Food Chemistry 202 (2016) 254–261

Contents lists available at ScienceDirect

Food Chemistry

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

Analytical Methods

Determination of antioxidant capacity and phenolic content of chocolate by attenuated total reflectance-Fourier transformed-infrared spectroscopy

Yaxi Hu [a], Zhi Jie Pan [a,b], Wen Liao [a], Jiaqi Li [a], Pierre Gruget [c], David D. Kitts [a], Xiaonan Lu [a,*]

[a] Food, Nutrition and Health Program, Faculty of Land and Food Systems, The University of British Columbia, Vancouver, V6T 1Z4 BC, Canada
[b] Food Science and Technology Program, Department of Chemistry, National University of Singapore, Singapore 117543, Singapore
[c] CHOCOLAT NATUREL, 5971 Marine Drive, West Vancouver, V7W 2S1 BC, Canada

ARTICLE INFO

Article history:
Received 8 June 2015
Received in revised form 23 January 2016
Accepted 29 January 2016
Available online 2 February 2016

Keywords:
Chocolate
Antioxidant
Phenolic content
FT-IR
Chemometrics
PLSR

ABSTRACT

Antioxidant capacity and phenolic content of chocolate, containing different amounts of cacao (35–100%), were determined using attenuated total reflectance (ATR)-Fourier transformed-infrared (FT-IR) spectroscopy (4000–550 cm^{-1}). Antioxidant capacities were first characterized using DPPH (2,2-diphenyl-1-picrylhydrazyl) and ORAC (oxygen radical absorbance capacity) assays. Phenolic contents, including total phenol and procyanidins monomers, were quantified using the Folin–Ciocalteu assay and high performance liquid chromatography coupled with photodiode array detector (HPLC–DAD), respectively. Five partial least-squares regression (PLSR) models were constructed and cross-validated using FT-IR spectra from 18 types of chocolate and corresponding reference values determined using DPPH, ORAC, Folin–Ciocalteu, and HPLC assays. The models were validated using seven unknown samples of chocolate. PLSR models showed good prediction capability for DPPH [$R^2 - P$ (prediction) = 0.88, RMSEP (root mean squares error of prediction) = 12.62 μmol Trolox/g DFW], ORAC ($R^2 - P$ = 0.90, RMSEP = 37.92), Folin–Ciocalteu ($R^2 - P$ = 0.88, RMSEP = 5.08), and (+)-catechin ($R^2 - P$ = 0.86, RMSEP = 0.10), but lacked accuracy in the prediction of (−)-epicatechin ($R^2 - P$ = 0.72, RMSEP = 0.57). ATR-FT-IR spectroscopy can be used for rapid prediction of antioxidant capacity, total phenolic content, and (+)-catechin in chocolate.

1. Introduction

Worldwide consumption of chocolate and cocoa-containing products increased by 10% from 2002 to 2010 (Afoakwa, 2014), which might be attributed to consumer economic enhancement and increasing knowledge of potential health benefits derived from cocoa constituents (Cooper, Donovan, Waterhouse, & Williamson, 2008). Chocolate and cocoa-containing products are a source of non-nutrient bioactive polyphenols with potential health benefits including reduced risk of cardiovascular disease (Hooper et al., 2012) and prebiotic activity (Tzounis et al., 2011).

The majority of polyphenols in chocolate belong to the flavan-3-ols, a class of flavonoids. Approximately one third (ca. 37%) of flavan-3-ols are monomer units of proanthocyanidins (*i.e.* (+)-catechin and (−)-epicatechin), while proanthocyanidins make up the reminder (ca. 57%). The high phenolic content of cocoa contributes to the *in vitro* antioxidant capacity of hot chocolate compared with black and green teas, and red wine, which are generally considered polyphenol-rich (Lee, Kim, Lee, & Lee, 2003). Comparing studies and potential health benefits is difficult because not all polyphenols have the same bioactivity *in vivo*. Flavan-3-ol monomer concentrations increase rapidly in blood following consumption (Baba et al., 2000; Richelle, Tavazzi, Enslen, & Offord, 1999). Absorption of proanthocyanidins might also occur following transformation to monomers by gut bacteria (Spencer, Schroeter, Rechner, & Rice-Evans, 2001). Thus, accurate and descriptive analyses of chocolate antioxidant capacity and phenolic content (*i.e.*, total phenolic content and the content of proanthocyanidins monomers) are of great interest to researchers in the agri-food and biomedical industries.

Several assays, including the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging and oxygen radical absorbance capacity (ORAC) competitive assays, have been used to evaluate chocolate antioxidant capacity (Belščak, Komes, Horžić, Ganić, & Karlović, 2009; Lee et al., 2003; Miller et al., 2009; Ortega et al., 2008). The Folin–Ciocalteu assay indicates total phenolic content (Miller et al., 2009; Ortega et al., 2008) while high performance liquid chromatography (HPLC), coupled with photodiode array detector (DAD) or fluorescence detector, has been used to

* Corresponding author.
E-mail address: xiaonan.lu@ubc.ca (X. Lu).

http://dx.doi.org/10.1016/j.foodchem.2016.01.130
0308-8146/

determine flavan-3-ols with various degrees of polymerization (Miller et al., 2009; Robbins et al., 2012). Cell culture or animal models have also been useful for the evaluation of bioavailability of polyphenols in chocolate (Collodel et al., 2014; Rodríguez-Ramiro, Ramos, Bravo, Goya, & Martín, 2011; Yakala et al., 2013; Yasuda, Natsume, Osakabe, Kawahata, & Koga, 2011). Although these chemical methods and bioassays can measure individual components accurately, obtaining comprehensive profiles for chocolate antioxidant capacity and phenolic properties requires a range of analyses to be conducted. Having a simple, rapid and accurate method to simultaneously evaluate chocolate antioxidant capacity and phenolic content would be more relevant for the high throughput analysis used by the food industry and academic research.

