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Preparative separation of cacao bean procyanidins by high-speed counter-current chromatography

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ABSTRACT

In this work, an efficient method for preparative separation of procyanidins from raw cacao bean extract by high-speed counter-current chromatography (HSCCC) was developed. Under the optimized solvent system of *n*-hexane-ethyl acetate-water (1:50:50, v/v/v) with a combination of head–tail and tail–head elution modes, various procyanidins fractions with different polymerization degrees were successfully separated. UPLC, QTOF-MS and ^{1}H NMR analysis verified that these fractions contained monomer up to pentamer respectively. Dimeric procyanidin B2 (purity > 86%) could be isolated by HSCCC in a single run. Other individual procyanidins in these fractions could be further isolated and purified by preparative HPLC. The developed HSCCC together with preparative HPLC techniques appeared to be a useful tool for large preparation of different procyanidins from cacao beans. Furthermore, by antioxidant activity assays, it was proved that both fractions and individual procyanidins possessed greater antioxidant activities compared to standard trolox. The antioxidant activities of procyanidins increase as the increase of their polymerization degree.

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1. Introduction

Procyanidins are a subclass of flavonoids and are composed of the flavan-3-ol monomers that naturally occur in plants and are known to exhibit many physiological activities, including antioxidant, cardioprotective, anti-inflammatory, and enzyme inhibitory effects [1,2]. Various studies have shown that health benefits of procyanidins are related to their structures [3,4], and the structural diversity is due to the type of interflavanoid linkage, the kind and number of flavan-3-ol units. Most oligomeric proanthocyanidins (OPCs) and polymeric proanthocyanidins (PPCs) are linked through C4 → C8 and/or C4 → C6 interflavan bonds as (+)-catechin and/or (−)-epicatechin as basic unit.

Procyanidins are found in various plant-derived foods, such as apple, pear, berries, wine, cacao, and nuts [5–9]. Cacao beans are the fruit of the *Theobroma cacao* L., which is a plant originated in the rain forests of South and Central Americas. Though there are over 20 species in the genus, only *Theobroma cacao* L. is widely cultivated [10–12]. Nowadays cacao and its associated products are consumed widely throughout the world.

Cacao beans are rich in polyphenols which account for about 12 to 18 in percent of the dry weight [13]. The presence of dimeric procyanidins B1 to B7, trimeric procyanidin C1, tetrameric procyanidin (cinnamtannin A2) and pentameric procyanidin (cinnamtannin A3) in cocoa products has been reported [14,15]. A number of studies have shown that the content of procyanidins in cocoa products is higher than those in blueberry and cranberry on a dry weight basis and cocoa products has a higher antioxidant capacity than tea and red wine [16,17]. However, at present, there are few studies concerning raw cacao beans.

Procyanidins have been isolated from natural sources by extraction, fractionation and purification using traditional methods, such

Abbreviations: ABTS, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EC_{50}, concentration for 50% of maximal effect; FRAP, ferric reducing antioxidant power; HSCCC, high-speed counter-current chromatography; mDP, mean degree of polymerization; OPC, oligomeric proanthocyanidin; PBS, phosphate buffered saline; PPC, polymeric proanthocyanidins; TPTZ, 2,4,6-tris (2-pyridyl)-*s*-triazine.

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as liquid-liquid extraction [18], microwave extraction [19], ultrasound extraction [20], thin-layer chromatography [21], column chromatography [18,22] and semi-preparative and preparative HPLC [23]. These methods are inevitably accompanied with some disadvantages. Sun et al. [18] used a combined solvent system for liquid-liquid extraction of stilbenes from grape skins. The established method permitted large preparation of crude polyphenols fractions, but very time- and solvent-consuming. Microwave extraction was used for preparation of polyphenols from apple pomaces [19], but the proposed method has disadvantages of low yield and high cost. The polyphenols were extracted from the unripe apple assisted using ultrasound [20], but accompanied with weakness of complex process and solvent-consuming. Thin-layer chromatography was applied for separation of flavan-3-ols from oak bark and green tea [21], however, this method was limited to the qualitative analysis and needed other techniques consociated for large scale preparation. Column chromatography permitted fractionation of proanthocyanidins from grape and wine [22], but complex process and very time-consuming lead it inefficiency. Semi-preparative HPLC was used for isolation of procyanidins from grape seeds [23], but complicated pretreatment and low yield were inevitable. Moreover, all of these methods presented disadvantage of secondary pollution because of the solid support matrix and/or repetitive sample injection. Compared to traditional techniques, in recent decades, a new chromatographic separation technique called high-speed counter-current chromatography (HSCCC) was developed, which is a support-free liquid-liquid partition chromatography based on hydrodynamic equilibration of the two-phase solvent system in the separation column. HSCCC technique provides several advantages such as higher partition efficiency in a shorter elution time [24–26], high sample recovery and large loading capacity. In previous work in our laboratory, by only one run of HSCCC for about 4 h, with a loading of 200 mg of monomer proanthocyanidins, (+)-catechin and (−)-epicatechin were succeeded in isolation as high as 78.9 mg and 52.5 mg, respectively. As compared, using traditional column chromatography, followed by semi-preparative HPLC, much less yields of polyphenols were obtained [23]. Another advantage of HSCCC technique over conventional column chromatography is no irreversible adsorption of sample onto the solid support [27]. Moreover, the high repeatability of HSCCC technique permits the established method in laboratory-scale applicable for large or industrial scale production. As a consequence, HSCCC technology has been widely used as an excellent method in the separation and purification of phenolic compounds from natural plants [24–26,28].

