Food Chemistry 208 (2016) 177–184

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Assessment of flavanol stereoisomers and caffeine and theobromine content in commercial chocolates

M.E. Alañón *, S.M. Castle, P.J. Siswanto, T. Cifuentes-Gómez, J.P.E. Spencer

Food and Nutritional Sciences Department, School of Chemistry, Food and Pharmacy, University of Reading, Whiteknights, RG6 6AP Reading, United Kingdom

ARTICLE INFO

Article history:
Received 21 December 2015
Received in revised form 16 March 2016
Accepted 29 March 2016
Available online 1 April 2016

Keywords:
Chocolate
Flavanols
Epicatechin
Catechin
Stereoisomers
Methylxanthines

ABSTRACT

Assessment of the flavanol composition of 41 commercial chocolates was by HPLC-DAD. Among individual flavonols ranged from 0.095 to 3.264 mg g^{-1}, epicatechin was the predominant flavanol accounting for 32.9%. Contrary to catechin, epicatechin was a reliable predictive value of the polyphenol content. Conversely the percentage of theobromine used as a proxy measure for nonfat cocoa solids (NFCS) was not a good predictor of epicatechin or flavanol content. In a further chiral analysis, the naturally occurring forms of cocoa flavanols, (−)-epicatechin and (+)-catechin, was determined joint the occurrence of (+)-epicatechin and (−)-catechin due to the epimerization reactions produced in chocolate manufacture. (−)-Epicatechin, the most bioactive compound and predominant form accounted of 93%. However, no positive correlation was found with% cocoa solids, the most significant quality parameter.

1. Introduction

In recent decades, nutrition research has focused on the investigation of bioactive dietary flavonoids, widely found in many plant-based foods and beverages, in order to elucidate their beneficial properties to human health. Cocoa (*Theobroma cacao L.*) and chocolate products appear to be one of the most promising foods due to their high polyphenol content, which evidently highlights the link with health-promoting properties (Andújar, Recio, Giner, & Ríos, 2012).

Cocoa is regarded to be a rich source of polyphenols, in particular flavanols, also known as flavan-3-ols or catechins. Predominant monomers include (−)-epicatechin and (+)-catechin, whilst these also act as oligomeric counterparts to form longer chain procyanidins. Human intervention studies have demonstrated that the consumption of cocoa or chocolate polyphenols can yield significant improvements in certain long-term cardiovascular health outcomes: blood pressure, vascular tone, endothelial function, insulin resistance, glucose tolerance, platelet reactivity and immune and antioxidant defense system (Fisher, Hughes, Gerhard-Herman, & Hollenberg, 2003; Heiss et al., 2003; Hopper et al., 2012; Lettieri-Barbato et al., 2012; Ostertag et al., 2013; Sies, Schewe, Heiss, & Helm, 2005).

More evidence suggests consumption of cocoa flavanols may improve aspects of cognitive function through enhanced neuronal activity, visual performance and cerebral blood flow (Field, Williams, & Butler, 2011; Francis, Head, Morris, & Macdonald, 2006; Sorond, Lipsitz, Hollenberg, & Fisher, 2008); the latter of which could in part be associated to cardiovascular-related properties previously mentioned. Additionally, cocoa is also rich in methylxanthines, namely theobromine and caffeine. These alkaloids are pharmacologically active, with the physiological capacity to modulate the central nervous system gastrointestinal tract inducing increased diuresis (Franco, Oñatibia-Astibia, & Martínez-Pinilla, 2013; Smit, Gaffan, & Rogers, 2004).

Among the flavanols, (−)-epicatechin has been identified as the more active compound responsible for the vascular health benefits associated with cocoa and chocolate (Schroeter et al., 2006). The chiral nature of monomeric catechins should be noted when considering bioactivity as this unique property enables epimerization processes that lead to conversion of (−)-epicatechin to (−)-catechin and (+)-catechin to (+)-epicatechin. Conditions associated with manufacturing processes of chocolate such as fermentation, drying, roasting and alkalinisation, and also standard cooking processes seem to impact upon rates of stereochemical conversion of catechins (Andres-LaCueva et al., 2008; Hurst et al., 2011; Kothe, Zimmermann, & Galensa, 2013; Stahl et al., 2009). More key evidence from a recent human study demonstrates that the bioactivity of flavanols is significantly influenced by their stereochemical configuration. They show that after oral administration of

* Corresponding author.
E-mail address: a.p.elena@reading.ac.uk (M.E. Alañón).

http://dx.doi.org/10.1016/j.foodchem.2016.03.116
0308-8146/

(−)-epicatechin the vascular response produced is nearly six times higher than that of (−)-catechin (Ottaviani et al., 2011). Therefore, based on this research, the distinction of stereochemical forms of flavanols in chocolate is relevant not only for the process manufacturing industry, but also due to their nutritional relevance and potential health effects.

Despite the numerous achiral analytical methodologies developed for the characterization and quantification of polyphenols from cocoa samples, few enantioselective methods have been reported in the bibliography. The chiral analysis has been carried out by means of chiral capillary electrophoresis (CEE) (Kofink, Papagiannopoulos, & Galensa, 2007; Kothe et al., 2013), micellar electrokinetic chromatography (MEKC) (Gotti, Furlanetto, Pinzauti, & Cavrini, 2006), ultra-high performance liquid chromatography (UPLC) (Cooper et al., 2007) and high performance liquid chromatography (HPLC) (Machonis, Jones, Schoneberg, & Kwik-Uribe, 2012), using as chiral selectors cyclodextrins in all the cited techniques. However, the occurrence of these compounds in chocolate has scarcely been reported showing contradictory results since the presence of (+)-epicatechin in cocoa beans has not been detected by some authors (Kofink et al., 2007; Kothe et al., 2013) while the presence of the four stereoisomers have been reported in other surveys (Gotti et al., 2006).

