LC-MS identification and bioaccessibility of free and melanoidins-bound phenolic compounds in chocolate

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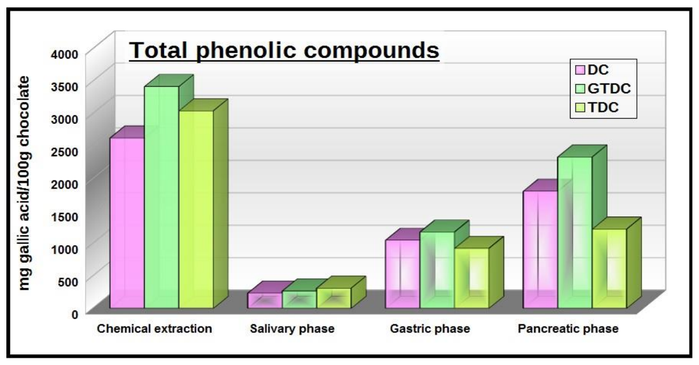
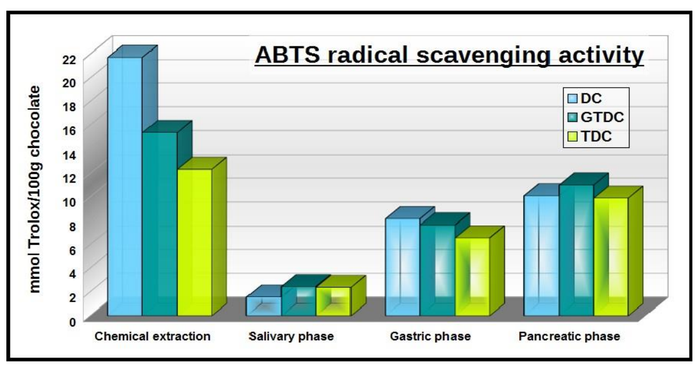
Introduction and aim

Polyphenols have become an intense focus of research interest because of their perceived health-beneficial effects such as anti-atherogenic, anti-inflammatory and anti-tumoural effects. Cocoa (*Theobroma cacao*) is a major economically important international crop and has been associated with several nutritional benefits [1]. Moreover, emerging evidence from *in vitro* and *in vivo* studies suggested that the gastrointestinal tract may be the key site for the biological action of food melanoidins [2]. Despite the poor knowledge regarding melanoidins structure, it is well known that phenolic compounds, present in the raw material, can be incorporated into the melanoidins skeleton [3]. Transport of antioxidant compounds through the gastrointestinal tract can be an essential physiological function of dietary melanoidins that has received little attention so far. The aim of the study was to carry out a comprehensive characterization of the phenolic profile of three different types of dark chocolate using LC-ESI-QTOF-MS/MS, before and after *in vitro* digestion, in order to evaluate the bioaccessibility index of the polyphenols. We also characterized high molecular weight melanoidins (HMWM) extracted from dark chocolate for their ability to release low molecular weight polyphenols after simulated *in vitro* digestion.

Experimental methods

Three different types of dark chocolate (dark 70% cocoa (**DC**), dark 70% cocoa and 8% turmeric (**TDC**), dark 62% cocoa and 2% Sakura green tea (**GTDC**)) were *in vitro* digested with a simulated gastro-intestinal procedure, which mimicked the physiochemical conditions of the gastro-intestinal tract [4]. Polyphenols were extracted from un-digested samples with methanol-water-formic acid solution (70:28:2 v/v) followed by a second extraction step with acetone. Total polyphenols were determined by Folin-Ciocalteau assay whereas antioxidant activities by FRAP and ABTS methods [4]. Water soluble high molecular weight melanoidins (HMWM) were extracted by ultrafiltration (10 kDa) from the three different types of dark chocolate. HMWM have been characterized for their content in total polyphenols and antioxidant activity [5]. HMWM were *in vitro* digested [6] and separated from low molecular weight fractions (LMWF) by ultrafiltration. Digested HMWM and LMWF were characterized for their total phenolic content and antioxidant activity. Finally, phenolic compounds in chocolates, digested chocolates and LMWF from digested melanoidins were identified and quantified by liquid chromatography-electrospray ionization-quadrupole-time of flight mass spectrometry (LC-ESI-QTOF-MS/MS).