Infrared (IR) spectroscopy has potential for this specific purpose. IR spectroscopy provides a spectrum of IR energy (between the microwave and visible light region, wavelength range: 700 nm–1 mm) absorbed by molecules that possess a permanent dipole moment and is indicative of the functional groups in the molecules present. With the development of FT-IR (Fourier transformed-IR) spectroscopy and attenuated total reflectance (ATR) techniques, use of IR spectroscopy has increased significantly and sample preparation has become much less involved. Multivariate statistical analyses, including principal component analysis, hierarchical cluster analysis, and partial least square regression (PLSR), are commonly used in chemometrics to construct models for sample classification and calibration (Lu & Rasco, 2012). As a result of the rapid, accurate, and less destructive features of IR spectroscopy, there are an increasing number of applications using IR and chemometrics for the investigation of food adulteration (Ozen & Mauer, 2002), bacteria contamination in foods (Al-Qadiri, Lin, Cavinato, & Rasco, 2006) and quality assurance (Shiroma & Rodriguez-Saona, 2009) as well as compositional analysis (Halim, Schwartz, Francis, Baldauf, & Rodriguez-Saona, 2006).

The current study developed a high-throughput, accurate method for antioxidant capacity, total phenolic content, and identification of specific phenolic monomers in chocolate using FT-IR spectroscopy. To the best of our knowledge, this is the first study that has used FT-IR spectroscopy to determine these properties in chocolate simultaneously.

2. Materials and methods

2.1. Chemicals and reagents

DPPH (2,2-diphenyl-1-picrylhydrazyl), AAPH (2,2′-Azobis(2-methylpropionamidine) dihydrochloride), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), fluorescein sodium salt, Folin–Ciocalteu reagent, gallic acid, (+)-catechin (99%), and (−)-epicatechin (90%) were purchased from Sigma–Aldrich (St. Louis, USA). Sodium carbonate, sodium dihydrogen phosphate, potassium phosphate dibasic, *n*-hexane, methanol, acetic acid and folic acid were obtained from Fisher Scientific (Ottawa, Canada). All the chemical agents were of at least chemical grade. Methanol was HPLC grade. Deionized water (18.2 MΩ/cm) was prepared using a Millipore system.

2.2. Pre-treatment of chocolate samples

Chocolate samples for this study were purchased from local grocery stores in Vancouver (CA) or provided by "CHOCOLAT NATUREL" (Vancouver, Canada) and were stored at −80 °C until experimental use. The chocolate samples had no visible bloom. Samples included milk and dark chocolate, with the cacao content ranging from 35% to 100% (Table 1). Eighteen samples were used for the construction of PLSR calibration and cross-validation models, and seven additional samples were used as unknowns to evaluate the prediction capability of PLSR calibration models.

The chocolate was defatted before extraction of the phenolics as described by Belščak et al. (2009) with the following modifications. Briefly, samples were sliced into small pieces and 2 g of each extracted with 10 ml of *n*-hexane, twice, by shaking at 22 °C, 150 rpm for 40 min. After each extraction, the mixtures were centrifuged for 10 min at 4000×*g*, and the supernatant discarded. After the second extraction, samples were dried in a vacuum dryer for 24 h at 22 °C. The defatted samples were weighed and stored at −80 °C until further extraction. Two aliquots were taken from each sample to be defatted.

2.3. Determination of total phenolic content by Folin–Ciocalteu assay

The phenolics were extracted with 10 ml of 70% methanol from the defatted samples. Both defatted samples from each type of chocolate were extracted, and the results averaged. Total phenolic content of the chocolate was determined following the procedures of Singleton, Orthofer, and Lamuela-Raventos (1999). Diluted extract (0.1 ml) or blank (0.1 ml deionized water) was mixed with 7.9 ml water and 0.5 ml Folin–Ciocalteu reagent for 5 min at 22 °C. Then, 1.5 ml saturated sodium carbonate solution was added. Reagents were mixed thoroughly by shaking vigorously for 10 s by hand. The mixture was incubated in the dark at 22 °C for 2 h before determination of the absorbance at 765 nm using an UV/Visible spectrometer (Shimadzu Corporation Japan, UV-1700). Gallic acid in 70% methanol was diluted (0.05–1.5 g/L) to create a calibration curve. Total phenolic content is expressed as mg of gallic acid equivalents/g defatted chocolate (mg GAE/g DFW). Each extract was analyzed in triplicate.

2.4. Determination of antioxidant capacity by DPPH assay

The DPPH free radical assay was selected because it has been used extensively for different food constituents and matrices.

Table 1
Description of chocolate samples analyzed in the current study.

Sample	Product description	% Cacao
M80	Produced by CHOLAT NATUREL, sweetened by maple syrup	80%
S60	Produced by CHOLAT NATUREL, sweetened by sucrose	60%
LSM	Lindt Seasalt Milk	30%
LSD	Lindt Seasalt Dark	47%
RSD	Ritter Sport Dark	73%
RSM	Ritter Sport Milk	35%
HSD	Hershey's Special Dark	50%
Butl	Butlers Milk Chocolate	32%
CBCR	Chocolat Bonnat Cacao Real	75%
CDMO	Cadbury Dairy Milk Orange	N/A[a]
Chrisball	Christmas Ball	N/A[a]
HL	Honey Love	90%
LTC	Les Tropiques du Chocolat	100%
TB	Theobroma Chocolat	72%
TJD	Trader Joe's Dark	85%
TCTM	Thomashaas Chai Tea Milk	N/A[a]
TRD	Thomashaas Rooibos Dark	67%
VLNA	Valrhona Le Noir Amer	71%
CD	Cadbury Dark	N/A[a]
HCM	Hershey's Creamy Milk	N/A
S80	Produced by CHOLAT NATUREL, sweetened by sucrose	80%
LE	Lindt Excellence	70%
Peru	Perugina Bittersweet Chocolate	70%
MCGL	Michel Cluizel Grand Lait	45%
RSAM	Ritter Sport Alpine Milk	30%

[a] Not labeled on the package.

DPPH was dissolved in 70% methanol, and the absorbance at 515 nm adjusted to 1.1 in a UV/Visible spectrometer. Extracts were prepared as described for the Folin–Ciocalteu analysis (Section 2.3). Diluted extract (0.1 ml) or 70% methanol (0.1 ml) were added to 2 ml of DPPH solution. The absorbance at 515 nm was determined after incubation for 30 min at 22 °C in the dark. Trolox in 70% methanol (50–1000 μmol/L) was used to create a calibration curve. Results were expressed as μmol of Trolox equivalents/g defatted chocolate (μmol Trolox/g DFW). Each chocolate extract was analyzed in triplicate.

