1 validation (test set)				
		Mean	Min.–max.	SD	R^2	Slope	Bias	SECV	RPD	R^2	Slope	Bias	SEP	RSD
White skin mixture														
TP[d]	4000–400	0.78	0.15–1.77	0.33	0.88	0.88	0	0.12	2.9	0.86	0.84	0.02	0.13	16
	4000–1100				0.90	0.90	0	0.11	3.1	0.84	0.83	0.02	0.13	17
	1500–400				0.89	0.89	0	0.11	3.0	0.83	0.84	0.33	0.14	20
	1500–1100[b]				0.87	0.87	0	0.12	2.7	0.85	0.88	0.02	0.13	17
	1457–1168[a, c]				0.87	0.87	0	0.12	2.7	0.79	0.83	0.01	0.16	20
C^*[d]	4000–400	30.85	5.47–76.66	12.47	0.90	0.89	0	4.01	3.1	0.85	0.76	− 0.06	5.40	18
	4000–1100				0.90	0.90	0	3.94	3.2	0.84	0.79	− 0.38	5.33	17
	1500–400				0.90	0.90	0	4.02	3.1	0.84	0.84	0.06	5.45	18
	1500–1100[b]				0.87	0.87	0	4.52	2.8	0.86	0.86	− 0.59	5.08	16
	1457–1168[c]				0.88	0.88	0	4.34	2.9	0.86	0.86	0.00	4.77	15
Red skin mixture														
TP[d]	4000–400	0.86	0.21–1.80	0.37	0.90	0.90	0	0.12	3.2	0.69	0.92	0.02	0.14	16
	4000–1100				0.90	0.90	0	0.12	3.1	0.83	0.87	0.04	0.14	16
	1500–400				0.88	0.88	0	0.13	2.9	0.83	0.88	0.02	0.15	17
	1500–1100[b]				0.90	0.90	0	0.12	3.2	0.86	0.99	0.00	0.13	15
	1457–1168[c]				0.90	0.90	0	0.12	3.2	0.88	0.93	−0.01	0.13	15
C^*[d]	4000–400	50.45	1.84–77.08	21.94	0.91	0.91	0	6.65	3.3	0.88	0.85	−0.49	7.53	15
	4000–1100				0.93	0.93	0	5.75	3.8	0.90	0.87	0.58	6.91	14
	1500–400[a]				0.90	0.90	0	7.03	3.1	0.95	0.89	−1.15	6.76	13
	1500–1100[b]				0.85	0.85	0	8.27	2.7	0.86	0.81	−1.90	8.15	16
	1457–1168[a, c]				0.90	0.90	0	6.91	3.2	0.92	0.89	−1.68	6.32	13

[a] Raw spectra were used instead of their second derivative

[b] Main phenolic spectra range

[c] Selected by Fragoso et al. (2011) spectra range

[d] Averaged (combined MAE and CTE) standard error of the laboratory method SEL was 0.04 g GAE/L for TP and 0.64 for C^* for the white skin mixture, and 0.03 g GAE/L for TP and 0.92 for C^* for the red skin mixture

R^2, coefficient of determination; *SECV*, standard error in cross-validation; *RPD* = *SD/SECV*, residual predictive deviation; *SEP*, standard error of prediction; *RSD* = *SEP/mean*, relative standard deviation

The highest regression coefficients for both TP and C^* were observed in the main phenolic range of 1500–1100 cm^{-1} (stronger ethanol than water absorption, group II) and in the range of 1800–1500 cm^{-1} (stronger water than ethanol absorption, group I). Regarding the former, the large peaks were at 1100 cm^{-1}, 1176, cm^{-1}, 1260 cm^{-1}, 1356 cm^{-1} and 1448 cm^{-1}. Those peaks could be associated (Stuart et al. 1996) with aromatic rings (C–H in-plane bending at 1300–1100 cm^{-1} and ring stretching at 1450–1500 cm^{-1}), alcohols and phenols (C–OH at 1300–1100 cm^{-1}), as well as carboxylic acids (C–O–H at 1400 cm^{-1} and C–O at 1300–1200 cm^{-1}). The peaks in the range of 1500–1100 cm^{-1} identified in Fig. 4 were similar to those reported by other authors (Musingarabwi et al. 2016; Ristic et al. 2016; Cozzolino et al. 2011; Fragoso et al. 2011).

The peak at 1650 cm^{-1} could be linked with the C=C stretching band, overlapping with strong absorption of the C=N stretching band (Stuart et al. 1996). The regression coefficients at 1725 cm^{-1} represented the absorption of the C=O group stretching associated with carboxylic acids (Cozzolino et al. 2011; Shah et al. 2010; Stuart et al. 1996) and it showed a strong impact of pH (either pH 1.5 or pH 3.5, Table 1) on phenolic extraction. The higher values of TP and C^* were obtained for the skin extracts at pH 1.5 than at pH 3.5 (Kwiatkowski et al. 2020).