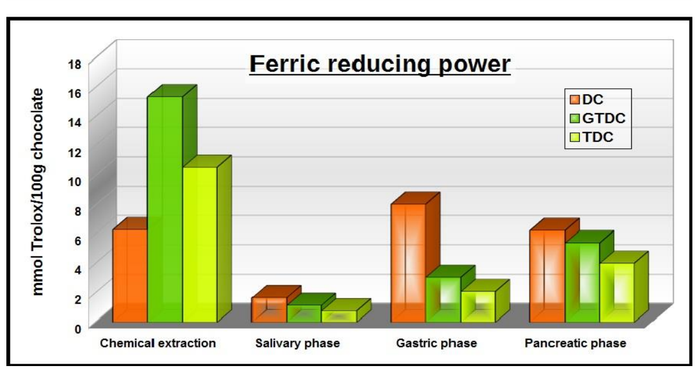
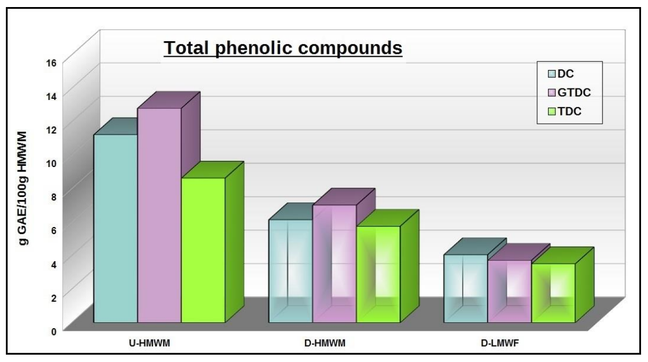


Figure 1. *Total phenolic determination (Folin-Ciocalteau assay), ABTS radical scavenging activity and ferric reducing power (FRAP) tested after chemical extraction and in vitro digestion in DC, GTDC and TDC.*

Results

Figure 1 shows total phenolic concentrations (Folin-Ciocalteau assay), ABTS radical scavenging activity and ferric reducing power (FRAP) assayed on chemical extracted chocolates and *in vitro* digested chocolates in DC, GTDC and TDC. As reported in **Figure 1,** total phenolic compounds, antioxidant activity and ferric reducing ability decreased during the *in vitro* digestion depending on the type of chocolate and the formulation. *In vitro* digestion of melanoidins (HMWM) resulted in the release of phenolic compounds in the low molecular weight fractions (LMWF) (**Figure 2**). *In vitro* digestion of melanoidins (HMWM) resulted in a decrease in the antioxidant activity of HMWM with a concomitant increase in the antioxidant activity of the low molecular weight fraction (LWWF).

Total polyphenol concentrations were also evaluated by LC-ESI-QTOF-MS/MS, which showed that chemically extracted samples varied from 807.9 ± 5.9 to 1099.8 ± 5.1 mg/100 g of chocolate in DC and TDC, respectively. More than **140** phenolic compounds were identified in this study, among these **45** have been identified for the first time in chocolate (**Figure 3**). **Figure 4** shows the percent composition in phenolic compounds of the different chocolate types after chemical extraction, *in vitro* digested chocolates, and those released from melanoidins after chocolate *in vitro* digestion. Flavan-3-ols was the most representative class in each chocolate types followed by hydroxycinnamic acids. Catechin, epicatechin and epigallocatechin were the mains flavan-3-ols detected in the three chocolates. A remarkable amount of B and A types dimers, trimers, tetramers, pentamers and hexamers of procyanidins and derivatives were found, marking out the flavan-3-ols profile. Ferulic acid, caffeoyl-aspartate and coumaroyl-aspartate were the main found hydroxycinnamic acids. Caffeoyl-aspartate and coumaroyl-aspartate showed the highest values in TDC, 53.1 ± 0.6 and 21.8 ± 0.2 mg/100 g of chocolate, respectively. A great contribution was given by ellagitannins: ellagic acid, HHDP-galloyl-hexoside and ellagic acid-galloyl-hexoside. Some phenolic acids (caffeoylquinic, protocatechuic, vanillic acids) and derivatives (homovanillic acid glycoside, syringic acid glycoside, protocatechuic acid glycoside) were also identified. The addition of green tea and turmeric resulted in the presence of typical compounds related to the two ingredients. Typical green tea gallate flavan-3-ols were found: epigallocatechin-3-gallate and epicatechin gallate contributed with concentration values of 33.5 ± 2.1 and 9.1 ± 0.1 mg/100 g of chocolate, respectively. Theasinensin C, quercetin-3-rutinoside, galloylquinic acid and gallic acid were also found in GTDC. Bisdemethoxycurcumin, demetoxycurcumin and curcumin pinpointed the turmeric addition with concentration values of 115.6 ± 2.2, 82.7 ± 1.3 and 74.6 ± 0.5 mg/100 g of chocolate, respectively.