2.5. Determination of antioxidant capacity by $ORAC_{FL}$ assay

Chocolate extracts for the ORAC assay were prepared as described elsewhere (Robbins et al., 2012) with the following modifications. Briefly, defatted samples were extracted in duplicate with 20 ml acetone:water:acetic acid (70:29.5:0.5), and stored at −80 °C for further analysis. ORAC-fluorescein method was used as described previously (Chen & Kitts, 2008). A 75 mM phosphate buffer (pH 7.4) prepared with monosodium phosphate and potassium hydrogen phosphate was used for the dilution of reagents and samples. Samples and Trolox (100 μL) was mixed with 60 μl fluorescein (200 nmol/L) and 40 μl AAPH (60 mmol/L) in 96-well plates. Fluorescence (excitation = 485 nm; emission = 527 nm) was read every minute up to 60 min using a fluorescent microplate reader (Tecan Austria GmbH, Tecan infinite M200 Pro). Area-under-the-curve was calculated by integration, and the linear relationship with sample or Trolox concentrations was calculated ($ORAC_{FL}$ = slope of sample/slope of Trolox). Results were expressed as μmol of Trolox equivalents/g defatted chocolate (μmol Trolox/g DFW). Each chocolate extract was analyzed in triplicate.

2.6. Determination of phenolic monomers by high performance liquid chromatography

Extracts were prepared as previously described for total phenolic content, and then analyzed using reverse phase HPLC for two proanthocyanidins monomers, namely, (+)-catechin and (−)-epicatechin. The chromatographic conditions were as described previously (Belščak et al., 2009) with the following modifications. HPLC analyses were conducted using an Agilent 1100 Series HPLC–DAD at 278 nm. Extracts were passed through a 0.22-μm nylon syringe filter (Thermo Scientific, Rockwood, TN, USA) before injection on to a Phenomenex Kinetex C_{18} column (100 × 3.00 mm, 2.6 μ, 100 Å). The mobile phase consisted of 3% formic acid (solvent A) and HPLC grade methanol (solvent B). Elution was performed using a linear gradient at 30 °C with a flow rate of 0.2 mL/min as follows: 2% B was increased to 32% B over 17 min, increased to 40% B at 25.5 min, and again to 95% B over 30 min before being maintained for 4 min. Before injecting each sample, the column has been equilibrated at 2% B for 10 min. Each extract was injected three times and the average results are expressed as mg/g defatted chocolate (mg/g DFW).

2.7. FT-IR instrumentation and spectral collection

FT-IR spectra of the whole samples were collected at room temperature using a Perkin Elmer model spectrum 100 FT-IR spectrometer based on a universal attenuated total reflectance sensor. Samples and the (+)-catechin standard were scanned over the range 4000–550 cm^{-1} with a resolution of 2 cm^{-1}. Each spectrum was an average of 16 scans (*i.e.* 16 accumulations). The chocolate was grated into small pieces and deposited directly on to the crystal sensor, and the force gauge was set at 145. Spectra were collected for each chocolate sample using different sub-samples (n = 13) and for (+)-catechin powder (standard).

2.8. Spectral analysis and chemometric models

The FT-IR spectral fingerprinting region (1800–700 cm^{-1}) was used for further analyses because the targeted functional groups appeared primarily in this region. Automatic baseline correction was applied to FT-IR spectra by using OMNIC software version 7.0 (Thermo-Nicolet, Madison, WI, USA).

Using Matlab 2014a (MathWorks Inc, Natick, MA, USA), PLSR models were constructed based on the processed FT-IR spectra to determine if a linear relationship existed between FT-IR spectral information and the reference values for antioxidant capacity, total phenolic content and proanthocyanidins monomers. A sample set of 18 chocolates was used to establish the calibration models, and ninefold cross-validation was used to evaluate the prediction power of the calibration models, *i.e.* one ninth of the data was removed from the dataset sequentially for validation and the remaining data calibrated. Prediction powers of these calibration models were also evaluated using seven unknown chocolate samples. Correlation of determination (R^2) and root mean squares error of calibration (RMSEC), cross-validation (RMSECV) and prediction (RMSEP) were calculated to assess the performance of the calibration models.

3. Results and discussion

3.1. Phenolic profile of chocolate

Antioxidant capacities, along with phenolic contents including total phenolic content and procyanidins monomers, of 25 chocolate samples are presented in Table 2. Relative antioxidant capacities and phenolic compositions for the chocolates examined in this study were similar to previous reports (Belščak et al., 2009; Haytowitz & Bhagwat, 2010). Although dark chocolate, with more cacao, exhibited higher antioxidant capacity, total phenolic content, and procyanidins monomers than milk chocolate (Belščak et al., 2009; Haytowitz & Bhagwat, 2010; Miller et al., 2006), we could not establish a good correlation between cacao content and the various factors measured (Table S1). We attribute this to: (1) cacao content on the package represents total ingredients by weight derived from cacao beans including cacao solids and cocoa butter (Huang, Zou, Chen, Luo, & Kong, 2003). In the case of antioxidant capacity, therefore, our results represent activity derived from the hydrophilic components of chocolate, *i.e.* cacao solids (Gu, House, Wu, Ou, & Prior, 2006). If the product contains more cocoa butter, antioxidant capacity values would be lower than products with the same cacao content but more cacao solids. (2) Manufacturers use a variety of cacao cultivars as well as processing and storage parameters, and all of these impact antioxidant capacity and phenolic profiles of the final products. (3) Because of the complexity of chocolate, many components, other than phenols

Table 2
Antioxidant capacity and phenolic properties of chocolate samples.