The regression coefficients in the range of 2200–2000 cm^{-1}, particularly at 2136 $cm^{-1}$and 2197 cm^{-1}, were larger for the red than for the white skin extracts. The peak at 2136 cm^{-1} was related to the combination of the fundamental bands for C–H bending at 730 cm^{-1} and C–C stretching at 1400 cm^{-1} (Stuart

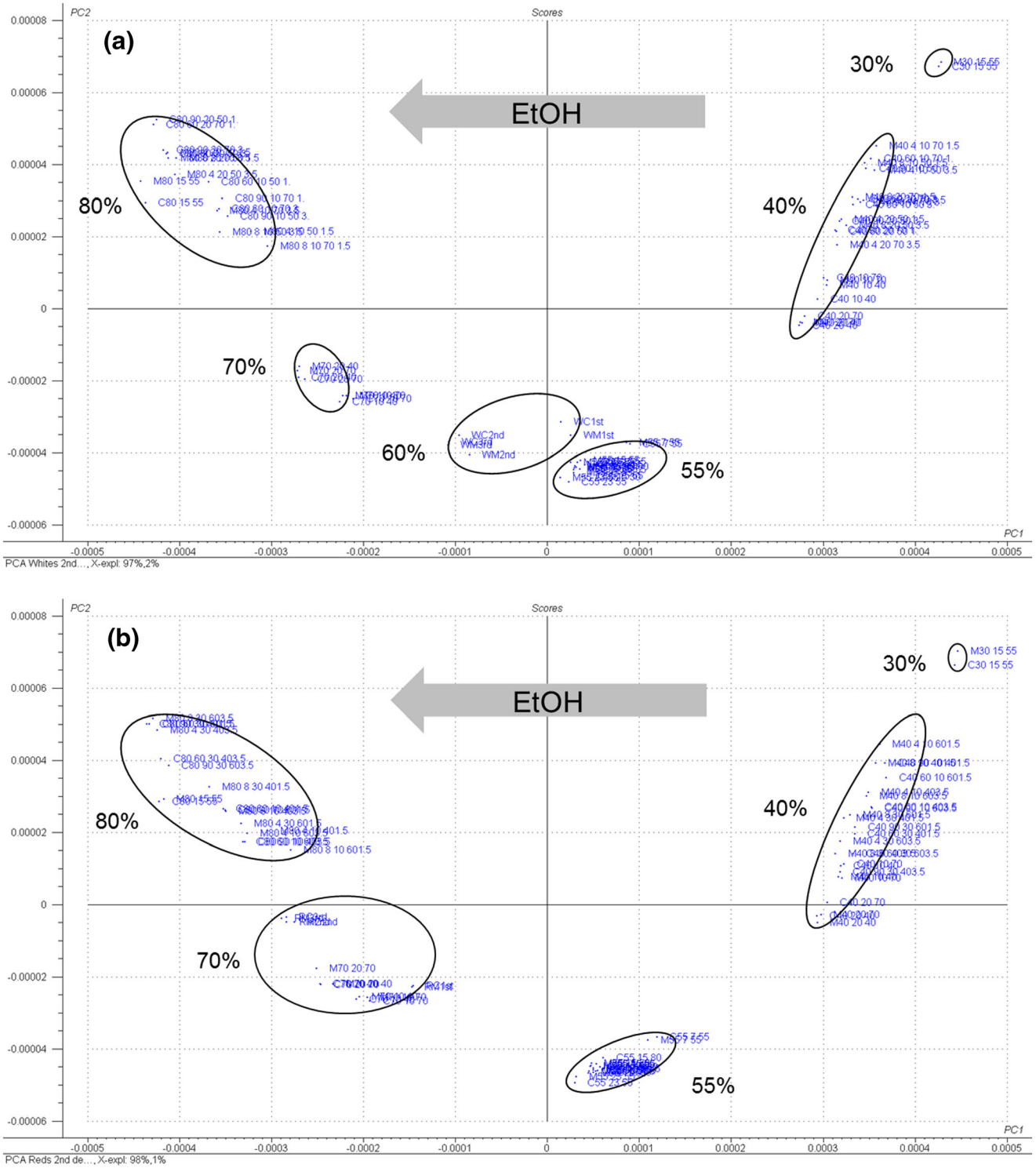

Fig. 3 PCA scores plots of the white (**a**) and red (**b**) grape skin mixture extracts, obtained using the second derivative of the ATR-MIR averaged spectra at the best range of 4000–1100 cm^{-1} during advanced screening, RS-optimisation and validation phases of the MAE and CTE methods. In the validation of the MAE (M) and CTE (C) phase, the 1st, 2nd and 3rd extractions were used for the white (W) and of the red (R) grape skins, respectively. Sample code 'M80 8 10 70 1.5' means that the extract was obtained using the MAE method, 80% ethanol aqueous solution, extraction time 8 min, LS 10 mL/g GS, temperature 70 °C and pH 1.5

et al. 1996). Similarly, the regression coefficients at 3000 cm^{-1} and 2940 cm^{-1} are larger for the red than for the white skin extracts, indicating a strong impact of ethanol on the TP and *C** predictions, however not as strong as at 1100 cm^{-1} and 1148 cm^{-1}. The peak at 2940 cm^{-1} represents the fundamental band for the C–H stretching of aromatics.

The range of 3700–2500 cm^{-1} was mainly related to the absorption of the O–H band in alcohols and phenols (Stuart et al. 1996; Gorinstein et al. 1993). In particular, the peak at 3400 cm^{-1} was associated with the O–H stretching in the COOH group, and the peak at 3680 cm^{-1} could be linked to the combination bands of C–H bending and C–H stretching.

The peaks between 4000 and 3000 cm^{-1}, as well as at 1640 cm^{-1}, could be related to the absorption of phenolics responsible for the colour of the white and red skins. Those peaks were larger for the red than for the white skin extracts, probably due to the presence of anthocyanins in the red skin extracts. The spectra of the Malvidin-3-glucoside (most abundant anthocyanin in red skins) 0.4 g/L in 0.05% acetic acid aqueous solution exhibited absorption (data not shown) related only to the regions of stronger water than ethanol absorption (group I), as anthocyanins were the water-soluble phenolics.

