Functional changes induced by extrusion during cocoa alkalization

Valverde, D.[1], Behrends, B.[2], Pérez-Esteve, É.[1*], Kuhnert, N.[2], Barat, J.M.[1]

[1]Departamento de Tecnología de Alimentos. Universitat Politècnica de València, Camino de Vera, s/n, 46022, Valencia, Spain

[2]Department of Life Sciences & Chemistry, Jacobs University, Campus ring 1, 28759, Bremen, Germany

*Corresponding author: edpees@upv.es

Abstract

Polyphenols, a group of secondary metabolites, have well-known relevant effects on human health. During traditional alkalization, this content dramatically lowers. We aimed to evaluate an alternative alkalization method based on extrusion on cocoa functional characteristics. The results showed that the antioxidant capacity and total phenolic values increased as alkali concentration and temperature did, and these values doubled under less extreme conditions. Comparing the functional properties between extruded and traditionally produced powders revealed that catechin, epicatechin and dimers B1 and B2 contents were 43%, 33%, 54% and 34% lower in the extruded samples, respectively. However, this reduction was partially balanced by increased clovamide content up to 50%. Thus the total phenol content and antioxidant capacity of the extruded samples were statistically above those of the commercial one. Hence extrusion alkalization should be considered a new processing alternative to avoid markedly reducing functional properties.

Keywords

Extrusion, alkalization, cocoa, Dutching, polyphenols, technology, flavanols

1. Introduction

In dry cocoa beans, polyphenols are secondary metabolites of plants that represent 10-15% of dry weight (Martín et al., 2013 and 2017; Aprotosoaie, Luca and Miron, 2015). Three main groups of polyphenols have been detected in cocoa: flavanols (catechin, epicatechin, gallocatechin, etc.), anthocyanins (leucoanthocyanins, etc.) and proanthocyanins (dimers, trimers and other polymers of flavan-3-ols). Apart from these groups, other compounds like flavones (vitexin, apigenin, luteolin, etc.), flavonols (avicularin, hyperoside, etc.) and phenolic acids (caffeic acid, chlorogenic acid, etc.) can be found at low concentrations in cocoa (Aprotosoaie, Luca and Miron, 2015). The classification of the different compounds in the polyphenol family tree, as well as the chemical structures of some of the compounds herein analyzed, are shown in Supplementary Figures 1 and 2.

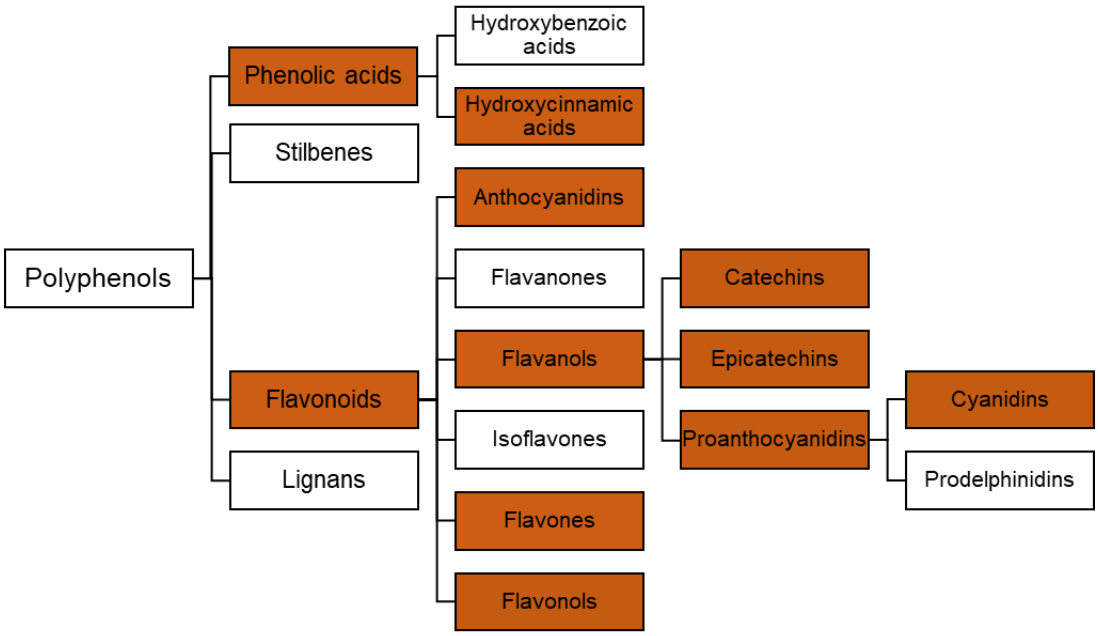

Figure S1. Polyphenol family tree. Compounds present in cocoa powders are highlighted in brown.