	DPPH (μmol Trolox/g DFW)	ORAC (μmol Trolox/g DFW)	Folin–Ciocalteu (mg GAE/g DFW)	(+)-catechin (mg/g DFW)	(−)-epicatechin (mg/g DFW)
Range of concentration	10.20–134.32	39.67–424.62	2.60–48.49	0.02–1.16	0.02–6.01

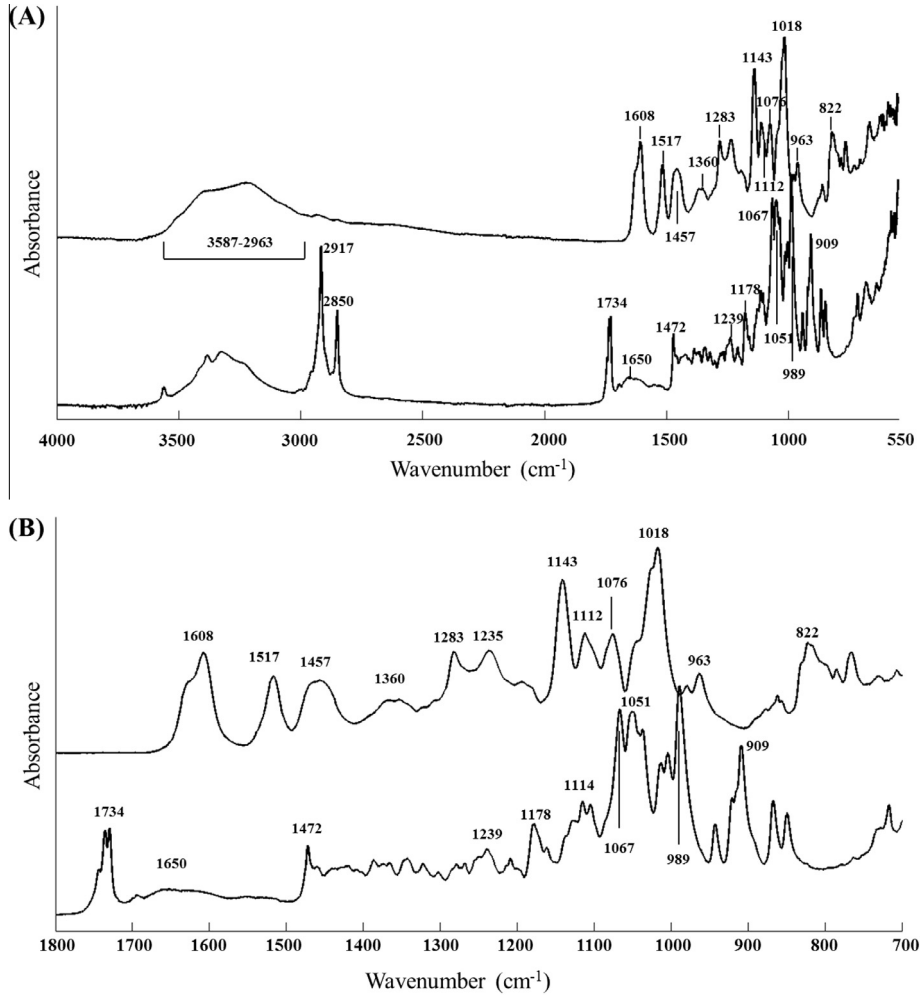

Fig. 1. (A) Representative full-range FT-IR spectrum of chocolate (bottom) and (+)-catechin (top). (B) Fingerprinting range (1800–700 cm^{-1}) of FT-IR spectra of chocolate (bottom) and (+)-catechin (top).

are present, that could influence the results in different ways (Arts et al., 2002). (4) Different types of phenolic compounds respond different in DPPH and ORAC analyses (Frankel & Meyer, 2000).

Thus, samples with higher cacao content might have lower DPPH values, and samples with higher antioxidant values could have lower total phenolic content values. These results support the viewpoint that it is important to develop an alternative multi-dimensional approach for the analysis of antioxidant capacity and the phenols content of chocolate (Frankel & Meyer, 2000). Antioxidant capacity and phenolic content can be predicted from FT-IR spectral features. Thus, calibration models constructed by correlating FT-IR spectral features with standard values measured using chemical-based assays (Table 2) were used for the development of robust regression models.

3.2. FT-IR spectral features of chocolate

Examples of the FT-IR spectral features of chocolate and (+)-catechin standard are shown in Fig. 1A. FT-IR spectra of different chocolates were similar (Fig. S2). (+)-Catechin is one of the flavan-3-ols in chocolate while other phenolic compounds share similar structures to (+)-catechin. Therefore, (+)-catechin FT-IR

Table 3
PLSR models for determination and prediction of antioxidant capacity, total phenolic content and procyanidins monomers in chocolate.

Assays		N[a]	n[b]	Factors	RMSEC[c]	R^2	RMSECV[d]	$R^2 - CV$	RMSEP[e]	$R^2 - P$
Antioxidant capacity	DPPH (μmol Trolox/g DFW)	234	104	13	8.46	0.95	10.87	0.92	13.07	0.89
	ORAC (μmol Trolox/g DFW)	234	104	15	30.97	0.95	45.52	0.89	37.92	0.90
Total phenolic content	Folin–Ciocalteu (mg GAE/g DFW)	234	104	11	3.21	0.96	4.21	0.93	5.08	0.88
Procyanidins monomers	(+)-catechin (mg/g DFW)	234	104	11	0.07	0.96	0.09	0.94	0.10	0.86
	(−)-epicatechin (mg/g DFW)	234	104	12	0.48	0.91	0.58	0.87	0.57	0.72

[a] N, number of spectra for calibration.
[b] n, number of spectra for prediction.
[c] RMSEC, root mean squares error of calibration.
[d] RMSECV, root mean squares error of cross-validation (9-fold).
[e] RMSEP, root mean squares error of prediction.

spectral features were used to represent chocolate and cacao phenolics. Bands in the FT-IR spectral fingerprinting region (1800–800 cm^{-1}) are critical to the identification of molecular structure (Lu & Rasco, 2012). Those at 963 cm^{-1}, 1018 cm^{-1}, 1076 cm^{-1}, and 1112 cm^{-1} were assigned to C–O and C–C stretching modes, while bands at 1283 cm^{-1}, 1360 cm^{-1}, and 1457 cm^{-1} are associated with O–C–H, C–C–H, and C–O–H bending modes (Wilkerson et al., 2013). Bands reflecting the ring structures occur at 822 cm^{-1} (ring C–H deformation), 1143 cm^{-1} (C–OH of phenyl), 1517 cm^{-1} (in-plane C–H bending of phenyl), and 1608 cm^{-1} (C–C stretching of phenyl) (Movasaghi, Rehman, & ur Rehman, 2008). The wide band at 3587–2963 cm^{-1} were assigned to O–H stretching (Lu & Rasco, 2012).