Figure 4 also showed the absorption region at 2358 cm^{-1}, which was associated with the phenolic absorption region when discussing Figs. 1a and 3. The peak at 2380 cm^{-1}, associated with the trough at 2358 cm^{-1}, was probably linked with the combination bands of C=N stretching (1650 cm^{-1}) and C–H bending (730 cm^{-1}). This combination overlapped the combination bands of C=C stretching (1650 cm^{-1}) and C–H bending (730 cm^{-1}). As expected, (Fig. 1), these regression coefficients at 2380 cm^{-1} and 2358 cm^{-1} were higher for the white than for the red skin extracts. In addition, the peak at 3840 cm^{-1} was probably associated with the combination bands of some phenolics (2380 cm^{-1}) with the first overtone C–H bending (1460 cm^{-1}); the peak was larger for the white than for the red skin extracts. Similarly, the peak at 3960 cm^{-1} could be associated with the combination bands (2380 cm^{-1}) and C=C stretching (1650 cm^{-1}) of some phenolics.

Future Trials

As the ATR-MIR technique is relatively new (Dambergs et al. 2015), the results of this study may help in better understanding of the applicability of the ATR-MIR spectroscopy in measuring phenolics and colour of the grape skin extracts. The methodology developed for the grape skin mixtures presented herein could be helpful in the future processing of skins isolated from pomace, as well as of whole pomace, in particular when procured after multivarietal winemaking. It could also help in further investigation of white grape skin colour, as well as differences between the white and red skins. The ATR-MIR methodology presented herein could be qualified as an efficient and environmentally friendly one. It can also be utilised in the future production of functional foods and nutraceuticals including natural colourants, the latter could be employed in dyeing of foods and textiles, on a laboratory, pilot and commercial scales when processing plant materials or the post-winemaking wastes. The methodology transfer on that subject was discussed in details in Kwiatkowski et al. (2020).

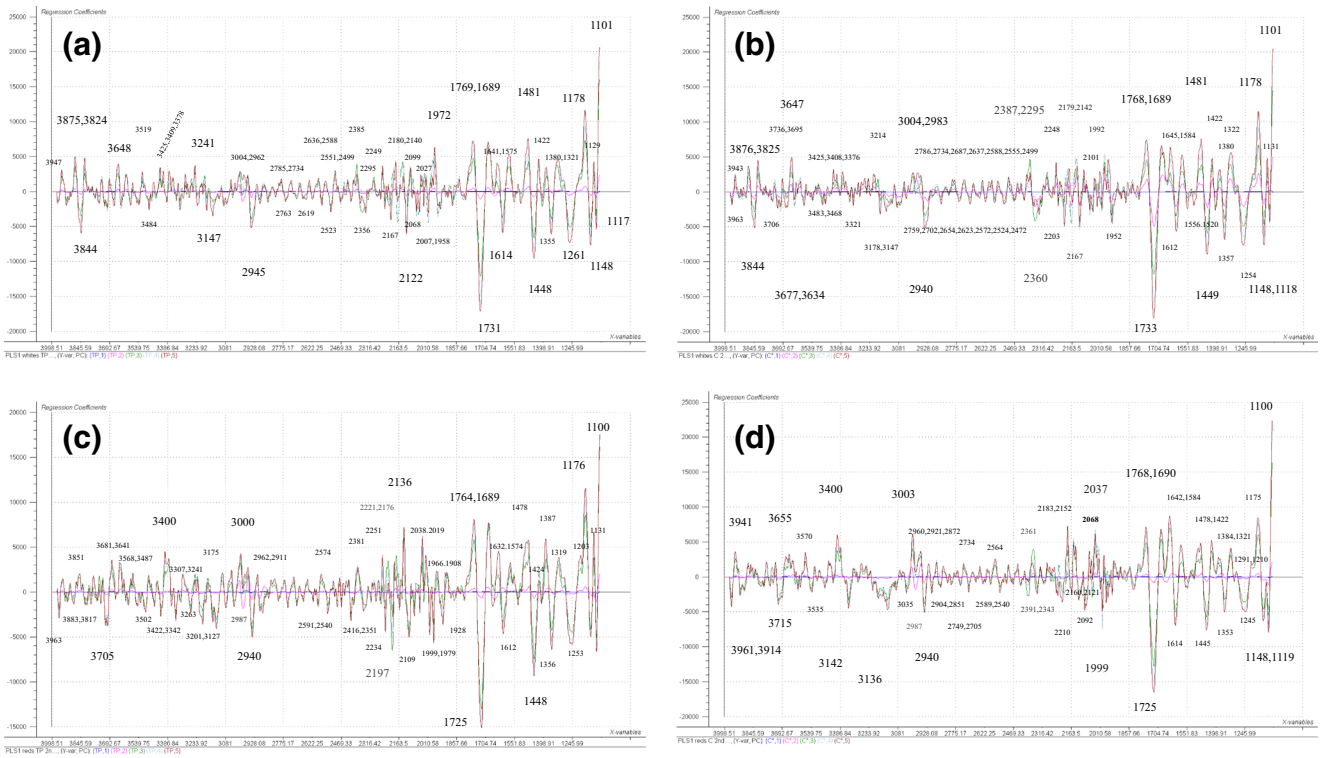

Fig. 4 Regression coefficients for PLS1 calibrations (training set) obtained using the second derivative of the ATR-MIR spectra in the best range of 4000–1100 cm^{-1} to predict TP (**a**) and *C** (**b**) of the white skin mixture extracts, and TP (**c**) and *C** (**d**) of the red skin mixture extracts, collected during advanced screening, RS-optimisation and validation of the MAE and CTE methods

Conclusions

This study presents a fast and simple ATR-MIR spectroscopy method developed as an alternative method for predicting the total phenolic content as well as colour (CIELAB chroma) of white and red skin mixture extracts. In comparison with the high-throughput, although still laborious, Folin-Ciocalteu reference method to measure total phenolics, the ATR-MIR spectroscopy produces adequate accuracy and can thus reduce time and costs of TP analysis.

