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# Quantification by UHPLC of total individual polyphenols in fruit juices

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ABSTRACT

The present work proposes a new UHPLC-PDA-fluorescence method able to identify and quantify the main polyphenols present in commercial fruit juices in a 28-min chromatogram. The proposed method improve the IFU method No. 71 used to evaluate anthocyanins profiles of fruit juices. Fruit juices of strawberry, American cranberry, bilberry, sour cherry, black grape, orange, and apple, were analysed identifying 70 of their main polyphenols (23 anthocyanins, 15 flavonols, 6 hydroxybenzoic acids, 14 hydroxycinnamic acids, 4 flavanones, 2 dihydrochalcones, 4 flavan-3-ols and 2 stilbenes). One standard polyphenol of each group was used to calculate individual polyphenol concentration presents in a juice. Total amount of polyphenols in a fruit juice was estimated as total individual polyphenols (TIP). A good correlation ($r^2 = 0.966$) was observed between calculated TIP, and total polyphenols (TP) determined by the well-known colorimetric Folin-Ciocalteu method. In this work, the higher TIP value corresponded to bilberry juice (607.324 mg/100 mL fruit juice) and the lower to orange juice (32.638 mg/100 mL fruit juice). This method is useful for authentication analyses and for labelling total polyphenols contents of commercial fruit juices.



## 1. Introduction

Polyphenols are secondary metabolites present in all vegetal tissues, as well as in flowers and fruits. Several thousand of plant polyphenols are known, including a wide variety of molecules that contain at least one aromatic ring with one or more hydroxyl groups in addition to other constituents. They are important antioxidants of human diet (El Gharras, 2009).

Recent findings put forward the role of polyphenols in preventing diseases such as chronic inflammatory diseases, some kind of cancers, cardiovascular diseases, antimicrobial and anti-cariogenic effects, or neurodegenerative diseases (Dai, Borenstein, Wu, Jackson, & Larson, 2006; De Pascual-Teresa & Sánchez-Ballesta, 2008; Pan, Lai, & Ho, 2010; Scalbert, Manach, Morand, Remesy, & Jimenez, 2005). Effects of polyphenols on health require full knowledge on their chemistry, occurrence in foods, metabolism and bioavailability, mechanisms of biological activity, or surrogate markers of health (Link, Balaguer, & Goel, 2010; Sutherland, Rahman, & Appleton, 2006; Yang, Sang, Lambert, & Mao-Jung, 2008).

Focusing on fruit juices, consumers are highly interested in health claims of polyphenols. There is an increasing market in antioxidant fruit juices and formulation of juice mixtures with high polyphenol concentrations. The main commercial antioxidant juices are based on the use of red fruits like bilberry, cranberry, strawberry, cherry, raspberry, mixed with other traditional juices like orange, apple or pineapple. Labelling of commercial fruit juices detailing polyphenol composition would be of great interest for consumers and these data could be also used for epidemiological studies. Juice industry is interested in an official analytical methodology to establish polyphenol profiles and its quantification in fruit juices. According to the CODEX-STAN 247-2005 for Fruit Juices and Nectars (http://www.codexalimentarius.net), the IFU method number 71 (1998) type I determine anthocyanins profile for red fruit juice authentication. Anthocyanins are the polyphenol compounds responsible of the red colour of these juices. This HPLC method use very acid mobile phases of formic acid with a pH value close to 2.3, which is needed for good anthocyanins resolution (Obón, Díaz-García, & Castellar, 2011). The International Fruit Juice Association (IFU) does not have a polyphenol method to analyse profiles and quantify polyphenols (http://www.ifu-fruitjuice.com/ifu-methods). Thus, it is crucial to have easy and powerful analytical methodology to measure polyphenol content of commercial fruit juices.

HPLC is the preferred method for separation and quantification of individual polyphenols in fruits, using detection systems based on spectrophotometry, fluorometry, and/or mass spectrometry. The amount and diversity of polyphenols in vegetal tissues show

*Abbreviations:* PDA-Fluo, photodiode array detector-fluorimetric detector; TIP, total individual polyphenols; TP, total polyphenols; UHPLC, ultra high-performance liquid chromatography; GAE, gallic acid equivalents; IFU, International Federation of Fruit Juice Producers; USDA, United States Department of Agriculture.

