Chocolate and other cocoa-containing products are a rich source of polyphenols. This paper describes an ultra-performance liquid chromatography (UPLC) method that can separate and quantify in 3 min six of the major chocolate polyphenols: catechin; epicatechin; B2 (epicatechin-4â€”8-epicatechin); B5 (epicatechin-4â€”6-epicatechin); C1 (epicatechin-4â€”8-epicatechin-4â€”8-epicatechin); and tetramer D (epicatechin-4â€”8-epicatechin-4â€”8-epicatechin-4â€”8-epicatechin). A survey of 68 chocolate samples indicated that there was a strongly predictive relationship between epicatechin and the other individual polyphenols, especially procyanidin B2 (R^2 = 0.989), even though the chocolates came from varied sources and manufacturers. The relationship was less strong with catechin, and so further work to explore the reasons for this difference was performed. Chiral analysis on a subset of 23 chocolates showed that (−)-epicatechin had a predictive relationship with (+)-catechin in line with the other polyphenols, but not with (−)-catechin (the predominant form). This indicates that (−)-catechin is the most affected by manufacturing conditions, possibly formed through epimerization from (−)-epicatechin during processing. The results show that epicatechin concentrations can be used to predict the content of other polyphenols, especially B2 and C1, and total polyphenols content. Finally, the (−)-catechin content is not predictable from the epicatechin content, and it is concluded that this is the main form of polyphenol that varies according to manufacturing conditions and cocoa origin.

INTRODUCTION

Cocoa beans, chocolate, and cocoa powder are known to be rich sources of polyphenols (1). Fresh Forastero cocoa beans contain 15–20% soluble polyphenols in dried fat-free mass, which corresponds to approximately 6% in air-dried beans and 5% in fermented beans (2). Monomers and polymers of up to 10 units constitute 95% of the polyphenol content in cocoa. The main monomer is (−)-epicatechin, with up to 35% of polyphenol content in the cocoa bean (2). A recent study claimed that epicatechin content is the main reason for the vascular health benefits associated with cocoa and chocolate (3). Other reported compounds are catechin, traces of procyanidin B1, procyanidin B2, procyanidin B5, and procyanidin C1 (2, 4), as well as resveratrol and piceid (5). The concentrations of all polyphenols can vary tremendously between cocoa-containing foods, and this can vary depending on the source of the beans, the processing conditions, and how the chocolates are manufactured. Due to these factors, the ratio and type of polyphenols found in the beans are unlikely to be exactly the same as those found in the final product.

The initial processing steps that cocoa beans must undergo at the site of cultivation are fermentation, drying, and deshelling. These processes are not as strictly controlled as factory-based steps. Beans (or broken beans called nibs) are sterilized, roasted, and ground to become cocoa liquor, and then this can be made into cocoa powder or used directly in chocolate products. The steps to make cocoa powder can include alkalinization (or Dutching), which is also known to reduce the polyphenol content considerably (6). Fermentation is also considered to be one of the major steps that affect polyphenol content, and the beans can be fermented under a variety of conditions depending on the desired flavor characteristics of the final cocoa. Ecuadorian beans are characteristically fermented for 3 days and West African beans for 5 days. Between days 2 and 3, epicatechin content has been shown to decrease sharply, which could indicate that it is either taken up in the formation of larger tannins or lost in the fluids that drain away during fermentation (7). There is a large variation in epicatechin after fermentation (range = 1.00–16.52 mg/g) (7, 8), indicating that judicious conditions, and how the chocolates are manufactured. Due to
choice of fermented beans for chocolate could be the most important step in ensuring a high-polyphenol chocolate, but this does have consequences as short fermentation results in higher bitterness and less flavor of the cocoa (8, 9) and therefore the resultant chocolate.

Chocolate and cocoa have been found to deliver potential health benefits, often related to their antioxidant effects. End points include blood pressure (10–12), flow-mediated dilation (FMD) (10, 13–15), LDL and HDL cholesterol (16), insulin sensitivity (10–12), platelet activity (17, 18), antioxidant status, oxidative stress, and nitric oxide (19). For instance, 100 g of dark chocolate (74% cocoa content) was shown to improve arterial function 60 min after consumption (13). Some studies have been performed with people with essential (10) or systolic hypertension (12). In another study, 40 g of dark chocolate (74% cocoa content) significantly improved FMD and total antioxidant status and reduced platelet function in healthy smokers 2 h after consumption (20). A recently published study, as part of the Zutphen Elderly Study, has discovered that high cocoa consumption in elderly men was related to lower blood pressure and cardiovascular mortality compared to low consumers (21).

