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Development and validation of a simple high performance thin layer chromatography method combined with direct 1,1-diphenyl-2-picrylhydrazyl assay to quantify free radical scavenging activity in wine

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ABSTRACT

The aim of this study was to: (a) develop a simple, high performance thin layer chromatographic (HPTLC) method combined with direct 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay to rapidly assess and compare free radical scavenging activity or anti-oxidant activity for major classes of polyphenolics present in wines; and (b) to investigate relationship between free radical scavenging activity to the total polyphenolic content (TPC) and total antioxidant capacity (TAC) in the wine samples. The most potent free radical scavengers that we tested for in the wine samples were found to be resveratrol (polyphenolic non-flavonoid) and rutin (flavonoid), while polyphenolic acids (caffeic acid and gallic acid) although present in all wine samples were found to be less potent free radical scavengers. Therefore, the total antioxidant capacity was mostly affected by the presence of resveratrol and rutin, while total polyphenolic content was mostly influenced by the presence of the less potent free radical scavengers gallic and caffeic acids.

1. Introduction

Wine is one of the oldest alcoholic beverages with its documented use extending back to 6000 BC (Rai, 2012). Ancient Egyptian Papyri detail the medicinal role of wine, especially red wine, making it the world's oldest documented man-made medicine. Wine played a major role in medicine throughout history until the late 19th and early 20th century, when medical research linked health risks to alcohol consumption. However, more recently medical opinion has changed and it is thought that there are significant health benefits in moderate wine consumption, due to the observation that French people have one of the lowest rates of coronary heart disease despite their diet rich in high saturated fats in the form of cheese, butter, cream, eggs, liver, meat (the French paradox) (Renaud & de Lorgeril, 1992). According to the World Health Organisation coronary heart diseases are the leading cause of death worldwide (Guilbert, 2003). The health benefits of red wine are associated with the presence of polyphenols, such as phenolic acids (mainly gallic acid and caffeic acid), flavonoids such as rutin, and non-flavonoids such as resveratrol (Kammerer, Claus, Carle, & Schieber, 2004). Phenolic molecules are found in higher concentrations in grape skin and therefore larger quantities are present in red wines than white wines due to the increased contact time of grape skins with the juice during vinification.

Resveratrol was originally thought to be the phenolic compound mainly responsible for the cardioprotective effects of moderate red wine consumption. However, compared with other polyphenols, concentrations of resveratrol are so small that in order to obtain a functional level within the body one would have to consume large quantities of red wine (Gerogiannaki-Christopoulou, Athanasopoulos, Kyriakidis, Gerogiannaki, & Spanos, 2006). Gallic acid and caffeic acid are present at higher concentrations in all wine samples (Agatonovic-Kustrin, Hettiarachchi, Morton, & Razic, 2015) and therefore may significantly contribute to the total antioxidant properties of red wine.

A large number of studies on the analysis of grape and wine phenolic compounds have been published over the past four decades. The diversity of methods and experimental procedures used reflects the complexity of phenolic analytes in grapes and wine. Due to the relatively low concentration and structural diversity of phenolics in wine, most of the analytical methods that are used to detect and to quantify them are costly, time consuming, and require sophisticated instrumentation. For instance, high performance liquid chromatography (HPLC) in conjunction with high resolution detectors (e.g. nuclear magnetic resonance (NMR) and

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mass spectroscopy (MS)) would be the best choice (Singleton, 1988). HPLC methods are precise, accurate, specific and sensitive for the simultaneous determination of phenolic compounds in wine (Porgali & Büyüktuncel, 2012). However, long equilibrium periods for the system together with relatively long analysis times are the main limitations of this method. High performance thin layer chromatography (HPTLC) when compared to on-line column chromatography offers several advantages, such as flexibility due to off-line operation, simplicity, simultaneous visual evaluation and comparison of multiple samples, rapid results, and cost effectiveness (Urakova, Pozharitskaya, Shikov, Kosman, & Makarov, 2008). Also the possibility of multiple evaluations of the plate both before and after derivatization without running another set of samples on a new plate is an added advantage. In addition to R_F values, bands may be characterised visually by observing the nature of fluorescence of bands under UV–Vis light which is useful for characterising and analysing components in complex samples.

The aim of this study was to: (a) develop a simple, high performance thin layer chromatographic (HPTLC) method combined with direct DPPH assay to rapidly assess and compare free radical scavenging activity or anti-oxidant activity of caffeic acid, gallic acid, rutin and resveratrol as representatives of the four major classes of phenolics that are found in wines (hydroxycinnamic acids, hydroxybenzoic acids, stilbenes and flavanols); and (b) to investigate the relationship between free radical scavenging activity, total polyphenolic content (TPC), and total antioxidant capacity (TAC) in the wine samples. The DPPH free radical, is a stable free radical that can react with antioxidants present in a sample (Agatonovic-Kustrin, Morton, & Yusof, 2014). Normally it forms a deep violet solution that becomes pale yellow when it reacts with antioxidant compounds. This change in colour allows both the qualitative and quantitative analysis of antioxidant analytes.