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the difficulty to obtain pure profiles with no peak overlapping in HPLC chromatograms. The use of different detection systems, besides spectrophotometry, as fluorometry and mass spectrometry are important to analyse and to corroborate the identification of every compound. If some unidentified compounds are not taken into account for HPLC quantification, an underestimation of total polyphenol content is obtained. To simplify peak identification and quantification analysis some authors hydrolyze juice polyphenols before HPLC analysis. For example Mattila, Hellstrom, McDougall, Dobson, and Pihlava (2011) made an alkaline hydrolysis in their studies to determinate polyphenol content in European blackcurrant juices. Sometimes it is needed a pre-concentration and a purification of polyphenols from its complex matrix before instrumental analysis by HPLC (Wang, Gong, Chen, Han, & Li, 2012).

In addition, different specific methods exist for analysis of juice profiles of the different polyphenol types, like anthocyanins, procyanidins, flavanones, flavonols, flavan-3-O-ols, flavones and phenolic acids (Ignat, Volf, & Popa, 2011). Special emphasis is focused on a general fast HPLC method for all polyphenol analysis (De Villiers, Kalili, Malan, & Roodman, 2010; Rodríguez-Medina, Segura-Carretero, & Fernández-Gutiérrez, 2009; Valls, Millán, Martí, Borrás, & Arola, 2009) valid to quantify a total phenolic index (Tsao & Yang, 2003), but in general no quantification of the resulting compounds in juices has been done.

Quantification of total phenolic in juices is usually done by colorimetric methods. These simple assays are used to determine different structural groups present in phenolic compounds. The Folin-Ciocalteu assay is widely used to measure total phenolics (Singleton, Orthofer, & Lamuela-Raventós, 1999), while the vanillin and proanthocyanidin assays are used to estimate total proanthocyanidins (Naczk & Shahidi, 2006). These assays suffer from non-specificity, for instance the non-phenolic compounds as ascorbic acid reacts with the Folin-Ciocalteu reagent. Although, these methods provide very useful qualitative and quantitative information, their main disadvantage is that they only give an estimation of the total phenolic content and do not give quantitative measurement of individual polyphenol content.

An important effort has been done by databases such as Phenol-Explorer or USDA Database to compile both, total and individual polyphenol contents in foods measured by different analysis methods. This information is easily accessible and very useful to standardize polyphenol profiles obtained for the same food by different authors (Scalbert et al., 2011; USDA Database).

The aim of this research was to present a unique, fast and reliable UHPLC method valid to identify all kind of polyphenols and other interesting compounds like ascorbic acid (vitamin C) present in fruit juices. The principal objective was to improve HPLC IFU method number 71 (1998) with the simultaneous use of PDA and fluorescence detection. Trifluoracetic acid was used instead of formic acid in mobile phases. Besides, HPLC–MS was used to check the identification of doubtful compounds. The method proposed was applied to quantify the total individual polyphenols (TIP) in seven pure fruit juices. The selection of analysed fruit juices was done to cover most of compounds of polyphenol groups. This method allows obtaining TIP value valid for labelling fruit juices and a "fingerprint" of the tested fruit juice. This is one way to fulfill quality criteria and to check any adulteration of fruit juices.

## 2. Materials and methods

### 2.1. Chemicals

Phenolic compounds classified as standard compounds used for quantification were: gallic acid (assay HPLC $\geqslant$99%) for hydroxybenzoic acids, p-Coumaric acid (assay HPLC $\geqslant$98.0%) for hydroxycinnamic acids, (+)-catechin hydrate (assay HPLC $\geqslant$99%) for monomeric flavan-3-ols, resveratrol (assay GC $\geqslant$99%) for stilbenes, being all supplied by Sigma–Aldrich (Madrid, Spain). Anthocyanins standard was pelargonidin 3-O-glucoside (assay HPLC $\geqslant$95%) (callistephin chloride), flavanones standard was hesperidin (assay HPLC $\geqslant$98.5%), flavonols standard was quercetin 3-O-glucoside (assay HPLC $\geqslant$90%), and dihydrochalcones standard was phloridzin (assay HPLC $\geqslant$95%), all were purchased from Extrasynthèse (Genay, France). Ascorbic acid (assay $\geqslant$99.7%) was purchased from Panreac Química S.A. (Barcelona, Spain).