Ultra-performance liquid chromatography (UPLC) shows improved speed, sensitivity, and resolution compared to HPLC (22). The same separation on reversed phase HPLC that takes over 20 min can be accomplished in under 3 min by reversed-phase UPLC (RP-UPLC). This study aimed to use the newly developed UPLC methodology on a wide range of chocolates from different countries and investigate the relationships between the individual polyphenol concentrations.

MATERIALS AND METHODS

Reference Compounds. The standards (+) catechin and (−) epicatechin were obtained from Sigma, and procyanidin B2 was obtained from Extrasynthese (Genay, France). A reference sample (SRM 2384 baking chocolate) was obtained from the National Institute of Standards and Technology (http//www.nist.gov/srm) with certified catechin and epicatechin values.

Chocolate Samples. A survey on 68 commercially available (finished product) chocolate samples (61 dark, 7 milk) sourced from different countries (Australia, Belgium, Brazil, Canada, France, Italy, Japan, The Philippines, Russia, South Africa, Switzerland, and the United States), covering the brand names of Auchan, Barry Callebaut, Cadbury, Cemoi, Cote d’Or, Hershey’s, Kraft, Lindt, Mars, Meiji, Nestlé, and Poulain, was conducted. The labeled cocoa contents were in the range of 20–99%, although only 40 chocolates declared cocoa content. The chocolates included finished products with wafer and nut ingredients, as well as tablet form chocolate.

Extraction Process. The chocolate sample extraction was based on the work of Adamson et al. (23), slightly modified as per Couret et al. (24). Samples were ground to obtain a homogeneous material. Approximately 0.5 g of ground chocolate was defatted three times with 5 mL of hexane, and the residue was dried under a gentle nitrogen stream. The residue was then extracted three times with 2.5 mL of aceton/ water/acetic acid (70:28:2 v/v/v) by sonication and centrifugation, each time combining the supernatant in centrifuge tubes. These extracts were then evaporated under vacuum at 35 °C to a volume of approximately 2–3 mL, and this was diluted with Milli-Q water up to 25 mL. Catechin and epicatechin solubility was checked up to 10 times higher than the limit of quantification to ensure full dissolution at these concentrations. After filtration using 2 μm membrane filters, the diluted extracts were then analyzed using RP-UPLC. Each extraction was carried out in duplicate, and a quality control sample was included in each batch, that is, NIST Certified Reference Material (SRM 2384 baking chocolate). We used the analysis of the SRM to calculate recovery for catechin and epicatechin, and these were found not to be different from 100%.

UPLC Analysis. The UPLC system was equipped with a binary gradient pump, a sample injector, a column oven, a photodiode array detector, and a degassing system and driven by Waters Empower software. Five microliters of the diluted extract was injected onto the UPLC system and separated by an Acquity UPLC BEH C18 column (1.7 μm, 50 × 2.1 mm). The binary system phases were (A) water/ THF/TF (98:2:0.1 v/v/v) and (B) acetonitrile with 0.1% TFA, with a flow rate of 0.3 mL/min, giving a maximum back pressure of 5600 psi, which is within the capabilities of the UPLC. The 8 min gradient was as follows: 0.0–0.8 min, 90–87% A (nonlinear); 0.8–3.0 min, 87–85% A; 3.0–3.1 min, 85–0% A; 3.1–5.0 min, 0% A linear; 5.0–5.1 min, 0–90% A; 5.1–8.0 min, 90% A re-equilibration time. Detection was at 220 nm, and a spectrum was recorded at 190–350 nm to aid identification. The wavelength 220 nm was selected as it was the best compromise for both resolution and sensitivity than the more standard 280 nm. Catechin, epicatechin, and procyanidin B2, epicatechin-4β-8-epicatechin, were identified by comparing the retention times and spectral characteristics of their peaks with those of standards (Figure 1a,c,e). B5, epicatechin-4β-6-epicatechin; C1, epicatechin-4β-8-epicatechin-4β-8-epicatechin; and tetramer D, epicatechin-4β-8-epicatechin-4β-8-epicatechin-4β-8-epicatechin, were identified by their UV spectra characteristics and their relative order of elution, because the order of elution is largely independent of minor variations in the solvent system of RP-HPLC (2). Catechin was quantified using the catechin standard, whereas epicatechin, procyanidins B2 and B5, trimer C1, and tetramer D were quantified as epicatechin equivalents. A linear response was obtained for catechin and epicatechin standards in the concentration ranges of 11.2–448.0 μg/g (R2 = 0.9999) and 62.5–2500 μg/g (R2 = 0.9967) of chocolate, respectively. Therefore, the limits of quantification were taken as 11.2 μg of catechin/g of chocolate and 62.5 μg of epicatechin/g of chocolate. The method was validated using the NIST Certified Reference Material.

