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Isolation of aspalathin and nothofagin from rooibos (*Aspalathus linearis*) using high-performance countercurrent chromatography: Sample loading and compound stability considerations☆

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ABSTRACT

Aspalathin and nothofagin, the major dihydrochalcones in rooibos (*Aspalathus linearis*), are valuable bioactive compounds, but their bioactivity has not been fully elucidated. Isolation of these compounds using high-performance countercurrent chromatography (HPCCC), a gentle, support-free, up-scalable technique, offers an alternative to synthesis for obtaining sufficient amounts. An HPLC-DAD method was adapted to allow rapid (16 min from injection to injection) quantification of the four major compounds (aspalathin, nothofagin, isoorientin, orientin) during development of the isolation protocol. The traditional shake-flask method, used to determine distribution constants (K_D) for target compounds, was also adapted to obtain higher repeatability. Green rooibos leaves with a high aspalathin and nothofagin content were selected as source material. Sample loading of the polyphenol-enriched extract was limited due to constituents with emulsifying properties, but could be increased by removing ethanol-insoluble matter. Furthermore, problems with degradation of aspalathin during HPCCC separation and further processing could be limited by acidifying the HPCCC solvent system. Aspalathin was shown to be fairly stable at pH 3 (91% remaining after 29 h) compared to pH 7 (45% remaining after 29 h). Aspalathin and nothofagin with high purities (99% and 100%, respectively) were obtained from HPCCC fractions after semi-preparative HPLC.

1. Introduction

Aspalathin, a dihydrochalcone *C*-glucoside (Fig. 1), is unique to and the most abundant phenolic compound in *Aspalathus linearis*, well-known for its use as the herbal tea, rooibos [1]. Its 3-deoxy derivative, nothofagin (Fig. 1), is the second most abundant dihydrochalcone in the plant material [2]. Nothofagin has thus far only been reported in two other species, namely *Nothofagus fusca* [3] and *Schoepfia chinensis* [4]. Both compounds display antioxidant [5] and antimutagenic activity [6], while aspalathin also displays α-glucosidase inhibitory activity [7] and hypoglycaemic activity [7,8].

The structural similarity of nothofagin (phloretin-3′-*C*-glucoside) to aspalathin (3-hydroxyphloretin-3′-*C*-glucoside) and the sodium glucose co-transporter inhibitor [9] phloridzin (phloretin-2′-*O*-glucoside), suggests its therapeutic potential for treatment of diabetes. This indicates that the antidiabetic potential of nothofagin merits investigation. Aspalathin and nothofagin represent valuable bioactive compounds, but their bioactivity have not been fully elucidated. Recently, two chemical synthetic routes for aspalathin have been published to provide sufficient quantities for investigation of its biological properties [10,11], while yeast modified to express a *C*-glucosyltransferase from rice was used to convert phloretin to nothofagin [12]. Selection of *Aspalathus linearis* plant material, based on the abundance of these compounds, combined with an up-scalable isolation procedure could offer an alternative means to obtain these compounds in pure form and in sufficient quantities for bioactivity testing.

Many different chromatographic techniques can be used to isolate phenolic compounds from crude plant extracts. Countercurrent chromatography (CCC) was selected as a suitable technique as it is

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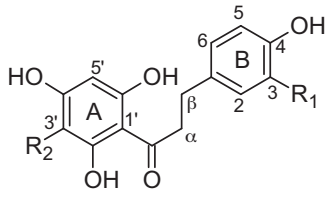

Aspalathin: R_1 = OH, R_2 = *C*-β-D-glucosyl
Nothofagin: R_1 = H, R_2 = *C*-β-D-glucosyl

Fig. 1. Chemical structure of aspalathin and nothofagin.

a gentle, support-free liquid chromatographic technique enabling complete recovery of the sample and very little degradation of the target compound [13]. As separation times are relatively short, degradation of unstable compounds are limited. In addition, due to the practically unlimited choice with regards to solvent system for separation, a suitable solvent system can be designed for almost any separation. Sutherland et al. [14] demonstrated the scalability of high-performance CCC (HPCCC) to process scale and its suitability as a platform technology for the pharmaceutical industry due to its high throughput and low solvent consumption compared to chromatography involving solid stationary phase.

The aim of this study was to develop a protocol including HPCCC for isolation of aspalathin and nothofagin with high purities, while maintaining high sample throughput for the HPCCC step. In order to facilitate method development, analytical methodology for fast quantification of the four major phenolic compounds in unoxidized ("green") rooibos using HPLC with diode-array detection (DAD) was developed. The standard shake-flask test used to determine the distribution constants (K_D) of the target analytes was also adapted to improve repeatability. Development of an HPCCC protocol mainly entailed determining the sample pre-treatment needed to obtain a high sample loading and finding a suitable solvent system to separate the target analytes from other rooibos constituents while maintaining stability of the target analytes.

