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A validated solid–liquid extraction method for the HPLC determination of polyphenols in apple tissues Comparison with pressurised liquid extraction

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Abstract

A solid–liquid extraction procedure followed by reversed-phase high-performance liquid chromatography (RP-HPLC) coupled with a photodiode array detector (DAD) for the determination of polyphenols in freeze-dried apple peel and pulp is reported. The extraction step consists in sonicating 0.5 g of freeze-dried apple tissue with 30 mL of methanol–water–acetic acid (30:69:1, v/v/v) containing 2 g of ascorbic acid/L, for 10 min in an ultrasonic bath. The whole method was validated, concluding that it is a robust method that presents high extraction efficiencies (peel: >91%, pulp: >95%) and appropriate precisions (within day: R.S.D. (n = 5) <5%, and between days: R.S.D. (n = 5) <7%) at the different concentration levels of polyphenols that can be found in apple samples. The method was compared with one previously published, consisting in a pressurized liquid extraction (PLE) followed by RP-HPLC-DAD determination. The advantages and disadvantages of both methods are discussed.
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Keywords: Polyphenols; Apple; Solid–liquid extraction; Pressurised liquid extraction; HPLC; Validation

1. Introduction

Apples present a wide diversity of polyphenols classified into several major classes. Flavan-3-ols are preponderant, being present in monomeric forms named catechins, and in oligometric and polymeric forms known as procyanidins. Hydroxycinnamic acids are the second class in concentration, and together with catechins, they are involved in the browning phenomena that takes place during apple fruit processing, being responsible for the yellow or orange coloration of apple products [1]. Dihydrochalcones, flavonols and anthocyanins are minor components that contribute to the pigmentation of apples and to the potential antioxidant activity of apples and their derived foodstuffs [2].

In cider apple cultivars, polyphenol interest is due to the fact that they are responsible for the colour and the balance of bitterness to astringency, which defines the 'overall mouth-

Abbreviations: ACY, anthocyanins; AVI, avicularin; CAT, (+)-catechin; CAT-2, unknown flavan-3-ol; CG-1, unknown anthocyanin; CQA, 5-caffeoylquinic acid; DHC, dihydrochalcones; EC, (−)-epicatechin; FA, flavan-3-ols; FO, flavonols; HCA, hydroxycinnamic acids; HYP, hyperoside; IDE, ideain; IQC, isoquercitrin; PB2, procyanidin B2; PCM, p-coumaric acid; PCQ, 4-*p*-coumaroylquinic acid; PLD-1, hydroxyphloretin diglycoside; PLD-2, hydroxyphloretin monoglycoside; PLG, phloridzin; PLXG, phloretin-2′-O-xyloglucoside; QCI, quercitrin; QG-1, unknown quercetin glycoside; RUT, rutin; GM, Geza miña; MK, Moko; MN111, Manttoni 111; MNEM7, Manttoni EM7; TX, Txalaka; UR, Urdin; DAD, diode array detector; DW, dried weight; HPLC, high-performance liquid chromatography; LOD, limit of detection; LOQ, limit of quantitation; nd, not detected; PLE, pressurized liquid extraction; PPO, polyphenoloxidase; R.S.D., relative standard deviation; S.D., standard deviation; SLE, solid–liquid extraction

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feel' of ciders [3]. Furthermore, they are implicated in the alcoholic and malolactic fermentations as metabolites, providing cider aroma, and as inhibitors of the microbiological growth, controlling fermentation rates and cider spoilage [4]. Phenolic compounds are also involved in the colloidal stability of cider [5]. In addition, polyphenols as natural antioxidant constituents of human diet are receiving increasing attention due to their health-protective properties [6].

The methodology used to analyse these phenolic compounds in apples generally includes extractions with solvents, such as methanol, ethanol, acetone or mixtures of these with water [7,8]; cleanup and further fractionation by liquid–liquid extraction (LLE), usually with ethyl acetate [9,10]; column chromatography (CC) [11,12] or solid phase extraction (SPE) [13]. Finally, after the extract is concentrated, polyphenols are separated by reversed-phase high-performance liquid chromatography (RP-HPLC) coupled with UV–vis detection or mass spectrometry [14]. In other works, samples are extracted by sonication with solvent, and the crude extract is directly injected into the HPLC system [15]. Generally, in publications about natural products in plants (such as those about the determination of polyphenols in apples), little information is given about the optimisation procedures of the analytical methods used, and frequently, these methods have not been validated.