The majority of energy absorbance information associated with functional groups of flavan-3-ols can be seen in Fig. 1B. However, the absorbances of specific bands were lower than in pure (+)-catechin due to the low concentrations of the phenolic compounds. Furthermore, additional bands were visualized in FT-IR spectrum from the chocolate samples, reflecting the complex chemical composition of chocolate. Most of the additional bands in FT-IR spectrum of chocolate were associated with lipids (909 cm^{-1}, 1239 cm^{-1}, 1472 cm^{-1}, 1734 cm^{-1}, 2917 cm^{-1} and 2850 cm^{-1}) (Che Man, Syahariza, Mirghani, Jinap, & Bakar, 2005; Movasaghi et al., 2008; Schulz & Baranska, 2007), which are consistent with the emulsion-like characteristic of chocolate – cocoa solid dispersion phase within cocoa butter or milk butter continuous phase (Afoakwa, Paterson, & Fowler, 2007). Other bands were assigned to carbohydrates (989 cm^{-1}, 1051 cm^{-1} and 1067 cm^{-1}) and proteins (1178 cm^{-1} and 1650 cm^{-1}) (Movasaghi et al., 2008).

3.3. Quantitative analysis of antioxidant capacity, total phenolic content and procyanidins monomers of chocolate

The fingerprinting region of FT-IR spectra (1800 cm^{-1} and 700 cm^{-1}) was selected for the quantitative analyses of chocolate antioxidant capacity and phenolic content in PLSR models. With 11–15 latent variables (*i.e.* PLSR factors), 91–96% of the spectral variances were explained and correlated with the reference values (Table S2). Parameters for the PLSR calibration models are summarized in Table 3. Apart from the (−)-epicatechin PLSR model (R^2 = 0.91, RMSEC > 32% of the average value), all the others had high R^2 values (~0.95), while the RMSECs were around 13–16% of the average reference values. These indicate a good linear relationship between FT-IR spectral features and the reference values.

Ninefold cross-validation was applied to avoid over-fitting of the PLSR models. The cross-validation models for each standard value were constructed five times, and the averaged parameters are summarized in Table 3. A representative cross-validation model is shown in Fig. 2. The R^2 – CV were lower and RMSECV were higher in all the models compared with the calibration models, which is in accordance with the principle of cross-validation (Lu & Rasco, 2012). Apart from the (−)-epicatechin model (R^2 – CV = 0.87, RMSECV > 38% of the average value), the high R^2 – CV (>0.90) and low RMSECV (<23% of the average values) of the PLSR models support the capability of FT-IR spectroscopy to predict antioxidant capacity, total phenolic content and (+)-catechin contents in chocolate.

PLSR loading plots were employed to identify the wavelengths of the original FT-IR spectra that explained more of the discrimination, and to assign the chemical structures that might be associated with the properties determined by different assays. The loading plots for the first two factors of the five PLSR calibration models were, in general, different, but had some subtle similarity (a representative plot is shown in Fig. 3). The wavelengths with loading values greater than zero were correlated positively with reference values, while wavelengths with negative values were correlated

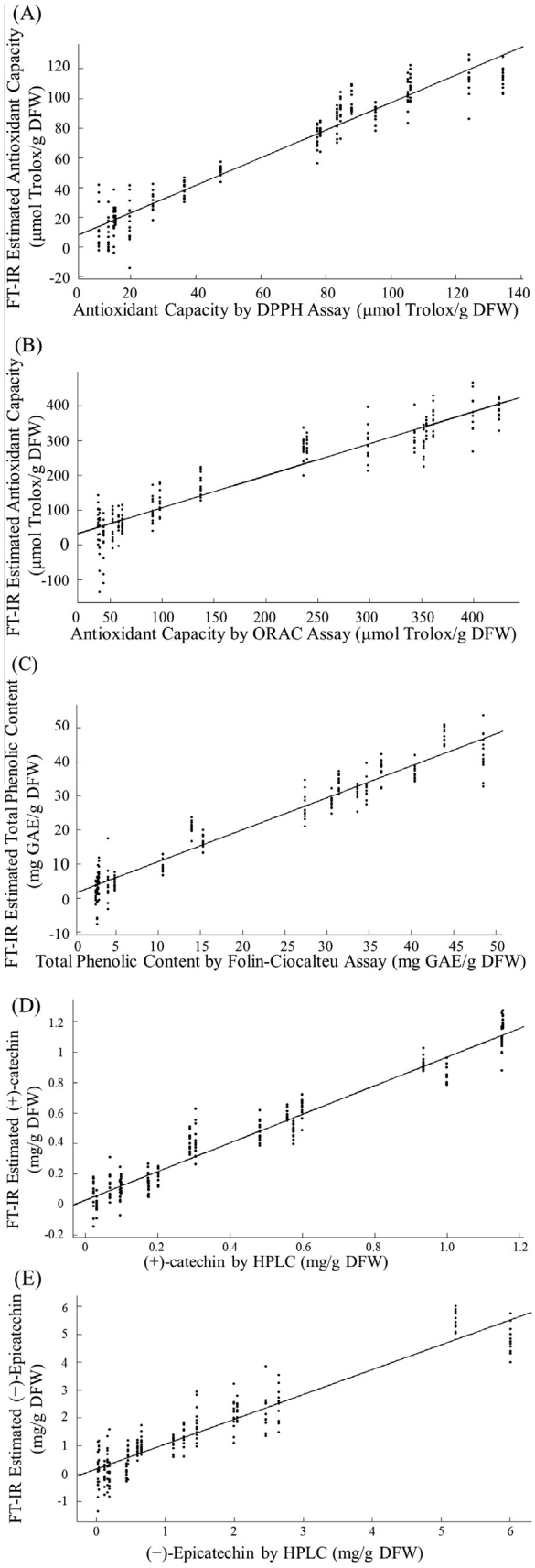

Fig. 2. Representative cross-validated (9-fold) PLSR models for antioxidant capacity, total phenolic content and procyanidins monomers in 18 types of chocolate using (A) DPPH assay, (B) ORAC assay, (C) Folin–Ciocalteu assay, (D) (+)-catechin by HPLC, and (E) (−)-epicatechin by HPLC (n = 13).