In light of all the evidence discussed, there is need for more data related to the phenolic profile and methylxanthine content of chocolate, in particular the enantiomereric flavanols. This has prompted us to analyse the detailed composition of commercially available chocolates by means of high performance liquid chromatography in order to assess their potential health benefits.

2. Materials and methods

2.1. Chemicals and reagents

The standards (−)-epicatechin, (+)-catechin, (−)-catechin, dimmer B2, theobromine and caffeic acid were obtained from Sigma-Aldrich (St. Louise, USA). Particularly (+)-epicatechin was provided by Nacalai Tesque INC (Kyoto, Japan). Solvents and reagents such as acetonitrile, methanol, hexane and acetone, acetic acid, trifluoro acetic acid, sodium phosphate monobasic, sodium acetate were purchased from Sigma Aldrich (St. Louise, USA) and water HPLC grade and tetrahydrofuran (THF) was obtained from Fisher Scientific (Leicester, UK).

2.2. Sample collection and preparation

Products selected for this study comprise dark and milk chocolate samples found in commercial markets of Reading (United Kingdom) between April and July of 2014 (Table 1). The sample set was intended to be the most representative possible covering a wide variety of product categories and cocoa solid content. The study was conducted with a total of 41 samples. The percentage of cocoa solids of dark chocolate and milk chocolate samples ranged between 47–90% and 20–39% respectively, except for two samples where the information was not declared on the label. Once the samples were purchased, they were coded and stored at −20 °C prior to their analysis.

2.3. Extraction process

Each sample was grated in order to obtain a homogeneous powder, which were well mixed and divided in three batches to carry out the analysis in triplicate. The isolation of the phenolic fraction of the chocolate samples was carried out according to the method proposed by Adamson et al. (1999) with some modifications.

Table 1
Sample set of the chocolate (n = 41).

Type of chocolate	Brand and product category (Cocoa%)[a]
Dark chocolate	Cavalier (55)
	Divine (70)
	Green and Black's (85, 70, 60)
	Lindt Excellence (90, 70, 49, 47)
	Morrison Savers (50, 40)
	Morrison Signature (85, 72, 70)
	Sainsbury's basic (50)
	Sainsbury's taste the difference (72)
	Tesco (74)
	Tesco finest (74, 72, 70)
Milk chocolate	Cadbury (20, 39)
	Cadbury (36)
	Galaxy (25)
	Green and Black's (34)
	Lindt Lindo (30, 31)
	Milka (30); Nestle (25)
	Morrison (32, 27)
	Morrison savers (28)
	Sainsbury's basic (27)
	Sainsbury's taste de difference (30)

[a] Brands have been listed alphabetically within product category. Chocolate order in this table has no relationship to the order shown in the following tables.

Before the extraction process, grated samples (0.50 g) were submitted to a defatting process to eliminate lipids adding 5 mL of *n*-hexane. The mixture was vortexed and sonicated for 5 min at room temperature, followed by centrifugation at 1500*g* for 5 min also at room temperature. The supernatant was discarded and the same defatting procedure was repeated twice more. Finally, the defatted samples were dried under a gentle stream of nitrogen.

Phenolic compounds were extracted from the defatted residue three times with 2.5 mL of acetone/water/acetic acid (70:28:2 v/v/v) by vortexing and subsequent sonication (10 min at room temperature). Samples were then centrifuged at 1500*g* for 5 min and supernatants were pooled. The extracts were concentrated under vacuum at room temperature to a volume of approximately 2 mL, which was diluted with 1 mL of 10% (v/v) acetonitrile with 0.5 mg/mL EDTA and ascorbic acid solution and water HPLC grade up to 5 final volume of 5 mL. Finally, diluted extracts were filtered through a 0.20 μm membrane filter to vials that were stored at −80 °C until their chromatographic analysis.

2.4. HPLC analysis of flavanols

HPLC separation and quantification of flavanol content from chocolate samples were performed on an Agilent 1100 series system (Agilent Technologies, Palo Alto, CA, USA), equipped with a DAD photodiode detector (G1316A), quaternary gradient pump (G1311A) and a degassing system (G1322A) coupled to an Agilent Chem Station (version B.01.03) for data processing. Extracts in triplicate, after filtration (0.20 μm polyester membrane, Sartorius Stedim Biotech GmbH, Goettingen, Germany) were injected (50 μL) on Nova-Pak C18 column (250 × 4.6 mm; 4 μm particle size; Waters Ltd., Elstree, UK) protected by a column guard (Phenomenex, Torrance, CA, USA), both thermostated at 30 °C. The binary system phases were solvent A water/THF/TFA 92:2:0.1 (v/v/v) and solvent B acetonitrile with 0.1% TFA, with a flow rate of 0.7 mL min^{-1} and a stop time of 28 min. The gradient was as follows: Initially, 15% B; 10 min, 17% B; 20 min, 100% B; 24 min, 15% B. Detection was made using the DAD chromatograms obtained at 220 nm. Catechin, epicatechin and procyanidin B2 (epicatechin-4-*β*-8-epicatechin) were identified by comparing the retention times and spectral characteristics of those provided by their standards. Meanwhile, dimmer B5 (epicatechin-4-*β*-6-epicatechin), trimmer C1 (epicatechin-4-*β*-8-epicatechin-4-*β*-8-epicatechin)