Sample extraction procedures are often regarded as bottlenecks in analytical methods. Moreover, classical sample preparation techniques are both time and solvent consuming, and sample handling can decrease the quality of the analytical results. In this sense, the sample preparation step accounts for at least one-third of the error generated by the analytical method [16]. Therefore, the importance of sample preparation in analytical methods should not be undervalued.

In this work, a method for the determination of polyphenols in apples was developed using a solid–liquid extraction (SLE) assisted by sonication and followed by RP-HPLC coupled with a photodiode array detector (DAD). This method was optimised and exhaustively validated by evaluating the selectivity, the linear range, the limits of detection and quantitation, the accuracy, the repeatabilities within day and between days, the robustness of the method and the polyphenol stabilities. In addition, the method was compared with a published validated method, which consists in a pressurised liquid extraction (PLE) followed by RP-HPLC analysis [17]. The advantages and drawbacks of both methods are commented.

2. Experimental

2.1. Reagents and standards

Methanol (Romil Chemical Ltd., Heidelberg, Germany) was of HPLC grade. Water was purified on a Milli-Q system from Millipore (Bedford, MA, USA). Glacial acetic acid and formic acid provided by Merck (Darmstdt, Germany) and ascorbic acid by Panreac (Barcelona, Spain) were of analytical quality. All solvents used were previously filtered through 0.45 μm nylon membranes (Lida, Kenosha, WI, USA).

Polyphenol standards were supplied as follows: (+)-catechin, (−)-epicatechin, rutin, phloridzin, 5-caffeoylquinic acid and *p*-coumaric acid by Sigma–Aldrich Chemie (Steinheim, Germany); hyperoside, isoquercitrin, avicularin, quercitrin and ideain chloride by Extrasynthèse (Genay, France). 4-*p*-Coumaroylquinic acid, phloretin-2′-*O*-xyloglucoside and procyanidin B2 were kindly provided by Dr. Guyot, Dr. F.A. Tomás–Barberán and Dr. C. Santos–Buelga, respectively. Stock standard solutions of (+)-catechin, (−)-epicatechin, rutin, phloridzin, 5-caffeoylquinic acid and *p*-coumaric acid at a concentration of 1 mg mL^{-1} and hyperoside, isoquercitrin, quercitrin and ideain at 0.6 mg mL^{-1} were prepared in methanol and stored at 4 °C in dark. The other standards were prepared in approximately concentrations and only used for chromatographic peak identification.

2.2. Plant materials

Apple cultivars used for the optimization and validation of the solid–liquid extraction of polyphenols from apple peel and pulp were some local varieties used for cidermaking: Urdin (UR), Txalaka (TX), Mantonni 111 (MN111), Manttoni EM7 (MNEM7), Geza Miña (GM) and Moko (MK). Apples were harvested in the Experimental Orchards of the Diputación Foral de Gipuzkoa in Hondarribia (Guipúzcoa, Spain) and the Diputación Foral de Bizkaia in Zalla (Vizcaya, Spain) during the 2000 season.

2.3. Apple powder preparation

Fruits were harvested at maturity, which was tested by the lugol index [18]. For each variety, fruits were mechanically peeled and cored, and sprayed with an aqueous solution of 3% formic acid in order to avoid polyphenol oxidation. Peels and pulps were immediately frozen in liquid nitrogen and then they were freeze-dried. An aliquot for each variety was used to determine the fresh/dry matter ratio. The dried tissues were crushed in closed vessels to avoid hydratation, obtaining an homogenous powder that was stored at room temperature in a desiccator until analysis. Aliquots of 0.5 g of freeze-dried apple peel or pulp were used for each analysis.

2.4. Analytical procedures

2.4.1. Solid–liquid extraction of freeze-dried samples

In broad outline, the procedure proposed for the solid–liquid extraction (SLE) of polyphenols from apple peel and pulp consisted in a direct extraction of the freeze-dried plant material with an appropriate solvent in an ultrasonic bath (Selecta, Barcelona, Spain) during a certain period of time.

The most influent experimental variables on the extraction procedure, which were the composition and the volume

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