negatively with reference values. Among all the bands (peaks or valleys), some appeared in all five loading plots (Figs. 3 and S2). Bands at 1729 cm^{-1}, 1472 cm^{-1}, 1029 cm^{-1} and 909 cm^{-1} were assigned to lipid structures, while the band at 989 cm^{-1} is associated with polysaccharides (Che Man et al., 2005; Movasaghi et al., 2008; Schulz & Baranska, 2007). With an increase in cacao solids, less lipids and carbohydrates will be present in a chocolate formulation. Thus, bands related to lipids and polysaccharides would be expected to have a negative correlation with the references values. Bands at 1029 cm^{-1}, 909 cm^{-1} and 989 cm^{-1} were correlated negatively with the reference values at least in factor one, which contributed most to the total variances explained. However, bands at 1729 cm^{-1} and 1472 cm^{-1}, which are assigned to the C=O stretching of lipids and CH_2 bending of methylene chains in lipids (Movasaghi et al., 2008), were correlated positively with the reference values in factor one of all the models and in factor two of some. We attribute this observation to the complex composition of chocolate. For example, unsaturated lipid constituents and structural analogs might not be removed completely and could be extracted by the methods used. Lipid components could contribute to antioxidant capacity in the form of tocopherol isoforms in soy lecithin and vegetable oils (Elisia, Young, Yuan, & Kitts, 2013), which are added to modify texture or maintain freshness. In addition, bands at 1729 cm^{-1} and 1472 cm^{-1} did not contribute significantly to the PLSR models because the positive loading values at these two wavelengths were low for the first factor.

Although the RMSECV generated by 9-fold cross-validation approach was reported to be more reliable than other approaches, such as leave-one-out cross-validation, it has some limitations (Baumann, 2003). Therefore, alternative prediction models were generated using additional samples, in this case seven "unknown" samples, the PLSR parameters of which are summarized in Table 3. The smaller $R^2 - P$ of different prediction models, compared to the ninefold cross-validation models, indicated an overly optimistic approach. However, apart for the (−)-epicatechin PLSR model ($R^2 - P = 0.72$, RMSEP > 72% of the average values), all of the PLSR calibration models had good prediction capability, as indicated by the high $R^2 - P$ ($R^2 - P > 0.86$) and low SEP (RMSEP < 31% of the average values). Furthermore, apart from the (−)-epicatechin PLSR models, the small decline in $R^2 - P$ compared to R^2 (less than 0.1) and the increase in RMSEP (around 15% of the average values) addressed the over-fitting of PLSR models with too many latent variables.

The presence of lipids and polysaccharides contributed significantly to the spectral features, which reduced the signal-to-noise ratio and the prediction capability of FT-IR spectroscopy for antioxidant chemicals at low concentrations in the chocolate. Chemical-based assays, such as the DPPH and ORAC assays, which provide a more comprehensive analysis of antioxidant moieties, would be expected to correlate better with predictive models based on FT-IR spectral features. Many components that could be present in chocolate contribute to antioxidant capacity profile, such as phenolics and natural lipid soluble antioxidants (*e.g.* tocopherols) as well as protein hydrolysates (Sarmadi & Ismail, 2010), have the capacity to scavenge free radicals. The Folin–Ciocalteu assay generates results that are closely associated with the phenolic content, but only partially reflect chocolate antioxidant capacity, while procyanidins monomers are specific to cacao the concentrations of which are low in chocolate. However, the relationships identified between FT-IR spectral features and standard values did not follow this pattern.

PLSR models for DPPH and ORAC were anticipated to have the best correlation with FT-IR spectral dataset. However, the ORAC PLSR calibration and cross-validation models were not as reliable as the models for DPPH and Folin–Ciocalteu (high R^2 and low RMSEC, RMSECV), and required a larger number of latent variables to correlate highly with FT-IR spectral features. This could be attributed to larger intra-sample variation for the ORAC assay. However, the ORAC calibration model has the best performance in terms of predicting values for the unknowns. Concentrations of (+)-catechin and (−)-epicatechin were both low in chocolate, and models for (+)-catechin were worse than models for Folin–Ciocalteu but significantly better than models for (−)-epicatechin. The differences in models performance for (+)-catechin and (−)-epicatechin might be related to variations that occur in chocolate processing. Significant loss of (−)-epicatechin (from 13.35 ± 2.24 to 0.66 ± 0.09 mg/g DFW) has been reported in cacao beans after drying, fermentation and roasting (Hurst et al., 2011), while the loss of (+)-catechin was much less (0.58 ± 0.10 to 0.08 ± 0.00 mg/g DFW). Although the percentage decrease in the two chemicals were not significantly different (95% for (−)-epicatechin and 86% for (+)-catechin), the total decrease in (−)-epicatechin was more obvious. Thus, differences in chocolate processing parameters adopted by chocolate manufacturers could represent an additional variable in the large amounts of (−)-epicatechin, compared to (+)-catechin, in the final product.

Taken together, the parameters for PLSR models indicate the feasibility of using ATR-FT-IR spectroscopy for the prediction of antioxidant capacity, total phenolic content, and (+)-catechin contents in chocolate. For the traditional chemical-based assays, sample pre-treatment took ca. 36 h and the analytical measurements required 30–120 min. In comparison, the same data collection by FT-IR spectroscopy took less than 2 min, and no sample pre-treatment. The non-destructive, simple, rapid and reliable