To predict the total phenolic content and chroma of the extracts, a number of ranges of both raw spectra and their second derivatives have been investigated. The second derivative of the spectra 4000–1100 cm^{-1} provided most satisfying results, regardless of the skin colour. RPD for this range was ≥ 3.0, but less than 4.9 for the four responses of interest: TP and C^* for white skins, and TP and C^* for red skins. The models provide fair classification, and hence can be recommended for screening.

A comparative analysis between the spectra of white and red skin mixture extracts was performed for the chosen spectra range. It was found that some specific spectra ranges (i.e., 2200–2000 cm^{-1} and 4000–3000 cm^{-1}) as well as peaks (i.e., 1100 cm^{-1}, 1148 cm^{-1}, 1640 cm^{-1}, 2136 cm^{-1}, 2358 cm^{-1}, 2940 cm^{-1}, 3000 cm^{-1} and 3840 cm^{-1}) showed noticeable differences in absorption of phenolics and compounds responsible for the colour of white and red skins.

The authors plan to investigate the use of the ATR-MIR spectra to predict the total phenolic content and colour of grape skin extracts of single white and red grape cultivars. A more in-depth investigation of differences between the white and red skins in the identified specific spectral ranges and peaks is also planned. Finally, the authors intent to use the outcomes of this study to research the applicability of the ATR-MIR technique in the optimisation of industrial experiments in food industry and waste management.

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Compliance with Ethical Standards

Conflict of Interest Dr. Daniel Cozzolino declares that he has no conflict of interest. Mariola Kwiatkowski declares that she has no conflict of interest. Dr. Dennis K. Taylor declares that he has no conflict of interest.

Ethical Approval This article does not contain any studies with human or animal subjects.

Informed Consent (In case humans are involved) Informed consent was obtained from all individual participants included in the study. (If not applicable on the study) Not applicable.

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Chapter 4

Supplementary material

ATR-MIR Spectroscopy Predicts Total Phenolics and Colour for Extracts Produced by Microwave-Assisted or Conventional Thermal Extraction Methods Applied Separately to Mixtures of Grape Skins from White or Red Commercial Cultivars