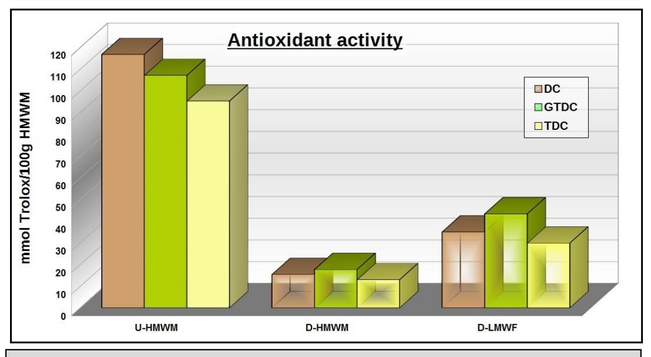


Figure 2. *Total phenolic determination (Folin-Ciocalteau assay) and ABTS radical scavenging activity tested on un-digested HMWM (U-HMWM), digested HMWM (D-HMWM) and digested LMWF (D-LMWF) extracted from DC, GTDC and TDC.*

The same LC-ESI-QTOF-MS/MS identification was carried out on low molecular weight fractions (LMWF), extracted by ultrafiltration after *in vitro* digestion of high molecular weight melanoidins (HMWM) (**Figure 4**). Total polyphenol concentrations in low molecular weight fractions varied from 558.2 ± 14.4 in DC to 980.1 ± 9.7 mg/100 g of high molecular weight melanoidins (HMWM) in GTDC. Ferulic acid is the most concentrated among phenolic acids, ranging from 51.3 ± 1.0 in TDC to 296.5 ± 3.1 mg/100 g of HMWM in GTDC. Ellagic acid gave an important contribution in the phenolic profile concentration, showing concentration values of 302.0 ± 9.2, 549.7 ± 9.1 and 769.4 ± 15.6 mg/100 g of HMWM in DC, GTDC and TDC, respectively. Quinic acid exhibited a significant contribution among phenolics related to organic acids, by varying its concentration from 147.3 ± 0.7 to 151.0 ± 0.6 mg/100 g of HMWM in DC and GTDC, respectively.

Mass spectrometry quantification confirmed the decrease over than 80% in the polyphenol concentrations in the three different kinds of chocolate due to the simulated gastro-pancreatic digestion suggesting that phenolic compound extraction and stability was significantly influenced by *in vitro* digestion (**Figure 4**). The bioaccessibility index is reported in **Figure 5.**