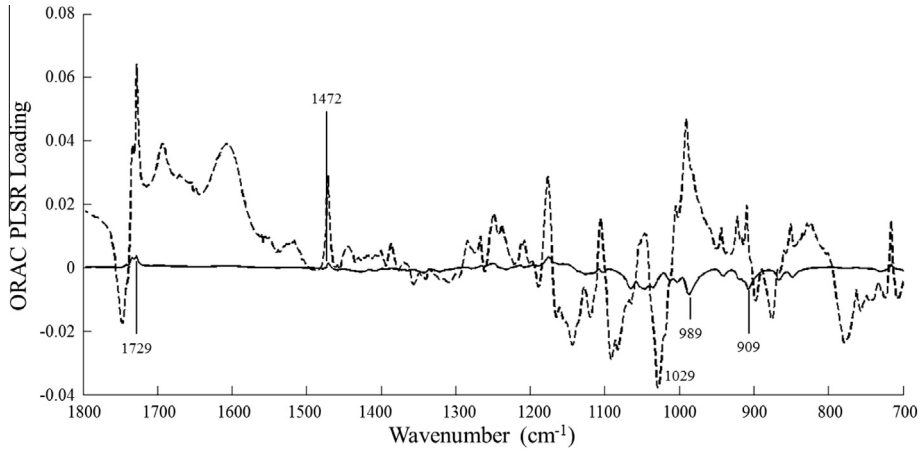

Fig. 3. Representative loading plot of ORAC PLSR calibration model. Solid line: PLSR factor 1; dashed line: PLSR factor 2.

attributes of ATR-FT-IR make it more suitable for the high-throughput analysis required by the food industry and in academic research, specifically for the detection of adulteration (*e.g.* replacement of cacao constituents) and/or prediction of antioxidant capacity.

This current study included a range of chocolate types (*i.e.* dark and milk chocolate with wide range of cacao contents) resulting in improved robustness for predicting the antioxidant profiles of samples of unknown composition. By increasing the variety of samples utilized in training PLSR calibration models, improved accuracy can be achieved.

4. Conclusion

FT-IR with ATR can be used to quantify and predict antioxidant capacity, total phenolic content and (+)-catechin concentration of chocolate. Compared with traditional chemical-based assays that take hours to days, FT-IR spectroscopy can obtain the same data in 2 min without any sample pre-treatment, saving time and reducing costs. As a high-throughput approach, this method would be expected to be more attractive to the food industry and commercial laboratories in food analysis. Further research should be performed to incorporate different types of chocolate for the development of more robust and precise prediction models. In addition, a portable IR spectrometer has the potential to be applied for on-site determination of chocolate content.

Acknowledgements

This work was supported by funds awarded to X.L. by Natural Sciences and Engineering Research Council of Canada (Engage Grant, EGP 463727-14). We thank Hilda Kisielewski and Yazheng Liu for assistance with techniques.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.01.130.

References

Afoakwa, E. O. (2014). World production, grinding and consumption trends of cocoa. In *Cocoa production and processing technology* (pp. 38–39). CRC Press.

Afoakwa, E. O., Paterson, A., & Fowler, M. (2007). Factors influencing rheological and textural qualities in chocolate–A review. *Trends in Food Science & Technology, 18*(6), 290–298.

Al-Qadiri, H. M., Lin, M., Cavinato, A. G., & Rasco, B. A. (2006). Fourier transform infrared spectroscopy, detection and identification of *Escherichia coli* O157: H7 and *Alicyclobacillus* strains in apple juice. *International Journal of Food Microbiology, 111*(1), 73–80.

Arts, M. J., Haenen, G. R., Wilms, L. C., Beetstra, S. A., Heijnen, C. G., Voss, H.-P., & Bast, A. (2002). Interactions between flavonoids and proteins: Effect on the total antioxidant capacity. *Journal of Agricultural and Food Chemistry, 50*(5), 1184–1187.

Baba, S., Osakabe, N., Yasuda, A., Natsume, M., Takizawa, T., Nakamura, T., & Terao, J. (2000). Bioavailability of (−)-epicatechin upon intake of chocolate and cocoa in human volunteers. *Free Radical Research, 33*(5), 635–641.

Baumann, K. (2003). Cross-validation as the objective function for variable-selection techniques. *Trends in Analytical Chemistry, 22*(6), 395–406.

Belščak, A., Komes, D., Horžić, D., Ganić, K. K., & Karlović, D. (2009). Comparative study of commercially available cocoa products in terms of their bioactive composition. *Food Research International, 42*(5), 707–716.

Che Man, Y., Syahariza, Z., Mirghani, M. E. S., Jinap, S., & Bakar, J. (2005). Analysis of potential lard adulteration in chocolate and chocolate products using Fourier transform infrared spectroscopy. *Food Chemistry, 90*(4), 815–819.

Chen, X. M., & Kitts, D. D. (2008). Antioxidant activity and chemical properties of crude and fractionated Maillard reaction products derived from four sugar–amino acid Maillard reaction model systems. *Annals of the New York Academy of Sciences, 1126*(1), 220–224.

Collodel, G., Moretti, E., Del Vecchio, M. T., Biagi, M., Cardinali, R., Mazzi, L., ... Castellini, C. (2014). Effect of chocolate and propolfenol on rabbit spermatogenesis and sperm quality following bacterial lipopolysaccharide treatment. *Systems Biology in Reproductive Medicine, 60*(4), 217–226.

Cooper, K. A., Donovan, J. L., Waterhouse, A. L., & Williamson, G. (2008). Cocoa and health: A decade of research. *British Journal of Nutrition, 99*(01), 1–11.

Elisia, I., Young, J. W., Yuan, Y. V., & Kitts, D. D. (2013). Association between tocopherol isoform composition and lipid oxidation in selected multiple edible oils. *Food Research International, 52*(2), 508–514.

Frankel, E. N., & Meyer, A. S. (2000). The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. *Journal of the Science of Food and Agriculture, 80*(13), 1925–1941.

Gu, L., House, S. E., Wu, X., Ou, B., & Prior, R. L. (2006). Procyanidin and catechin contents and antioxidant capacity of cocoa and chocolate products. *Journal of Agricultural and Food Chemistry, 54*(11), 4057–4061.

Halim, Y., Schwartz, S. J., Francis, D., Baldauf, N. A., & Rodriguez-Saona, L. E. (2006). Direct determination of lycopene content in tomatoes (*Lycopersicon esculentum*) by attenuated total reflectance infrared spectroscopy and multivariate analysis. *Journal of AOAC International, 89*(5), 1257–1262.

Haytowitz, D. B., & Bhagwat, S. (2010). USDA database for the oxygen radical absorbance capacity (ORAC) of Selected foods, Release 2. *US Department of Agriculture*.