Mass Spectrometric Analysis. Accurate mass and fragmentation pattern information was obtained using a Micromass Q-TOF II hybrid mass spectrometer equipped with an electrospray ionization (ESI) ion source. Major experimental parameters were as follows: nebulizer gas (nitrogen) maximum (approximately 900 L/h); auxiliary gas, 250 L/h; cone gas, 75 L/h; source block temperature, 150 °C; nebulizer temperature, 400 °C; time-of-flight potential, 9.1 kV; multichannel plate potential, 2200 V. In negative mode 3 kV needle voltage and 20 V cone voltage were used. Mass range was 50–1500. The scan time was 0.5 s. Argon was used as focusing/collision gas at a pressure of 12 psi. Transmitter quadrupole and other lens parameters were optimized for maximum sensitivity while maintaining a resolution of 10000. To maximize mass accuracy a calibrating solution (0.1% phosphoric acid) was pumped by a syringe pump into the ESI interface through a T-junction element installed between the UPLC column and the ESI interface. To obtain the fragmentation pattern of analytes, tandem mass spectrometric experiments were performed using collision-induced dissociation (CID). The collision energy was varied in the range of 20–50 eV to obtain representative product ion spectra.

Analysis of Catechin and Epicatechin Enantiomers. To determine the concentrations of both enantiomers of catechin and epicatechin in chocolate samples, a subset of 23 chocolate bars from the above study was selected (19 dark, 4 milk). The extraction method was principally the same as for UPLC except that sample concentration was achieved with evaporation with nitrogen gas to a volume of approximately 1 mL. Samples were then reconstituted to a volume of 5 mL with acetonitrile 10% prior to HPLC analysis. Concentrations were determined by comparison of values obtained from a standard curve prepared in commercial white chocolate, which did not contain detectable levels of flavanols. Chiral HPLC analysis was performed using a Waters 2690 HPLC (Waters, Milford, MA) using a Cyclodex 1-2000 RSP 250 × 4.6 mm (Advanced Separation Technologies, Whippany, NJ) containing derivatized β-cyclodextrin. Mobile phase A consisted of 50 mM NaH2PO4 at pH 3.0, and mobile phase B consisted of 80% acetonitrile in 30 mM NaH2PO4 at pH 3.0. The flow rate was 1 mL/min with a linear gradient from 10.0 to 13.5% B over 45 min and then increasing to 45% B at 70 min. Detection was by fluorescence monitoring (ex, 280 nm; em, 310 nm). A chromatogram of a representative chocolate is shown in Figure 2.
RESULTS

Panels a and c of Figure 1 show the chromatograms for catechin and epicatechin standards and for the certified reference material. It can be seen that all six polyphenols are separated within 3 min compared to 20 min with HPLC (Figure 1b,d). Table 1 shows the standard deviation of repeatability and intermediate reproducibility obtained by replicate analysis of a certified reference material (n = 8). To confirm the identity of the peaks found in the UPLC chromatogram, their accurate masses were determined. Comparing theoretical and experimentally measured masses, the mass errors were calculated and for each compound were found to be below 5 ppm (Table 2).