Other polyphenols used for identification purposes were flavonols aglycones (kaempferol, rhamnetin, isorhamnetin and myricetin) purchased from Sigma–Aldrich (Madrid, Spain), flavonols glycosides (kaempferol 3-O-glucoside, kaempferol 3-O-rutinoside, cynarin, quercetin 3-O-galactoside, kaempferol 7-O-glucoside, isorhamnetin 3-O-glucoside and isorhamnetin-3-O-rutinoside, purchased from Extrasynthèse (Genay, France). Quercetin 3-O-rutinoside and quercetin 3-O-rhamnoside were purchased from Sigma-Aldrich (Madrid, Spain). Hydroxybenzoic acids (3,4-dihydroxybenzoic acid, vanillic acid, 4-hydroxybenzoic acid), hydroxycinnamic acids (chlorogenic acid, caffeic acid, ferulic acid, syringic acid, cinnamic acid, ellagic acid), and monomeric flavan-3-ols ((−)-epigallocatechin, (−)-epicatechin, (−)-epigallocatechin 3-gallate) were purchased from Sigma–Aldrich (Madrid, Spain). Flavanones (narirutin, naringin, didymin) were purchased from Extrasynthèse (Genay, France).

Acetonitrile HPLC grade (assay 99.9%) was purchased from Panreac Química S.A. (Barcelona, Spain); trifluoroacetic acid for HPLC (assay 99%) and formic acid for HPLC (assay 98%) were purchased from Sigma–Aldrich (Madrid, Spain); Folin-Ciocalteu reagent was purchased from Merck (Darmstadt, Germany). Water was purified in a Milli-Q water purification system from Millipore (Bedford, MA, USA). All other chemicals employed were of analytical grade.

### 2.2. Fruit samples

Seven commercial fruit juices of well known polyphenol composition have been selected for this study. Strawberry puree (*Fragaria x ananassa*) with 6.8°Brix, orange juice (*Citrus sinensis* L. *Osbeck*) with 10.8°Brix, apple juice (*Malus pumila P. Mill.*) with 11.1°Brix, and black grape juice concentrate (*Vitis vinifera* L.) with 65.6°Brix, were kindly supplied by J. García Carrión S.A. (Jumilla, Spain). Bilberry juice concentrate (*Vaccinium myrtillus* L. *wild*) with 64.5°Brix, was kindly supplied by Grünewald Fruchtsaft (Stainz, Austria), and American cranberry juice concentrate (*Vaccinium macrocarpon Ait.*) with 62.2°Brix was supplied by Oceans Spray (Lakeville, Massachusetts, USA). Sour cherry juice concentrate (*Prunus cerasus* L.) with 60.2°Brix, was kindly supplied by Mondi Food (Rijkevorsel, Belgium). All fruit juices were frozen at −20 °C until use.

Concentrated fruit juice samples (60.2–65.6°Brix) were 5 times diluted in water to obtain a similar °Brix value to non concentrated fruit juices, while purees and fruit juices (6.8–11°Brix) were not diluted. In all cases, samples were centrifuged at 15,000*g* at 10 °C during 15 min in a Z383K Hermle centrifuge (Wehingen, Germany) to remove any solid residue. Supernatants were filtered with Teknokroma nylon filters of 0.45 μm (Barcelona, Spain) and used directly for juice analysis.

### 2.3. UHPLC-PDA-fluorescence analysis methods

Fruit juice analyses were performed in a UHPLC Agilent Technologies modular liquid chromatographic system serie 1200 (Santa Clara, CA, USA) equipped with a binary pump (G1312B), a photodiode array detector (PDA) with multiple wavelength (G1315C), a