Hooper, L., Kay, C., Abdelhamid, A., Kroon, P. A., Cohn, J. S., Rimm, E. B., & Cassidy, A. (2012). Effects of chocolate, cocoa, and flavan-3-ols on cardiovascular health: A systematic review and meta-analysis of randomized trials. *The American Journal of Clinical Nutrition, 95*(3), 740–751.

Huang, X., Zou, H., Chen, X., Luo, Q., & Kong, L. (2003). Molecularly imprinted monolithic stationary phases for liquid chromatographic separation of enantiomers and diastereomers. *Journal of Chromatography A, 984*(2), 273–282.

Hurst, W. J., Krake, S. H., Bergmeier, S. C., Payne, M. J., Miller, K. B., & Stuart, D. A. (2011). Impact of fermentation, drying, roasting and Dutch processing on flavan-3-ol stereochemistry in cacao beans and cocoa ingredients. *Chemical Central Journal, 5*, 53.

Lee, K. W., Kim, Y. J., Lee, H. J., & Lee, C. Y. (2003). Cocoa has more phenolic phytochemicals and a higher antioxidant capacity than teas and red wine. *Journal of Agricultural and Food Chemistry, 51*(25), 7292–7295.

Lu, X., & Rasco, B. A. (2012). Determination of antioxidant content and antioxidant activity in foods using infrared spectroscopy and chemometrics: A review. *Critical Reviews in Food Science and Nutrition, 52*(10), 853–875.

Miller, K. B., Hurst, W. J., Flannigan, N., Ou, B., Lee, C., Smith, N., & Stuart, D. A. (2009). Survey of commercially available chocolate- and cocoa-containing products in the United States. 2. Comparison of flavan-3-ol content with nonfat cocoa solids, total polyphenols, and percent cacao. *Journal of Agricultural and Food Chemistry, 57*(19), 9169–9180.

Miller, K. B., Stuart, D. A., Smith, N. L., Lee, C. Y., McHale, N. L., Flanagan, J. A., ... Hurst, W. J. (2006). Antioxidant activity and polyphenol and procyanidin contents of selected commercially available cocoa-containing and chocolate products in the United States. *Journal of Agricultural and Food Chemistry, 54*(11), 4062–4068.

Movasaghi, Z., Rehman, S., & ur Rehman, D. I. (2008). Fourier transform infrared (FTIR) spectroscopy of biological tissues. *Applied Spectroscopy Reviews, 43*(2), 134–179.

Ortega, N. d., Romero, M.-P., Macià, A., Reguant, J., Anglès, N., Morelló, J.-R. n., & Motilva, M.-J. (2008). Obtention and characterization of phenolic extracts from different cocoa sources. *Journal of Agricultural and Food Chemistry, 56*(20), 9621–9627.

Ozen, B. F., & Mauer, L. J. (2002). Detection of hazelnut oil adulteration using FT-IR spectroscopy. *Journal of Agricultural and Food Chemistry, 50*(14), 3898–3901.

Richelle, M., Tavazzi, I., Enslen, M., & Offord, E. (1999). Plasma kinetics in man of epicatechin from black chocolate. *European Journal of Clinical Nutrition, 53*(1), 22–26.

Robbins, R. J., Leonczak, J., Li, J., Johnson, J. C., Collins, T., Kwik-Uribe, C., & Schmitz, H. H. (2012). Determination of flavanol and procyanidin (by degree of polymerization 1–10) content of chocolate, cocoa liquors, powder (s), and cocoa flavanol extracts by normal phase high-performance liquid chromatography: Collaborative study. *Journal of AOAC International, 95*(4), 1153–1160.

Rodríguez-Ramiro, I., Ramos, S., Bravo, L., Goya, L., & Martín, M. Á. (2011). Procyanidin B2 and a cocoa polyphenolic extract inhibit acrylamide-induced apoptosis in human Caco-2 cells by preventing oxidative stress and activation of JNK pathway. *The Journal of Nutritional Biochemistry, 22*(12), 1186–1194.

Sarmadi, B. H., & Ismail, A. (2010). Antioxidative peptides from food proteins: A review. *Peptides, 31*(10), 1949–1956.

Schulz, H., & Baranska, M. (2007). Identification and quantification of valuable plant substances by IR and Raman spectroscopy. *Vibrational Spectroscopy, 43*(1), 13–25.

Shiroma, C., & Rodriguez-Saona, L. (2009). Application of NIR and MIR spectroscopy in quality control of potato chips. *Journal of Food Composition and Analysis, 22*(6), 596–605.

Singleton, V. L., Orthofer, R., & Lamuela-Raventos, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods in Enzymology, 299*, 152–178.

Spencer, J. P., Schroeter, H., Rechner, A. R., & Rice-Evans, C. (2001). Bioavailability of flavan-3-ols and procyanidins: Gastrointestinal tract influences and their relevance to bioactive forms in vivo. *Antioxidants & Redox Signaling, 3*(6), 1023–1039.

Tzounis, X., Rodriguez-Mateos, A., Vulevic, J., Gibson, G. R., Kwik-Uribe, C., & Spencer, J. P. (2011). Prebiotic evaluation of cocoa-derived flavanols in healthy humans by using a randomized, controlled, double-blind, crossover intervention study. *The American Journal of Clinical Nutrition, 93*(1), 62–72.

Wilkerson, E. D., Anthon, G. E., Barrett, D. M., Sayajon, G. F. G., Santos, A. M., & Rodriguez-Saona, L. E. (2013). Rapid assessment of quality parameters in processing tomatoes using hand-held and benchtop infrared spectrometers and multivariate analysis. *Journal of Agricultural and Food Chemistry, 61*(9), 2088–2095.

Yakala, G. K., Wielinga, P. Y., Suarez, M., Bunschoten, A., van Golde, J. M., Arola, L., ... Heeringa, P. (2013). Effects of chocolate supplementation on metabolic and cardiovascular parameters in ApoE3L mice fed a high-cholesterol atherogenic diet. *Molecular Nutrition & Food Research, 57*(11), 2039–2048.

Yasuda, A., Natsume, M., Osakabe, N., Kawahata, K., & Koga, J. (2011). Cacao polyphenols influence the regulation of apolipoprotein in HepG2 and Caco2 cells. *Journal of Agricultural and Food Chemistry, 59*(4), 1470–1476.