The aim of this work was to separate the procyanidins in a preparative scale from raw cacao bean extract by an efficient method using HSCCC. Moreover, the antioxidant activity of the HSCCC fractions and individual procyanidins were verified, and the three common methods, i.e., 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and ferric reducing antioxidant power (FRAP) assays, were used in this work.

2. Materials and methods

2.1. Standards and reagents

(+)-Catechin, (−)-epicatechin and procyanidin B2 were purchased from Chendu Must Bio-Technology Co., Ltd. (Chengdu, China). Phloroglucinol was purchased from Aladdin reagent (Shanghai, China). 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,4,6-tris (2-pyridyl)-s-triazine (TPTZ), (±)-6-hydroxy-2,5,7, 8-tetramethylchromane-2-carboxylic acid (trolox) were purchased from Sigma–Aldrich (St. Louis, MO, USA).

All organic solvents used for HSCCC, UPLC and HPLC were purchased from Chemical Branch of Shandong Yuwang industrial Co., Ltd. (Shandong, China).

2.2. Materials

Freeze-dried cacao beans were provided by Polyphenol Laboratory of Pólo Dois Portos/INIAV (Portugal). The preparation of the freeze-dried cacao beans were performed by using the fresh cacao purchased in a local market of Sao Tome and Principle, islands in the gulf of Guinea of the West African Coast. The cacao beans were isolated manually, washed with distilled water to eliminate residual pulp, frozen with liquid nitrogen, followed by lyophilization for 48 h.

2.3. Preparation of cacao bean phenolic extract

The cacao bean phenolic extract was prepared as report in our previous work [29]. Briefly, the desiccative raw cacao beans were finely ground using a high-speed miller (Tianjin TAISITE, type: FW 100). The powder was immediately used for the extraction of phenolic compounds and was defatted three times with *n*-hexane (solid/liquid 1:5, w/v), followed by sequential extraction using 80% aqueous methanol (v/v) and 75% aqueous acetone (v/v). The combined supernatants were evaporated at <30 °C to remove organic solvents, followed by three times extractions with an equal volume of chloroform to eliminate some alkaloidal compounds. The aqueous phenolic solution was lyophilized and the powder obtained was stored at −20 °C until used.

2.4. HSCCC separation of cacao bean phenolic extract

2.4.1. Selection of solvent system

The partition coefficient (K) value and separation factor (α) were used as evaluation parameters for selection of the two-phase solvent system in HSCCC separation. In this work, the K values of cacao bean phenolic extract were determined by UV spectrophotometer based on reported method [27] with (−)-epicatechin, purified grape seed oligomeric procyanidins (OPCs), purified grape seed polymeric procyanidins (PPCs) as references. The K value was defined as A_L/A_U, where A_L and A_U meant the absorbance value of objective samples in lower stationary phase and the upper mobile phase, respectively. The separation factor (α) was the ratio of K values between two samples. Six solvent systems were selected (given in Table 1) and the K value and α value were obtained as follows. 0.5 mg of samples was added to 10 mL test tube with 3 mL of each phase of the above pre-equilibrated two-phase solvent system. The test tube was vigorously shaken and left to stand at room temperature until the equilibrium of the sample between the two phases was reached. 2 mL of each phase was evaporated to dryness and the residue was dissolved in 5 mL methanol to determine the absorbance at 280 nm.

2.4.2. Preparation of solvent system and sample solution

The optimized two-phase solvent system composed of *n*-hexane-ethyl acetate-water (1:50:50, v/v/v) was used for HSCCC separation of cacao bean phenolic extract. The solvent system in separatory funnel was shaken vigorously for mixing thoroughly and equilibrated at room temperature. The two phases were separated and degassed by ultrasonic device for 15 min. The lower aqueous phase was used as the stationary phase, and the upper organic phase as the mobile phase.

The sample solution was prepared by dissolving 400 mg of the freeze-dried cacao bean phenolic extract into 20 mL of lower phase

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