2. Materials and methods

2.1. Chemicals

All chemicals were analytical grade and sourced from either Sigma-Aldrich (St. Louis, MO, USA) or Merck Millipore (Darmstadt, Germany) unless otherwise specified. HPLC gradient grade acetonitrile was purchased from Sigma-Aldrich. Authentic reference standards with purities > 95% were obtained from Extrasynthese (Genay, France: isoorientin), Carl Roth (Karlsruhe, Germany: orientin) and the Medical Research Council (PROMEC Division, Bellville, South Africa: aspalathin, nothofagin). Deionized water, prepared using an Elix water purification system (Merck Millipore), was further purified to HPLC grade using a Milli-Q Academic water purification system (Merck Millipore).

2.2. Plant material

Shoots of 2-year old rooibos (*Aspalathus linearis* (Burm.f.) Dahlg.; family: Fabaceae; tribe: Crotalarieae) plants, comprising of leaves and thin stems, were harvested from an experimental plantation (Barrydale, Western Cape, South Africa) established from cuttings made from commercially-cultivated plants. Plants were identified by Dr Hannes de Lange from the South African National Botanical Institute (Cape Town, South Africa). The shoots were harvested during winter, i.e. from non-flowering plants. The shoots were dried intact at 30 °C in a temperature-controlled drying tunnel with forced air circulation to 6.4% moisture content. After drying, the leaves were separated from the stems and finely ground with a Retsch mill (1 mm sieve, Retsch GmbH, Haan, Germany).

2.3. Extract preparation and enrichment

The extraction, enrichment and isolation steps evaluated are outlined in Fig. 2. A crude hot-water extract was prepared by adding 1.5 L boiling water to 150 g milled plant material. The mixture was placed in a waterbath at 95 °C for 30 min and stirred every 5 min. The warm extract was filtered using Whatman nr 4 filter paper and cooled to room temperature. A 1 L portion of the water extract was partitioned using 3 × 250 mL portions of *n*-BuOH. The *n*-BuOH layers were pooled, partially evaporated under vacuum and freeze-dried to obtain a polyphenol-enriched fraction (PEF1) (yield = 18.478 g). Further enrichment using a two-phase solvent system, namely EtOAc–*n*-BuOH–0.1% aq. formic acid (4:1:5, *v*/*v*), was obtained by dissolving 250 mg of PEF1 in 100 mL of the lower aqueous layer and partitioning with 10 × 20 mL portions of the upper organic layer. The process was repeated 6 times (i.e. 1.75 g of PEF1 partitioned). The upper organic layer fractions were pooled, partially evaporated under vacuum and freeze-dried to obtain a second polyphenol-enriched fraction (PEF2) (yield = 1.338 g). An alternative enrichment step entailed dissolving 1 g of PEF1 in 1 L of ethanol by ultrasonication for 30 min. The solution was filtered using Whatman nr 4 filter paper, partially evaporated under vacuum and freeze-dried to obtain a polyphenol-enriched fraction (PEF3) (yield = 900 mg) devoid of ethanol-insoluble matter.

2.4. Determination of distribution constants in selected solvent systems

A range of solvent systems (Table 1) was tested for suitability to isolate aspalathin and nothofagin from PEF1 using a shake-flask method adapted from Garrard [15]. The extract was dissolved in 50% MeOH containing 10% ascorbic acid to obtain a concentrated stock solution (ca 80 mg/mL). Ascorbic acid was added as it is known to prevent aspalathin degradation during the time elapsing from sample preparation to injection into the HPLC [16]. The solvent systems were prepared and pre-equilibrated for at least 30 min. A 20 μL aliquot of the stock solution was added in triplicate to HPLC autosampler vials and 750 μL each of the upper and lower layer of the solvent systems added. After gentle mixing the mixtures were equilibrated for at least 60 min prior to HPLC analysis of the lower layer, which was directly injected from the HPLC vial. Quantification of the analytes was performed using HPLC-DAD as described in section 2.8. The distribution constant (K_D) for a given compound was calculated assuming reversed phase HPCCC separation as follows:

$$K_D = \frac{C_S}{C_M} = \frac{C_T - C_M}{C_M}$$

where C_S = Concentration of the compound in the stationary phase (i.e. upper layer).
C_M = Concentration of the compound in the mobile phase (i.e. lower layer).
C_T = Total concentration of the compound in the stationary and mobile phase estimated by calculation from the concentration in the PEF1 stock solution as analysed by HPLC-DAD.

2.5. Determination of aspalathin and nothofagin pH stability

The stability of aspalathin and nothofagin in sodium phosphate buffers (0.2 M) at pH 7, 6, 5 and 3 was determined by diluting 20 μL of the respective aspalathin and nothofagin stock solutions in DMSO (ca 1 mg/mL) with 250 μL buffer. The resulting mixtures