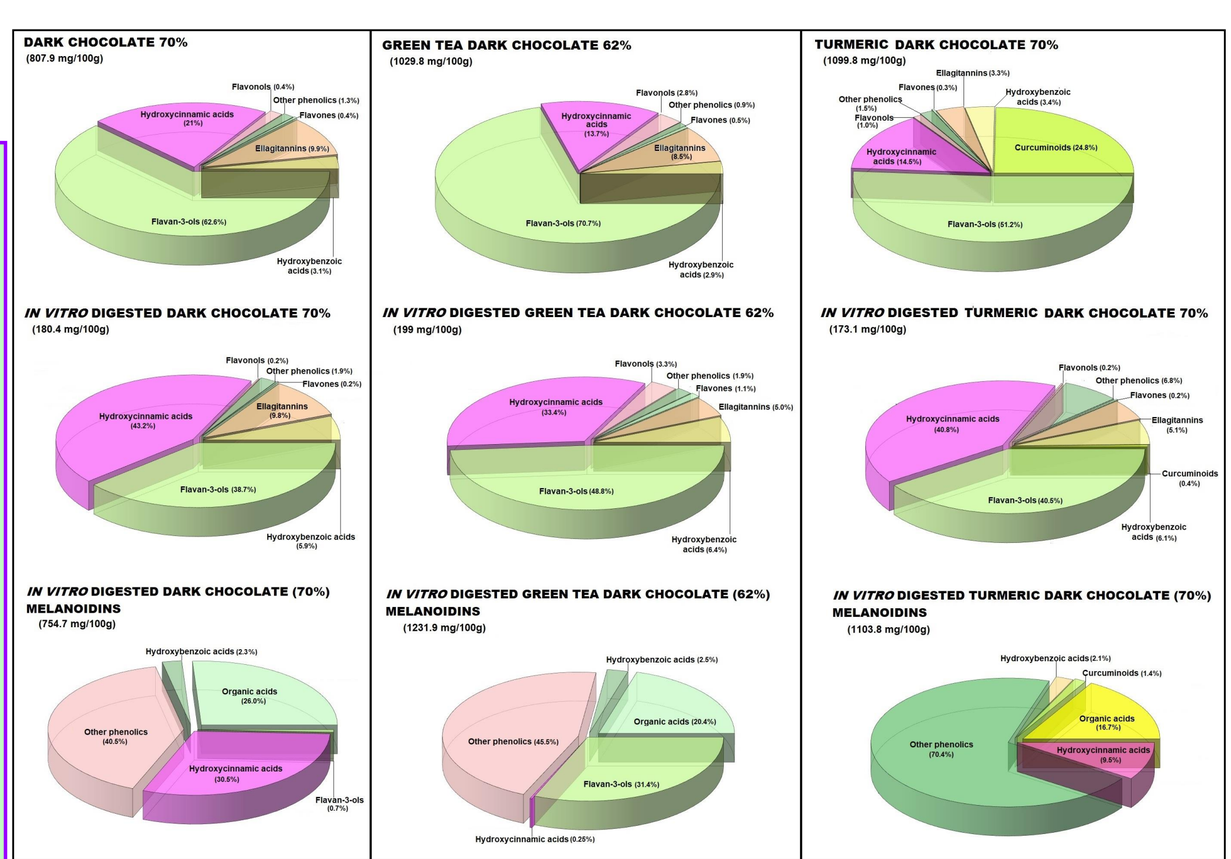


Figure 4. *Global percentage of flavan-3-ols, flavonols, hydroxycinnamic acids, hydroxybenzoic acids, flavones, ellagitannins, curcuminoids and other phenolics identified and quantified after chemical extraction and in vitro digestion in three different chocolate types and digested low molecular weight fractions from DC, GTDC and TDC.*

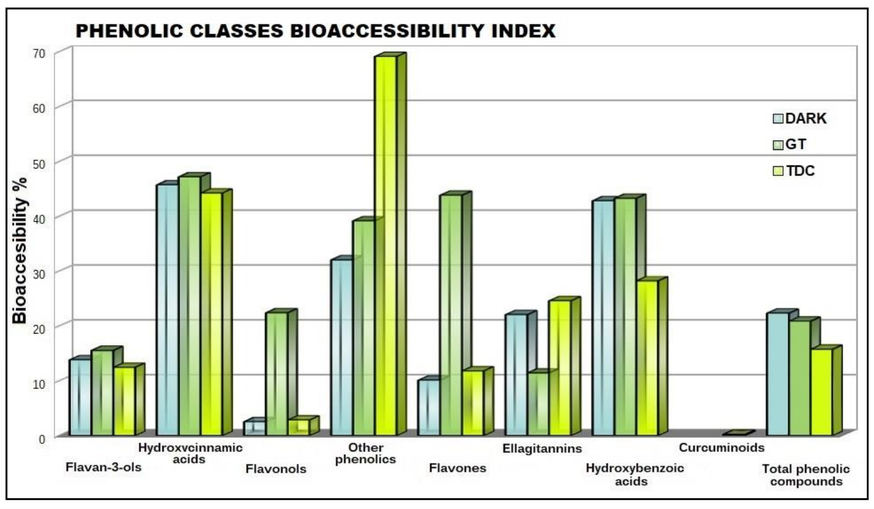


Figure 5. *Average bioaccessibility index (BI) of the chocolate samples and of the different phenolic classes in the three different chocolates (DC, GTDC and TDC). BI was calculated as follows:*

$$BI\ (\%) = (CC_D/CC_C) * 100$$

where CC_D is the concentration of a compound at the end of the in vitro digestion and CC_C is the concentration of the same compound in chocolate as determined with the chemical extraction procedure.

A	Quercetin 7-*O*-rhamnoside-3-*O*-rutinoside	Quercetin 7-*O*-hexoside-3-*O*-rutinoside	Kaempferol-7-*O*-rhamnoside-3-*O*-rutinoside	Kaempferol-7-*O*-hexoside-3-*O*-rutinoside	Myricetin-3*O*-(*O*-galloyl)-hexoside	Myricetin-7-*O*-hexoside-3-*O*-rutinoside
R1	-H	-H	-H	-H	-OH	-OH
R2	-OH	-OH	-H	-H	-OH	-OH
R3	-*O*-rut.	-*O*-rut.	-*O*-rut.	-*O*-rut.	-*O*-gall.-hex.	-*O*-rut.
R4	-*O*-rham.	-*O*-hex.	-*O*-rham.	-*O*-hex.	-OH	-*O*-hex.