Table 1. Standard Deviation of UPLC Repeatability and Intralaboratory Reproducibility Obtained by Replicate Analysis of a Certified Reference Material (n = 8)

<table>
<thead>
<tr>
<th>analyte</th>
<th>SD (r) (mg/g)</th>
<th>SD (R) (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>catechin</td>
<td>0.004</td>
<td>0.009</td>
</tr>
<tr>
<td>epicatechin</td>
<td>0.015</td>
<td>0.045</td>
</tr>
<tr>
<td>dimer B2</td>
<td>0.009</td>
<td>0.002</td>
</tr>
<tr>
<td>dimer B5</td>
<td>0.006</td>
<td>0.021</td>
</tr>
<tr>
<td>trimer C</td>
<td>0.006</td>
<td>0.013</td>
</tr>
<tr>
<td>tetramer D</td>
<td>0.011</td>
<td>0.026</td>
</tr>
</tbody>
</table>

The fragmentation patterns of all six peaks were also recorded by applying CID tandem mass spectrometry. By comparison of the experimentally found fragmentation patterns with the product ion spectra of standard compounds (catechin, epicatechin, and B2 dimer) and the information found in the literature (25), all characteristic fragments of all analytes have been detected, hereby further supporting their identity (Table 2).

Table 2. Results of Accurate Mass and Tandem Mass Spectrometric (Fragmentation Pattern) Experiments for the Six UPLC Peaks Shown in Figure 1c

<table>
<thead>
<tr>
<th>analyte</th>
<th>determined mass of compd (amu)</th>
<th>theoretical mass of compd (amu)</th>
<th>mass error (ppm)</th>
<th>confirmed fragments in MS/MS mode (amu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>catechin</td>
<td>290.079038</td>
<td>290.078213</td>
<td>−2.8</td>
<td>245</td>
</tr>
<tr>
<td>dimer B2</td>
<td>578.142427</td>
<td>578.142013</td>
<td>−0.7</td>
<td>425, 407, 289</td>
</tr>
<tr>
<td>epicatechin</td>
<td>290.079038</td>
<td>290.079713</td>
<td>2.3</td>
<td>245</td>
</tr>
<tr>
<td>trimer C</td>
<td>866.205815</td>
<td>866.202813</td>
<td>−3.5</td>
<td>577, 289</td>
</tr>
<tr>
<td>tetramer D</td>
<td>1154.269203</td>
<td>1154.270413</td>
<td>1.0</td>
<td>577, 289</td>
</tr>
<tr>
<td>dimer B5</td>
<td>578.142427</td>
<td>578.143713</td>
<td>2.2</td>
<td>425, 407, 289</td>
</tr>
</tbody>
</table>

Using the UPLC method, 68 different chocolates were analyzed. Epicatechin was in the range of 0.071–1.942 mg/g, whereas catechin was in the range of 0.043–0.519 mg/g, B2 in the range of 0.041–1.174 mg/g, B5 in the range of not detectable (ND)–0.236 mg/g, trimer C in the range of ND–0.905 mg/g, and tetramer D in the range of ND–0.387 mg/g. When the six measured polyphenols are combined as a total, the ranges found were 0.179–4.991 mg/g. Epicatechin accounted for a mean percentage of 39.0% of the total and catechin, 11.2%.

When the individual polyphenols contained in the chocolates are plotted against each other in scatter plots (Figure 3), it can be observed that epicatechin concentrations in the chocolate are strongly correlated to the concentrations of procyanidin B2 (R² = 0.989), B5 (R² = 0.931), trimer C (R² = 0.956), and, to a
Figure 3. Relationship of epicatechin content (a–d) and catechin content (e–h) to procyanidin B2 and B5, trimer C, and tetramer D contents within a sample set of 68 chocolates.
lesser extent, the tetramer D ($R^2 = 0.617$). However, these same chocolates did not have similar strong relationships between catechin and the four procyanidins, that is, B2 ($R^2 = 0.643$), B5 ($R^2 = 0.486$), trimer C1 ($R^2 = 0.566$), and tetramer D ($R^2 = 0.311$). The strong correlation between epicatechin and the sum of the six measured polyphenols (Figure 4) is evident, and even the weaker relationship between catechin and epicatechin (Figure 5) is not sufficient to affect the relationship.

