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Improved HPLC method for rooibos phenolics targeting changes due to fermentation

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ABSTRACT

The phenolic composition of rooibos (*Aspalathus linearis*) herbal tea is inherently linked to its health-promoting properties. "Fermentation" (oxidation) develops the characteristic flavour and colour of the herbal tea product. Changes to the phenolic composition during fermentation include conversion of aspalathin, a dihydrochalcone unique to rooibos and the major bioactive compound, to eriodictyol-glucopyranoside isomers. These compounds have not been quantified in rooibos plant material to date, due to separation challenges. In this study an improved HPLC method using a core shell column was developed to provide effective separation of a phenolic precursor (enolic phenylpyruvic acid-2-*O*-glucopyranoside, PPAG) and 15 rooibos tea phenolics (aspalathin, four eriodictyol-glucopyranoside isomers and 11 other major phenolic compounds) in a reasonable time. Compounds were tentatively identified using authentic reference standards and/or tandem mass spectrometry. The HPLC method utilising diode-array detection for quantitation was validated. After evaluation of different extraction solvents, a 40% aqueous acetonitrile solution was deemed optimal for extraction of phenolic compounds from rooibos plant material. Green, semi-fermented and fermented plant material from 10 sub-divided rooibos bushes were extracted and analysed. Water extracts, representing food ingredient extracts, were also analysed. The largest decreases with fermentation were observed for the dihydrochalcones, with moderate to small decreases for flavonols. Eriodictyol-glucopyranoside isomer concentrations increased following fermentation.

1. Introduction

Rooibos (*Aspalathus linearis* (Burm.f.) R.Dahlgren) is an endemic South African plant that grows naturally in the fynbos biome in the Cederberg area (Malgas et al., 2010). Plants are mostly cultivated for the purpose of herbal tea production and are processed to obtain a sought-after herbal tea that is extensively sold in South Africa. Major international markets are Germany, the Netherlands, the United Kingdom, the United States of America and Japan. The discovery in 1968 that administration of rooibos tea to an infant suffering from various allergic reactions helped to reduce colic, and the more recent advent of natural antioxidants as "lifespan

Abbreviations: ANOVA, analysis of variance; BPI, base peak intensity; ca, circa; DAD, diode array detector; DMSO, dimethylsulphoxide; ESI, electrospray ionisation; HPLC, high performance liquid chromatography; IHP, isocratic holding period; LC, liquid chromatography; LSD, least significant difference; MS, mass spectrometry; PCA, principal component analysis; PPAG, enolic phenylpyruvic acid-2-*O*-glucopyranoside; RP, reversed phase; Q-TOF, quadrupole time-of-flight; UPLC, ultra-high performance liquid chromatography.

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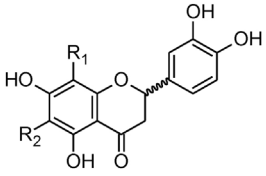
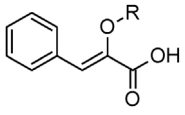
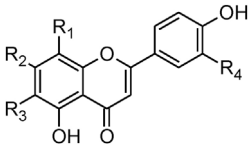
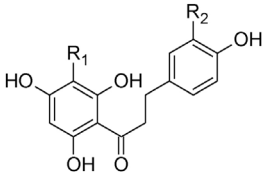
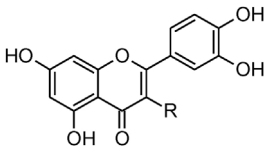
essentials", have led to further investigation of the beneficial health effects of rooibos products (Joubert and De Beer, 2011).

A major focus of the beneficial effects of rooibos and, in particular, its major flavonoid, the dihydrochalcone aspalathin, has been on its anti-diabetic properties (Muller et al., 2016). Aspalathin is exclusively found in rooibos plant material (Joubert et al., 2008; De Beer et al., 2016). Most of the herbal tea is consumed in "fermented" form. "Fermentation" of the plant material induces oxidation of the phenolic compounds as a result of cutting, bruising and exposure to air. During this process, the aspalathin content rapidly declines, with little aspalathin remaining at the end of fermentation (Joubert, 1996). Oxidation of aspalathin results in the formation of eriodictyol-glucopyranoside isomers (flavanones) as intermediate products (Table 1), with further oxidation of the flavanones producing the flavones iso-orientin and orientin (Krafczyk and Glomb, 2008). Aspalathin and its oxidation products may be further oxidised to form high molecular weight brown compounds via polymerisation reactions (Heinrich et al., 2012), thus contributing to the characteristic red-brown colour of rooibos. Other phenolic compounds present in unfermented rooibos, such as nothofagin, may also undergo enzymatic and/or chemical degradation, resulting in various phenolic reaction products.

In view of the contribution of the phenolic composition of rooibos tea in determining the properties of the product, accurate quantitative analysis of these compounds is of clear importance. To date, the most comprehensive analysis of rooibos phenolics has been achieved by the quantitative method of Beelders et al. (2012). However, the method was largely developed to quantify the major phenolic compounds in fermented rooibos, therefore little attention was given to the intermediate oxidation products of aspalathin, namely (*R*)- and (*S*)-eriodictyol-6-*C*-glucopyranoside and -8-*C*-glucopyranoside. Another important consideration in the quantitative analysis of the phenolic content of the plant material is the extraction solvent. Water extracts are mostly used to reflect the content of a cup of rooibos tea (Joubert and De Beer, 2012), but organic solvents such as acetonitrile or ethanol could be used to improve the extraction efficiency of phenolic compounds from the plant material (Pasrija and Anandharamakrishnan, 2015).

The objective of this study was therefore to develop and validate a reversed phase (RP) HPLC method using diode-array detection (DAD) on conventional HPLC instrumentation, specifically targeting the flavanone intermediate oxidation products of aspalathin. The method developed by Beelders et al. (2012) was used as a starting point for method development. Validation

Table 1
Structures of the phenolic analytes of interest for the analytical method.

Structure	Peak nr	Compound	Substituents
		Flavanones	
	1/2	(*S*)/(*R*)-eriodictyol-6-*C*-glucopyranoside	R_1 = H, R_2 = β-D-glucopyranosyl
	3/4	(*S*)/(*R*)-eriodictyol-6-*C*-glucopyranoside	R_1 = β-D-glucopyranosyl, R_2 = H
		Phenylpropenoid (phenolic precursor)	
	5	Phenylpyruvic acid-2-*O*-glucopyranoside (PPAG)	R = β-D-glucopyranosyl
		Flavones	
	6	Iso-orientin	R_1 = H, R_2 = R_4 = OH, R_3 = β-D-glucopyranosyl
	7	Orientin	R_1 = β-D-glucopyranosyl, R_2 = R_4 = OH, R_3 = H
	10	Vitexin	R_1 = β-D-glucopyranosyl, R_2 = OH, R_3 = R_4 = H
	13	Isovitexin	R_1 = R_4 = H, R_2 = OH, R_3 = β-D-glucopyranosyl
	15	Luteolin-7-*O*-glucopyranoside	R_1 = R_3 = H, R_2 = β-D-glucopyranosyloxy, R_4 = OH
		Dihydrochalcones	
	8	Aspalathin	R_1 = β-D-glucopyranosyl, R_2 = OH
	16	Nothofagin	R_1 = β-D-glucopyranosyl, R_2 = H
		Flavonols	
	9	Quercetin-3-*O*-robinobioside	R = α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranosyloxy
	11	Hyperoside	R = β-D-galactopyranosyloxy
	12	Rutin	R = α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyloxy
	14	Isoquercitrin	R = β-D-glucopyranosyloxy