2. Materials and method

2.1. Chemicals, solutions and samples

All samples and mobile phases were of Analytical Reagent grade. Wine samples were purchased from a local distributor and local supermarkets. Antioxidant standards, caffeic acid (98%), gallic acid (97%) and resveratrol (99%) were purchased from Sigma Chemicals (Balcatta WA, Australia), while rutin (97%) was purchased from Alfa Aesar (Ward Hill, Massachusetts). A gradient elution method was developed using the following mobile phase components: dichloromethane (Merck), methanol (Merck), formic acid (Ajax Chemicals), sodium dodecyl sulphate (Sigma), butanol (Chem supply), Milli-Q water (Millipore), and heptane (BDH). 2-Aminoethyl diphenylborinate (natural reagent) was purchased from Alfa Aesar (Heysham, UK) while DPPH was purchased from Sigma–Aldrich (Munich, Germany). 1 mg mL^{-1} standard solutions of resveratrol and 0.1 mg mL^{-1} standard solutions of caffeic acid, gallic acid and rutin, were made using absolute ethanol. A total of 24 wine samples (22 red wines and 2 white wines) were collected from different regions of Australia and overseas. Samples of wine were used without any pre-treatment. All samples and standards were refrigerated at 4 °C to prevent degradation (Spangenberg, Poole, & Weins, 2011). A 1% (w/v) 2-aminoethyl diphenylborinate solution and a 0.4% (w/v) DPPH solution were prepared in methanol, stored at 2–8 °C, and protected from light.

2.2. HPTLC procedure

HPTLC was performed on HPTLC Silica gel 60 F_{254} 20 × 10 cm glass plates (Merck, Germany). First, the plates were prewashed with methanol and then activated by placing in an oven at 105 °C for 15 min. Samples (10 μL of each wine sample and varying volumes of standards) were then sprayed onto the plates as10 mm narrow bands using a 100 μL syringe with a semi-automatic sample applicator, Linomat 5 (CAMAG, Muttenz, Switzerland), 8 mm from the lower edge, with 10 mm distance from each side, and a track distance of 7 mm (14 applications per plate). Three replicates of each standard were applied to the plate at low, medium and high volumes. In order to minimise errors arising from inter-plate variability due plate development and derivatization, bands containing caffeic acid, gallic acid, rutin, and resveratrol standards were included on each plate together with the wine samples.

2.3. HPTLC plate development and visualisation

HPTLC plates were developed in an Automated Multiple Development Chamber (AMD2, CAMAG) by the use of two step elution method with developing distance of: (a) 90 mm for step 1; and (b) 50 mm for step 2. Step 1 was performed with dichloromethane: methanol: formic acid (7: 20: 7) as mobile phase and step 2 was a water-in-oil microemulsion consisting of sodium dodecyl sulphate: butanol: water: heptane (8 g: 25 mL: 8 mL: 160 mL).

Images of plates were captured using a TLC Visualizer (CAMAG, Muttenz, Switzerland) with a 12 bit camera (CAMAG) under UV light at 366 nm both before and after derivatization. Capture parameters (focal length, focus and aperture) were fixed to ensure the quality of images and reproducibility of results between plates. VideoScan Digital Image Evaluation software (CAMAG 2003) was used for quantitative evaluation of plates and to transform images into chromatograms. Plates derivatized by spraying with 1% (w/v) 2-aminoethyl diphenylborinate solution were heated at 50 °C in an oven for 30 min in order to complete derivatization after which the plate image was then taken for later analysis. As the reaction for plates derivatized with DPPH solution is time dependent and light sensitive, after spraying with DPPH solution all plates were stored in the dark for 30 min before plate images were taken for later analysis.

2.4. Method validation

The method used to determine the concentrations of caffeic acid, gallic acid, resveratrol and rutin in wine samples was validated according to the current International Conference on Harmonisation (ICH) guidelines (ICH, 2005). Note that for resveratrol, peak area was determined before plate derivatization, while caffeic acid, gallic acid and rutin peak areas were determined once the plate had been derivatized with 1% (w/v) 2-aminoethyl diphenylborinate solution. All plate images were recorded at UV 366 nm. The method was assessed based on linearity, specificity, precision, limit of detection (LOD), and limit of quantification (LOQ).

The working range of the method for determination of caffeic acid, gallic acid, resveratrol and rutin was assessed by plotting chromatographic peak areas versus amount of standard over a concentration range of 0.04–0.4 μg for caffeic acid; 0.15–1.5 μg for gallic acid; 0.2–6 μg for resveratrol and 0.02–0.4 μg for rutin. Linear ranges were established using the least squared method. Specificity was assessed by the capacity of the optimised mobile phase to separate the four standards. Repeatability was assessed by applying three repetitions of each standard at three different concentrations within the calibration curve. Variance between repetitions was expressed as a relative standard deviation (%RSD). The LOD was calculated by multiplying the standard deviation of multiple measurements ($n = 3$) by 3 and then dividing by the slope of the calibration curve (Rubinson, 1986) using Eq. (1).

$$\mathrm{LOD} = \frac{3 \times Sd}{\mathrm{Slope}} \quad (1)$$