Both enantiomers of catechin and epicatechin from chocolate samples were separated using chiral HPLC (Figure 2). The chocolate samples contained $0.954 \pm 0.537$ mg/g of total monomeric flavanols (both enantiomers of catechin and epicatechin). Epicatechin in chocolate was predominantly in the (−) form, which accounted for 95 ± 1% of epicatechin present in the chocolate samples. The concentration of (−)-epicatechin in chocolate samples was $0.679 \pm 0.413$ mg/g, whereas (+)-epicatechin was $0.033 \pm 0.022$ mg/g. Catechin in chocolate was also predominantly in the (−) form, which accounted for 89 ± 3% of catechin present in the chocolate samples. The mean concentration of (−)-catechin was $0.218 \pm 0.126$ mg/g compared to a mean concentration of $0.025 \pm 0.015$ mg/g for (+)-catechin.

**DISCUSSION**

The content of epicatechin in chocolate according to other publications falls within the range found in this study. For instance, it has been found that milk and dark chocolate ($n = 8$) have a range of $0.18-1.25$ mg/g (6) compared to the current range of $0.071-1.942$ mg/g. For catechin, the same study found a range of $0.05-0.33$ mg/g, whereas the current study found a range of $0.043-0.519$ mg/g. The wider range is to be expected as 68 chocolates were used in this study compared to 8 in the previous study. A wide range of epicatechin content in chocolate was also found in a study of epicatechin contents of different foods, the average being $0.022$ mg/g ($n = 3$) (26), which is considerably lower than the mean found in the current study. However, there was a coefficient of variation of 146% among the data for chocolate, indicating that the three types chosen for that comparison were extremely dissimilar.

The strong correlation between epicatechin and the other polyphenols, excluding catechin (Figures 3a–d and 4), is most unexpected considering the diverse sources and types of chocolate in the sample set. These relationships suggest that whenever cocoa beans are processed into chocolate, these polyphenols are all affected to the same degree. For example, Dutching (alkalinization) of a cocoa powder could equally
reduce the amount of epicatechin as compared to procyanidin B2. This means that the epicatechin content could be used to predict the content of the other procyanidins. The relationships between catechin concentration and other polyphenols are much less linear and predictive (Figure 5), and this could indicate that catechin is the most susceptible of the six measured procyanidins to differences in manufacturing, cocoa bean source, and other factors. As it is known that there are two different forms of the two monomers from cocoa, this aspect was investigated further by measuring (+)- and (−)-catechin and epicatechin in a subset of 23 chocolates.

The data indicated that the (−)-form of catechin was predominant in all chocolates, with an average ratio of 1.0:1.1 (−)-catechin/(+)-catechin. The epicatechin content of chocolate correlated well with (+)-catechin content, but not with (−)-catechin (Figure 6). This signifies that (−)-catechin may be the main form of polyphenol that varies with manufacturing conditions. A previous publication showed that fresh cocoa beans contain (+)-catechin with undetectable levels of the (−)-form (27). The source of the (−)-catechin in chocolate could be epimerization of the (−)-epicatechin during processing. This study also found large variation in the concentrations of the natural forms of epicatechin and catechin in the raw beans at different maturation stages, but (−)-catechin was undetectable. If indeed there is epimerization, it does not appear to occur during the aging of the bean but only during cocoa processing. Previous work from our group has shown that the (−)-form of catechin is less bioavailable than the (+)-form (28). Since (−)-catechin is the predominant form in chocolate, this indicates lower potential absorption of catechin from chocolate.

The method reported here can be used to quickly screen chocolates for polyphenol content. Epicatechin concentrations can be used to predict the content of other polyphenols, especially B2 and C1 and the total polyphenol content, with a high degree of certainty. (−)-Catechin content is not predictable from epicatechin content, and it is concluded that (−)-catechin is the main polyphenol of those measured that varies with manufacturing conditions and therefore by brand.

**SAFETY**

Apart from standard caution with all solvents and acids, there are no specific safety criteria for this work.

**ABBREVIATIONS USED**

NIST, National Institute of Standards and Technology; RP-UPLC, reversed phase ultra-performance liquid chromatography; HPLC, high-performance liquid chromatography; HDL, high-density lipoprotein; THF, tetrahydrofuran; TFA, trifluoroacetic acid; ND, not detectable; ESI, electrospray ionization; CID, collision-induced dissociation.

**LITERATURE CITED**

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