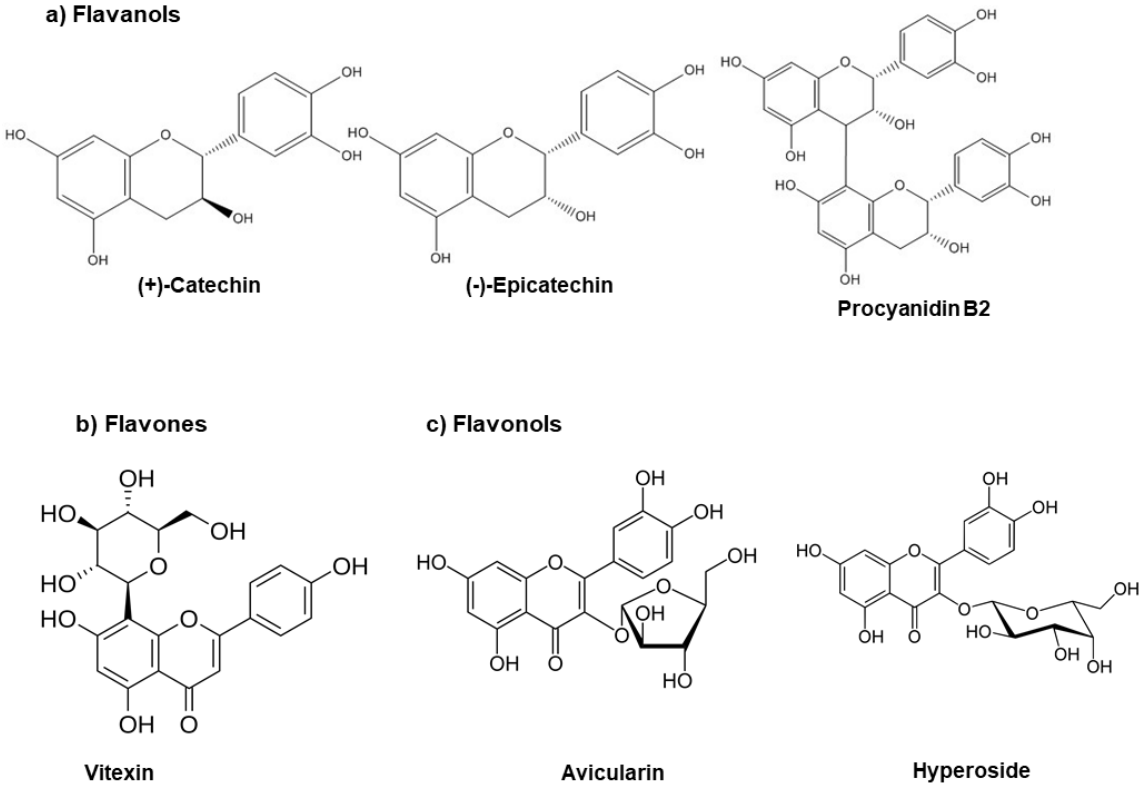

Figure S2. Chemical structure of some of the compound analysed in this word grouped by families.

The importance of polyphenols is related to their sensory and functional properties. For sensory features, some polyphenols have been identified as pigments, astringent and bitter compounds, or molecules able to modulate flavor (El Gharras, 2009). In functional activity, several polyphenols have different *in vitro* beneficial health effects, such as protection of neurons, stimulation of vasodilation, improvement of insulin secretion and inhibition of cancer cell proliferation (Del Rio et al., 2009).

Cocoa alkalization is an additional step of the cocoa production chain, in which material is treated with an alkali solution, pressure and temperature inside closed pressurized vessels. This treatment aims to darken cocoa color, increase the solubility of powder and reduce both the astringency and bitterness of natural material (De Zaan cocoa, 2006).

In addition to the desired modifications in the physico-sensory features of cocoa, alkalization has been reported to reduce the presence of polyphenols, methylxanthines, vitamins, amino acids and sugars, among other compounds (Brandon and Terink, 1981; Ellis, 1990; Wissgott, 1985; Li et al.,

2012; Huang and Barringer, 2010). For example, Gültekin-Özgüven et al (2016) analyzed the total polyphenol, flavanol content and antioxidant activity of traditionally alkalized cocoa liquors. They found that the above features lowered by 87%, 83% and 50%, respectively. In another work, Gu et al (2006) reported a reduction of 51% in antioxidant activity and one of 78% in procyanidins content in commercial cocoas, while Jolić et al (2011) observed a loss of 64% of total polyphenols, 59 % of total procyanidins and 39% of antioxidant activity when cocoa nibs were alkalized.