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Appendix A

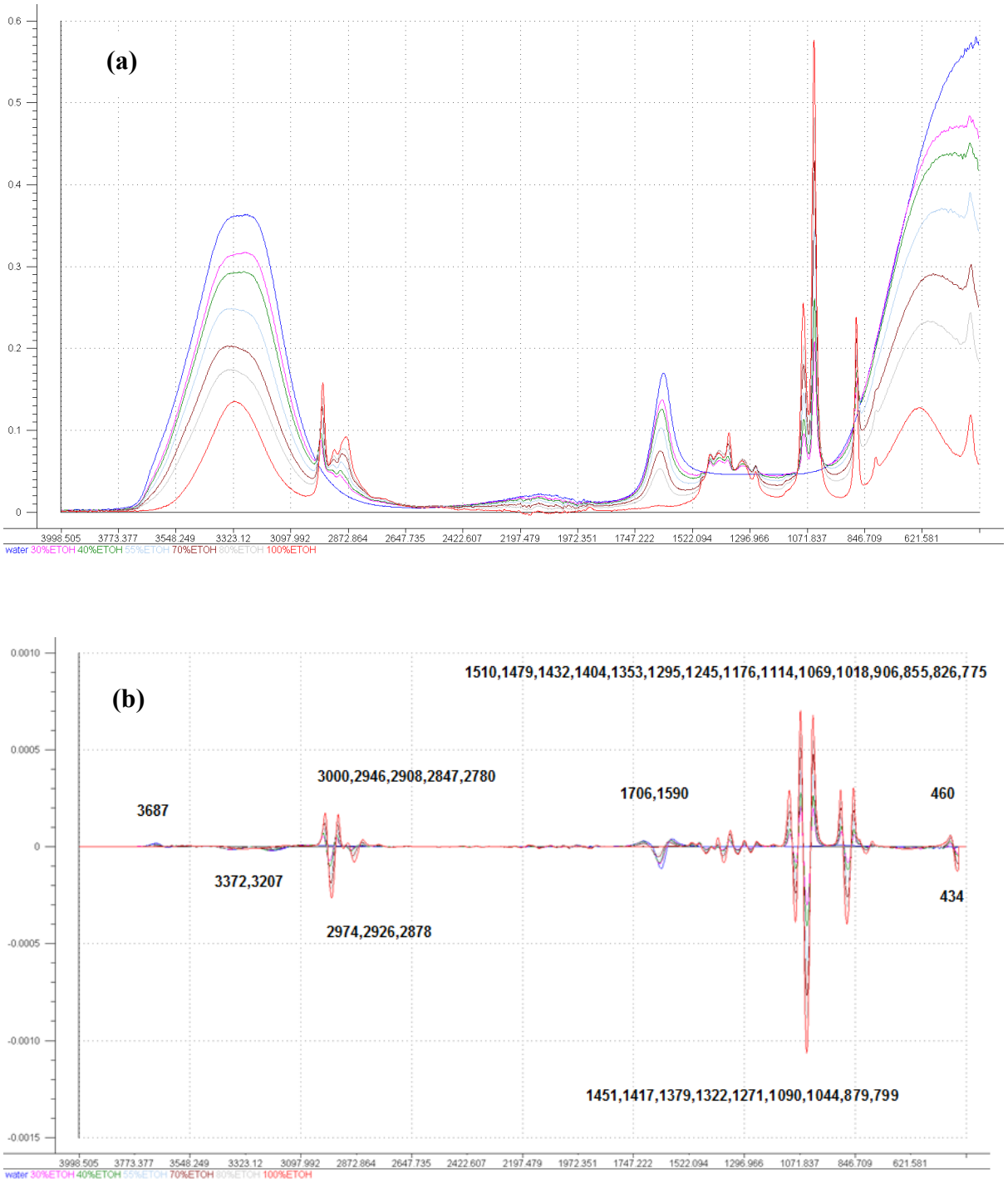
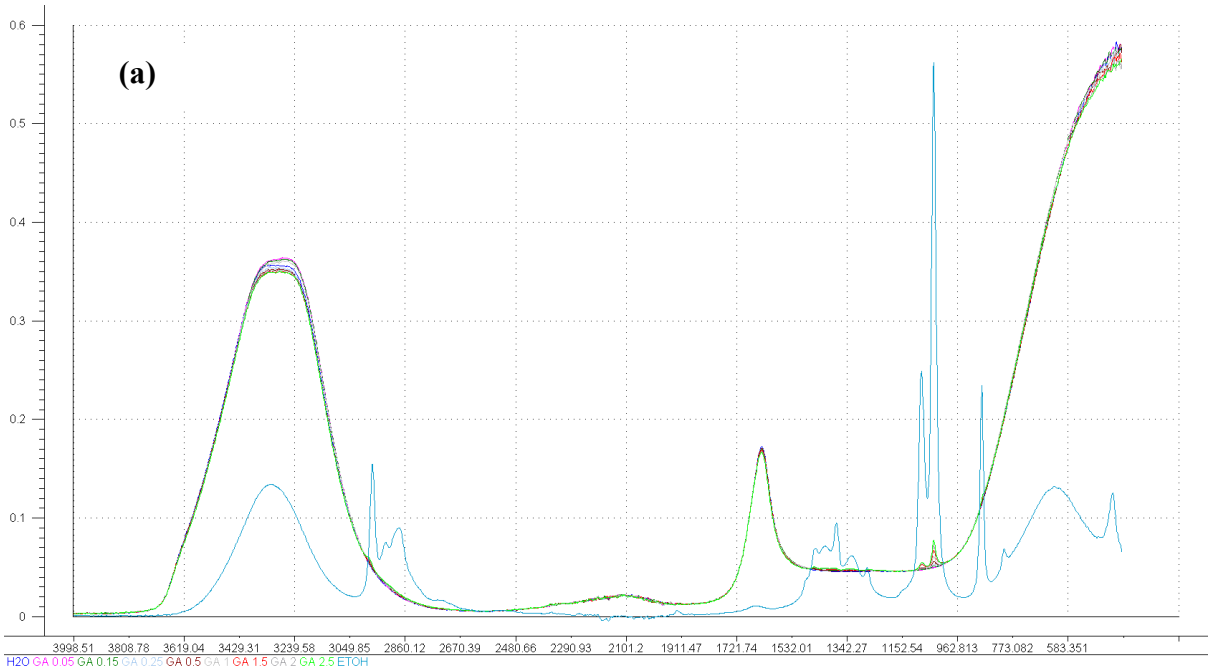
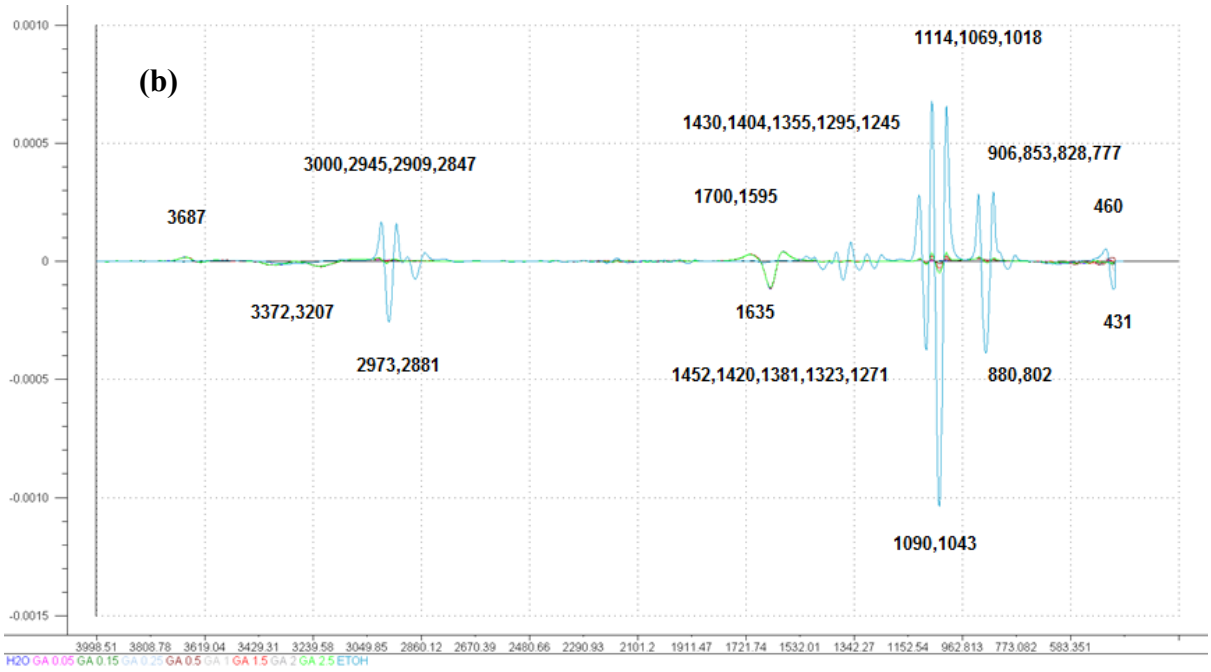


