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Determination of flavanols by liquid chromatography with fluorescence detection. Application to the characterization of cranberry-based pharmaceuticals through profiling and fingerprinting approaches

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ABSTRACT

In this work, a new method based on reversed-phase high-performance liquid chromatography (HPLC) with fluorescence detection (FLD) was established for the determination of catechins and related oligomeric proanthocyanidins (PACs) in cranberry-based pharmaceuticals. Compounds were recovered by liquid extraction using methanol/water/hydrochloric acid (60:39:1, *v:v:v*) as the extraction solvent. The chromatographic separation was carried out using a core-shell C18 column under an elution program based on 0.1% formic acid in water and methanol as the components of the mobile phase. The flow rate was 0.4 mL min^{-1} and the injection volume was 5 μL. Chromatograms were acquired at 280 nm by UV–vis absorption and at λ_{ex} 280 nm and λ_{em} 347 nm by fluorescence spectroscopy. Compared to UV detection, FLD demonstrated both increased sensitivity and selectivity to avoid interfering signals from other phenolic compounds present in the samples. Data resulting from the analysis of cranberry-based products was exploited to tackle an exploratory characterization and classification using principal component analysis. Samples were clustered according to their compositions and those enriched with PACs with antibacterial activity were clearly distinguished from the others.

1. Introduction

American red cranberries (*Vaccinium macrocarpon*), typical from the United States and Canada, are increasingly consumed worldwide as fresh fruit or fruit derivatives (e.g. raisins, juices, antioxidant juice cocktails, etc.) as a type of functional food. Besides, cranberries are currently used as raw material to obtain purified extracts to be introduced as the active ingredients of several pharmaceutical products (e.g., capsules, tablets, etc.). Cranberries are rich sources of dietary flavonoids and phenolic acids that may provide a variety of health benefits such as anti-proliferative, antioxidant, anti-inflammatory and antimicrobial properties [1–6]. One of the most appreciated activity is the inhibition of the growth of pathogenic bacteria such as *Escherichia coli* and *Helicobacter pylori* so that cranberry products have traditionally been used to treat and prevent urinary- and digestive-tract infections [3,5–7]. More recently, the antitumor properties of cranberries have made them a popular dietary component to prevent neoplastic diseases [8].

Cranberries contain high concentrations of polyphenols including flavanols, anthocyanins, flavonols and phenolic-acid derivatives [1,4,9]. Regarding flavanols, catechin and epicatechin are the main monomeric components occurring in concentrations of 10–100 mg kg^{-1}. Gallic acid derivatives such as gallocatechin, epigallocatechin, gallocatechin gallate and epigallocatechin gallate are found in similar amounts [4,10]. The monomeric flavanol units may be linked in two ways to form oligomers and polymers, the so-called proanthocyanidins (PACs). The most common linkage between the flavan rings is a 4β → 8, which is known as a B-type bond. The A-type linkage is less common and consists of both 4β → 8 and 2β → O → 7 bonds (see Fig. S1 in Supplementary material). The presence of A-type linkages provides an additional structural sta-

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bility to PAC molecules, thus being more resistant to cleavage under harsh conditions such as high temperature or extreme pH values. PACs with A-type linkages and degree of polymerization (DP) up to 10 are abundant in cranberries and have also been found in peanut skin, plum, avocado and curry [11,12].

One of the major protective features of PACs is their ability to bind to and subsequently precipitate proteins, which gives them astringency and makes them unpalatable to potential predators. Recent studies have pointed out the antiadhesive properties of A-type PACs, which seem to confer antimicrobial activity, while B-type counterparts do not exhibit such an activity [3–6].

The characterization and quantification of most of the polyphenolic families in cranberry have been well described elsewhere [1,12–15]. The study of PACs is more challenging due to their oligomeric nature and the lack of commercially available standards so further research strategies are needed [16]. The determination of PACs in matrices of plant origin is a complex issue that requires, in general, an initial extraction step and further determination by liquid chromatography [12–16]. PACs can be recovered from fresh, frozen or dried plant matrices by liquid extraction using acidified hydro-organic mixtures. Solvents such as acetone and methanol, and acids such as acetic, formic, citric or hydrochloric are typically used [13,14]. Plant samples can be subjected to air-drying or freeze-drying processes before grinding, sieving and homogenizing in order to facilitate the extraction and improve the yield. Extract solutions consist of a complex mixture of different classes of phenolic and concomitant substances such as sugars, amino acids, proteins, etc., that may lead to chromatograms with multiple unresolved peaks and overlapping "humps". As a result, further purification steps by adsorption or size-exclusion may be required for the removal of interference [17,18].