B	Protocatechuic acid-4-*O*-hexoside	Syringic acid-4-*O*-hexoside	Homovanillic acid-4-*O*-hexoside	Di-hydro-Coumaric Acid	Di-hydro-Caffeic Acid	Ferulic acid pentoside
R1	-COOH	-COOH	$-CH_2-COOH$	$-CH_2-CH_2-COOH$	$-CH_2-CH_2-COOH$	-CH=CH-COOH
R2	-OH	$-OCH_3$	$-OCH_3$	-H	-OH	$-OCH_3$
R3	-*O*-hex.	-*O*-hex.	-*O*-hex.	-OH	-OH	-*O*-pen.
R4	-H	$-OCH_3$	-H	-H	-H	-H

C	Apigenin-7-*O*-pentoside	Apigenin-6-*C*-hexoside-2'-*O*-rhamnoside	Apigenin-6,8-di-*C*-hexoside	Apigenin-8-*C*-hexoside-4'-*O*-hexoside	Apigenin-8-*C*-hexoside-6-*C*-pentoside
R1	-H	-hex.-rham.	-hex.	-H	-pen.
R2	-*O*-pen.	-OH	-OH	-OH	-OH
R3	-H	-H	-hex.	-hex.-hex.	-hex.

D	Naringenin-6-*C*-hexoside-7-*O*-hexoside	Eriodictyol-7-*O*-hexoside	Eriodictyol-6-*C*-hexoside	Eriodictyol-6-*C*-hexoside-7-*O*-hexoside
R1	-H	-OH	-OH	-OH
R2	-hex	-H	-hex.	-hex.
R3	-*O*-hex.	-O-hex.	-OH	-*O*-hex.

E	Phloretin-3'-*C*-hexoside	Phloretin-2'-*O*-hexoside-pentoside
R1	-OH	-*O*-hex.-pen.
R2	-hex.	-H

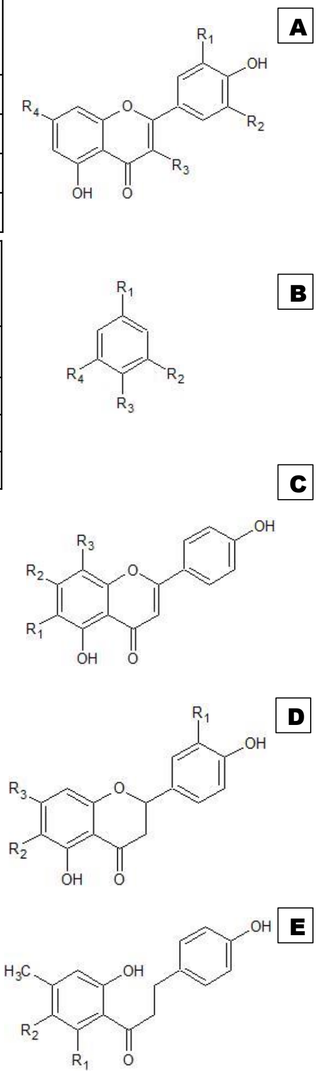


Figure 3. *Examples of newly identified phenolic structures belonging to flavonols, flavones, hydroxybenzoic and hydroxycinnamic acids classes. Gall: galloyl; hex: hexoside; pen: pentoside; rham: rhamnoside; rut: rutinoside.*

References

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Conclusion

This study allowed a tentative identification of **140** individual phenolic compounds, **45** newly identified in chocolate (**Figure 5**). The addition of Sakura green tea leaves or turmeric powder influenced and modified the phenolic profile, resulting in an increase in the phenolic concentration. Mass spectrometry confirmed that this increase was tightly connected to the food matrix, showing typical compounds belonging to green tea and turmeric. Finally, bioaccessibility index of total phenolic compounds was similar for each chocolate types; nevertheless, it was partially influenced by the formulation especially among the single phenolic classes. *In vitro* digestion of high molecular weight melanoidins (>10 kDa) resulted in the release of phenolic compounds non-covalently linked to the melanoidins skeleton. This could be ascribed to the high ionic strength of the gastro-intestinal fluids, which may promote the disruption of the electrostatic interaction and of the hydrogen bonds between melanoidins and phenolic compounds. In addition, the presence of bile salts may interfere with the hydrophobic interaction between phenolic compounds and melanoidins. Free phenolic compounds, released during the *in vitro* digestion, can exert their protective effect at the intestinal level or can be absorbed to various extents through the intestine and exert their health benefits at systemic level.