Fig. A.1. ATR-MIR spectra at the range of 4000-400 cm^{-1}: (a) raw and (b) second derivative, of the ethanol aqueous solutions at various concentrations (0, 30, 40, 55, 70, 80 and 100%) and pH 1.5, used in the RS-optimization phase of the MAE and CTE methods.


(a)
0.6
0.5
0.4
0.3
0.2
0.1
0
3998.51 3808.78 3619.04 3429.31 3239.58 3049.85 2860.12 2670.39 2480.66 2290.93 2101.2 1911.47 1721.74 1532.01 1342.27 1152.54 962.813 773.082 583.351
H2O GA 0.05 GA 0.15 GA 0.25 GA 0.5 GA 1 GA 1.5 GA 2 GA 2.5 ETOH


(b)
0.0010
0.0005
0
-0.0005
-0.0010
-0.0015
1114,1069,1018
1430,1404,1355,1295,1245
906,853,828,777
3000,2945,2909,2847
1700,1595
460
3687
3372,3207
1635
431
2973,2881
1452,1420,1381,1323,1271
880,802
1090,1043
3998.51 3808.78 3619.04 3429.31 3239.58 3049.85 2860.12 2670.39 2480.66 2290.93 2101.2 1911.47 1721.74 1532.01 1342.27 1152.54 962.813 773.082 583.351
H2O GA 0.05 GA 0.15 GA 0.25 GA 0.5 GA 1 GA 1.5 GA 2 GA 2.5 ETOH

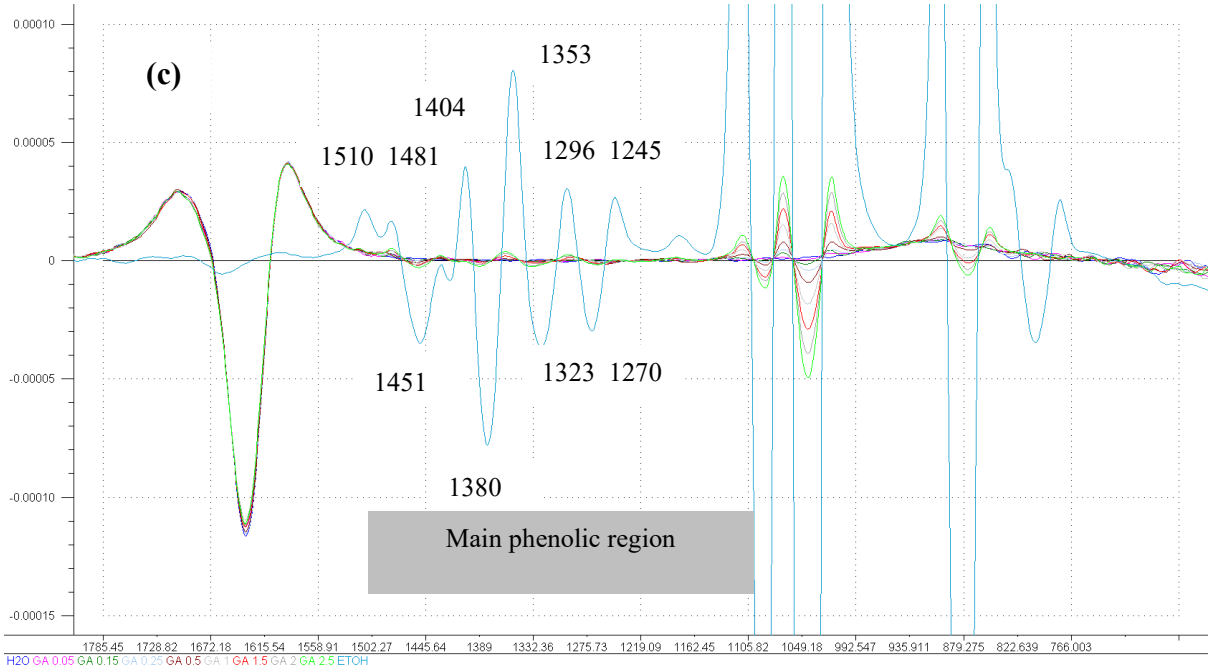


Fig A.2. ATR-MIR spectra at the range of 4000-400 cm^{-1}: (a) raw and (b and c (enlarged main phenolic region)) second derivative, of water, 100% ethanol and of the 10 % ethanol aqueous standard solutions of gallic acid (0.05, 0.15, 0.25, 0.5, 1.0, 1.5, 2.0 and 2.5 g GAE/L) used for the standard curve in TP measurement.