HPLC methods with UV and/or fluorescence detection are often used for PAC quantification [19–23]. However, because of the polymeric structure and the wide molecular mass range of flavanols, their characterization and determination remains as a big analytical challenge. PAC oligomers are hardly separated under reversed-phase mode, thus resulting in broad overlapping bands. Normal-phase mode seems to be more suitable for their separation, leading to peak clusters eluted as a function of DP throughout the chromatogram. Regarding detection, in comparison to UV spectroscopy, fluorescence detection (FLD) provides additional advantages dealing with improved selectivity and sensitivity [19,23]. Interferences from other phenolic compounds can notably be reduced in FLD from an appropriate selection of excitation and emission conditions. The analytical performance can also be enhanced by HPLC–MS [23–28]. The negative ionization in MS is compatible with the anionic nature of analytes although the larger oligomers are poorly detected due to the intense fragmentation under this mode. In contrast, positive ionization with electrospray (ESI) and atmospheric-pressure chemical ionization (APCI) sources seems to be preferred for large PAC molecules. Matrix-assisted laser desorption/ionization coupled with time-of-flight (MALDI-TOF) mass spectrometry has also been introduced for the characterization of flavanols from cranberry, apple and grape [29,30]. In this way, PAC molecules with high DP can be determined and the structure of monomer building unit and type of inter-flavan linkage (A/B-type) can be differentiated.

This paper is focused on the development of a new HPLC-FLD analytical method for the characterization and quantification of flavan-3-ols in cranberry extracts and pharmaceutical products. It should be pointed out that estimation of PAC amounts is still a controversial issue as values obtained may differ significantly as a function of the method used for the determination. Besides, an overall PAC index may be inefficient to assess the product activity to combat urinary tract infections. Here, a simple and efficient approach for product evaluation according to contents of A-type and B-type molecules was established which seem to be essential from the point of view of the assessment of the antibacterial features. Samples consisting of raw cranberry extracts, antimicrobial pills and capsules and dietary supplements were subjected to an extraction procedure using a methanol/water/hydrochloric acid (60:39:1 *v/v/v*). Analytes (catechin, epicatechin, procyanidin dimers and trimer) from the resulting extracts were separated by reversed-phase HPLC on a core-shell C18 column using an optimized elution gradient based on methanol. The method was applied to the characterization of cranberry-based products under both profiling and fingerprinting approaches using analyte concentrations and FLD chromatograms, respectively. Data was treated chemometrically using principal component analysis and related methods [31]. Here, commercial products can easily be differed according to the flavanol patterns, and samples with high contents of molecules with antimicrobial properties can be identified. The method could be extended to authentication of American cranberry from other types of cranberries or berries that may not contain proper levels of active ingredients.

2. Experimental

2.1. Reagents and solutions

The reagents and solvents used for the preparation of HPLC mobile phase and sample analysis were formic acid (>96%, Sigma-Aldrich, St Louis, USA), methanol (99.9%, UHPLC PAI-ACS SuperGradient, Panreac, Castellar de Valles, Barcelona, Spain) and Mili-Q Water, purified using an Elix 3 coupled to a Milli-Q system (Bedford, USA) and filtered through a 0.22 μm nylon filter integrated into Milli-Q system.

The polyphenolic standards used in this work were epicatechin and catechin from Sigma-Aldrich, procyanidin A2 and procyanidin C1 from PhytoLab (Vestenbergsgreuth, Germany) and procyanidin B2 (>98%) from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China). Stock standard solutions of polyphenols were prepared at ~1000 mg L^{-1} in methanol using amber glass vials. Intermediate working solutions for method optimization and calibration were prepared by proper dilution in 50% methanol at 1, 5, 10, 20, 40, 60, 80 and 100 mg L^{-1}. All stock and standard solutions were stored at 4 °C. Apart from flavanols, other common polyphenolic compounds were also assayed to assess chromatographic and spectral selectivity issues including gallic acid, 3,4-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, 4-hydroxybenzoic acid, homovanillic acid, vanillic acid, syringic acid, salicylic acid, piceid, resveratrol, chlorogenic acid, caffeic acid, coumaric acid, ferulic acid, quercetin, fisetin and kaempherol, all of them from Sigma-Aldrich.

2.2. Samples and sample treatment

A total of 2 raw cranberry extracts and 17 anti-cystitis products and dietary supplements were analyzed in this work. Raw cranberry extracts referred to as E01 and E02 were kindly provided by Deiters S.L. (Barcelona, Spain). These products were used as the main materials to prepare some pharmaceuticals. Dietary supplements and parapharmaceuticals were purchased from pharmacies and specialized shops in Gdansk (Poland) and Barcelona (Spain). They were coded as follows: C01 and C02 were tablets containing ca. 250 mg of cranberry extracts (equivalent to 130 mg PACs) together with other natural plant extracts; C3 to C14 consisted of gelatin capsules filled with amounts of cranberry extracts ranging from 175 to 425 mg and, depending on the cases, with some co-adjuvants such as ascorbic acid, grape, heather and salvia extracts; Sa01 and Sa02 were sachets containing 240 mg PAC; Sy01 was a syrup with 12 mg mL^{-1} PAC.