Chapter 5

Thesis Conclusions, Contribution to Knowledge and Future Directions

Thesis Conclusions

This thesis presents an investigation of how to extract maximal content of phenolic compounds from grape skins using microwave technology. Additionally, active research on phenolics in grapes was undertaken, as well as new robust analytical methods being developed. As presented in Chapter 1, to date, there have been many studies undertaken to extract phenolics employing conventional and novel methods. However, no extensive research has been done on novel versus conventional extraction methods, optimised parallelly using response surface methodology and sharing the same protocol, to find the conditions to maximise total phenolics and colour extracted from white and red skins. To this end, Chapter 1 of this thesis comprises a detailed summary of our current understanding on how phenolics and the compounds responsible for the skin colour can be extracted from white and red grape skins.

The first major study compared a microwave-assisted extraction (MAE) with a conventional thermal extraction (CTE) method in terms of total phenolic (TP) content and colour (CIELAB chroma *C**) from separate white and red skin mixtures of six commercial grape cultivars collected at veraison and harvest, each. The design of experiment (DoE) was based on five experimental factors: ethanol content, liquid to solid ratio, temperature, time and pH, which were investigated through preliminary, advanced screening, response surface (RS) optimisation and the model validation phases. The comparison of methods was done under the

optimal practical conditions for each method, which were identified and validated in this research.

Highlights

- To the best of our knowledge, no previous studies have presented MAE and CTE methods, both parallelly optimised using RS methodology, and sharing the same protocol in order to minimise the method-to-method experimental error, in extracting TP and *C** separately from white and red grape skin mixtures.
- A 2^3 central composite RS-optimisation was employed to find the optimal conditions for maximising TP, and to save time and resources, to assess the maximum *C** using the models built for maximising TP.
- A high throughput Folin-Ciocalteu assay utilizing 96-well plates and a liquid delivering robot was especially developed in this study to measure TP of skin extracts.
- A fast and simple CIELAB tristimulus method was used for the colour measurement of the skin extracts.
- The validation phase confirmed the reproducibility of a single extraction at the optimal practical levels chosen for both methods after taking into account practical constraints of safety and thermal stability of components.
- While the efficiency of a single extraction was comparable, the extraction time required for MAE was 15-fold shorter (4 min instead of 60 min), and 2.7-fold more energy efficient (63W instead of 167W for the equipment used in the study) than for CTE. Hence the MAE method was found more carbon friendly, i.e. “cleaner and greener”, than the CTE method.

Chapter 5

Continuing the research by Kwiatkowski et al. (2020), **the second study** demonstrated the application of the MAE and CTE methods at the optimal practical conditions found using RS-optimized for maximum TP and *C** in the first study. Both methods were compared in terms of TP and *C** extracted from the skins of six white and six red skin commercial cultivars collected at veraison and harvest under the optimal practical conditions for each method, which were identified for skin mixtures in the previous research. Adding to the extremely limited information in the literature for white and red commercial cultivars worldwide, the current study quantified the variation in phenolics and colour in individual skin extracts, which could be attributed to the joint effects of extraction method, cultivar and berry development. For each method, the discrepancies were discussed between the average TP (and *C**) obtained for individual skin extracts and the TP (and *C**) extracted from the corresponding white or red skin mixtures. Another aim of this study was to find if the phenolics extracted from individual cultivars would simply add up when the extractions are done under the same fixed and controlled settings, optimized for the mixtures of skins.

Highlights

- To save time and resources, phenolics and colour were extracted from six white and six red skins of commercial cultivars collected at veraison and harvest utilizing the MAE and CTE methods developed for the skin mixtures (veraison and harvest combined).
- The extraction efficiencies were found comparable for microwave and conventional methods. For white skins, CTE was statistically ($p < 0.01$) more efficient (15%) than MAE in phenolic and colour extraction (if veraison and harvest were combined), which was in agreement with Kwiatkowski et al. (2020). However, the method effect was not significant for TP in red skins, and TP under CTE was only 4% larger (in

comparison to 11% in Kwiatkowski et al. (2020)); consistently with Kwiatkowski et al. (2020), no statistical difference was found in colour extraction (except that for Shiraz, *C** under CTE exceeded by 19% *C** under MAE).

- For red cultivars except Grenache, the average results for TP and *C** (at harvest only) were aligned along the results for TP and *C** of the red skin mixture (veraison and harvest combined). For the white cultivars the average results for TP for Chardonnay (veraison and harvest), Gewürztraminer (harvest) and for Riesling, and Semillon (veraison), were well aligned with the average TP results for skin mixture; and for *C** at harvest: under CTE Viognier and Semillon, and under MAE Sauvignon Blanc and Riesling, were well aligned with the average *C** for the white skin mixture.
- Ranges of phenolics and colour were quantified among cultivars at veraison and harvest, with the potential value-returning winners for extracting maximum TP and *C**: under MAE, the potential value-returning winners for extracting maximum TP from white skins were Gewürztraminer at harvest and Sauvignon Blanc at veraison, and from red skins Merlot, Cabernet Sauvignon and Nebbiolo at harvest; for extracting colour, Chardonnay and Gewürztraminer, and Merlot, Shiraz and Cabernet Sauvignon, all at harvest were the potential value-returning winners.

The final study investigated the feasibility of attenuated total reflectance mid-infrared (ATR-MIR) spectroscopy to measure TP and *C** of the extracts obtained separately from white and red grape skin mixtures, each of six commercial cultivars collected at veraison and harvest, under MAE and CTE methods optimised for maximal TP content. This ATR-MIR method was developed after the advanced screening, RS-optimisation and methods' validation phases of the MAE and CTE methods were completed, during which the ATR-MIR spectra were

collected in order to find more robust analytical methods to speed up the TP and *C** analyses in future projects on phenolic extraction from grape skins.

ATR-MIR spectroscopy was chosen as the next step in obtaining a more robust method, particularly for TP analysis, with the TP results obtained using the high throughput, although still laborious, Folin Ciocalteu method employing a 96 well-plate with robotic manipulation as a reference method.

Highlights

- ATR-MIR scans were collected in the full range of 4000-400 cm^{-1} for 480 individual extracts from the white and red grape skin mixtures, and their TP and *C** were predicted from the spectra.
- Models built between the reference data for TP and *C** and the raw spectra or their second derivative (Savitzky-Golay, 20 points) were examined using the full range of spectra 4000-400 cm^{-1}, and sub-ranges of: 4000-1100 cm^{-1}, 1500-400 cm^{-1}, 1500-1100 cm^{-1} and 1457-1168 cm^{-1}.
- The PLS1 models built between the TP and *C** refrence data and the second derivative of the spectra range of 4000-1100 cm^{-1} provided the best results for both TP and *C**for each skin colour, offering fair classification, and hence suitable for screening purposes.
- To the best of our knowledge, no previous studies have reported the use of MIR spectroscopy to evaluate the TP content and CIELAB chroma *C** of skin mixture extracts obtained under RS-optimised MAE and CTE methods.
- Some spectral ranges and peaks have been identified to flag differences between white and red skins.

- ATR-MIR spectroscopy can be employed to replace the still laborious high throughput Folin-Ciocalteu method, as well as colour measurements using CIELAB chroma *C** on phenolics and colour extraction separately from white and red grape skin mixtures.

Contribution to Knowledge and Future Directions

The research reported in this thesis was targeted to investigate phenolic extracts using microwave technology. The new contributions of this thesis to knowledge with regard to future directions are as follows:

- It has been pointed out herein the importance of employing a multifactorial design of experiment (e.g. fractional factorial design in order to reduce the workload, and hence costs) and response surface methodology to find the optimal conditions for maximising phenolic content and colour extracted from grape skins using novel and conventional extraction methods.
- This research is novel in contrasting the performance of MAE, employing an industrial microwave reactor, to CTE when optimising all factors in order to maximize TP extraction levels, as well as utilizing the same protocols in order to minimise method-to-method experimental error.
- The ratio of MAE to CTE energy usage can be further improved if a more efficient industrial microwave, for example a multimode reactor CEM/MARS 6, is employed, in comparison to a monomode reactor CEM Discover S-Class, (CEM Co., USA) used in this thesis.
- Under the extraction settings maximising TP, this research further optimised conditions to maximize colour (chroma *C**) extraction from white and red grape skin mixtures separately, hence it has been cost effective.

- The methodology presented herein, with MAE as a superior extraction method, can be utilized in developing methodologies in phenolic and colour extractions in the following situations:
    - manufacturing supplements, food additives (incl. natural colourants), skincare and non-alcoholic beverages, all of great importance perceived by consumers in recent years,
    - viticultural trials conducted using commercial/boutique grape cultivars from different regions/countries in investigating the effects of terroir on phenolic content, which may help in winemaking decisions followed by consumer studies,
    - as well as grape plant materials, skins and whole fruit can be researched using our methodology.
- Following the Circular Economy recommendations (i.e. 'closing a loop'), as an alternative to the linear economy (i.e. raw materials used-product made-waste dumped) and to reduce landfill, the MAE methodology shown in this research may be relevant for future studies at laboratory, pilot and commercial scales on value-adding to white and red winemaking waste (pomace/marc accounts for approximately 10-30% of the mass of crushed grapes), in grape processing (particularly when grapes are in oversupply), as well as in emerging waste-to-energy developments.
- The results are of interest to researchers in grape waste management (post-winemaking and post-non-alcoholic production, food waste) as well as in oenology and manufacturing of nutraceuticals, supplements, skincare and textile dyes.
- The findings of this thesis may be also valuable in economic decision making about when (regarding the time-point of berry development) and which cultivar to choose for

future processing of skins and whole grapes, particularly when grapes are in over-supply.

- Using spectral assessment of phenolics would enable a quick process of optimization of extraction conditions.
- This thesis demonstrates that the valorization of skins would further add to bio-waste processing by enabling the extraction of cultivar-specific colour compounds and phenolics. These compounds would have high market value, as they are sought-after by the manufacturers of functional foods and nutraceuticals including natural food colourants.
- ATR-MIR spectroscopy can be employed to measure the phenolic content, as well as colour using CIELAB chroma *C**, for future research on phenolics and colour extraction separately from white and red grape skin mixtures. The PLS1 calibration and validation statistics for these models can be improved in comparison to those in this thesis when larger number of samples are used.
- Spectral ranges and peaks identified in this research can be applied in future in-depth investigations of differences between white and red skins, in particular of the compounds responsible for the colour of white skins.
- We plan to investigate the use of ATR-MIR spectra to predict TP and *C** of grape skin extracts of single white and red grape cultivars.
- Finally, the authors intend to use the outcomes of this study to research the applicability of the ATR-MIR spectroscopy in the optimisation of industrial experiments in the food, skincare, and dye industries and waste management.
- The methodology presented in this thesis can be utilized in the preliminary phase of future trials on extracting phenolics and colour from skins separated from pomace/marc

or whole pomace on a laboratory scale, followed by experiments on pilot and production scales.

- Future commercial scale waste management may be able to be based on microwave-assisted extractions as “green and clean” in manufacturing natural colourants, skincare and nutraceuticals.