One technique that has been applied as an alternative to cocoa alkalization, whose effects have not yet been studied, is extrusion (Chalin, 1974). This technology has been widely applied by the food industry to generate different kinds of products like pasta, chocolate, chewy gums, breakfast cereals and baby foods, among others (Fellow, 2000). Extrusion is based on placing a powdered material in an extruder and its continuous shearing, heating and pressurization, which results in a compacted product.

Extrusion has been reported to negatively affect the content of polyphenols of different food matrices, but increased antioxidant activity has also been documented in relation to the lysis of cells and the formation of Maillard reaction products with enhanced antioxidant activity (Sharma et al., 2016; El-Hady and Habiba, 2003; Shih, Kou and Chiang, 2009; Nayak et al., 2011).

As information about the effects of extrusion alkalization on the functional characteristics of cocoa is lacking, the first goal of this study was to assess the effects of different processing variables (water content, temperature, alkali type, concentration) on the functional features of cocoa. Our second goal was to determine the effect of extrusion on these characteristics compared to that of the conventional alkalization method.

2. Material and Methods

2.1. Materials

The cocoa employed as a raw material for extrusion experiments was a natural powder from Ivory Coast. The employed commercial samples used as the control are: three natural, one dark natural, three light, two medium and two strongly alkalized cocoas. They were all provided by Olam Food Ingredients SL (Cheste, Spain). Trolox was supplied by Across Organics (Geel, Belgium). (-)-Epicatechin, (+)-Catechin, avicularin, procyanidin dimers B1 and B2, trimer C1, tetramer A2, clovamide and hyperoxide were acquired from Phytolab (Vestenbergsgreuth, Germany). Clovamide was provided by Biozol (Eching, Germany) and vitexin came from Merck (Darmstadt, Germany). Potassium carbonate, sodium carbonate, sodium hydroxide, Gallic acid, analytical grade methanol, HPLC-grade acetonitrile, Folin-Ciocalteu reagent and analytical grade acetone were supplied by Scharlau (Sentmenat, Spain).

2.2. Experimental design

A response surface methodology was used to establish the combination of conditions to be applied and to determine the relations between the selected relevant process variables for alkalization (alkali concentration (X_1), water content (X_2), temperature (X_3)) and the response parameters (antioxidant activity, total phenol content, the concentration of 10 different polyphenols). Statistical modeling and analyses were performed by the design assistant of the experiments in Statgraphics Centurion (Manugistics Inc., Rockville, MD, USA). The design selected for surface response modeling was an orthogonal central composite design 2^3+star. The experimental conditions for the analysis are shown in Table 1.

After the data analysis, the behavior of each response variable in relation to the evaluated independent parameters was fitted in a quadratic polynomial model as shown in Eq. 1.

$$y = a_0 + \sum_{i=1}^{3} a_i X_i + \sum_{i=1}^{3} a_{ii} X_{ii} + \sum_{i \neq j=1}^{3} a_{ij} X_i X_j \quad (Eq. 1)$$

where "y" represents the response variable, "a_o" is the constant, "a_i", "a_{ii}" and "a_{ij}" are the linear coefficients and their interactions, and "X_i" and "X_j" are the experimental data for each variable.

The previous surface response methodology was carried out separately for the two alkali agents herein employed: NaOH and K_2CO_3. For all the models, the R^2 statistical values were obtained to evaluate their suitability.

2.3. Cocoa extrusion

Before extrusion, each cocoa powder was properly mixed with the corresponding amounts of water and alkali in a Blixer (Robot Coupe, Mataró, Spain). Mixtures were then placed inside a single screw extruder 19/25 (Brabender, Duisburg, Germany). The data of the screw barrel were 1.9 cm diameter, a 25:1 length to diameter ratio, regular lights (1:1) and no mixing elements. The die was a single 4 mm round die head. To study the influence of the concentration and type of alkali (NaOH and K_2CO_3), temperature and water content, the operational conditions were set: feeding speed (13 g) and extrusion speed (156 g). The temperature in the extruder was: 37ºC in module 1, 65ºC in module 2, 60ºC or 100ºC in module 3, depending on the assay temperature and the corresponding temperature in module 4. These extruder conditions were selected for being the most frequently found ones in different alkalization patents (Chalin, 1974; Ellis, 1992; Wiant et al., 1989; Wissgott, 1985; Brandon and Terink, 1981; Kopp et al., 2009). Treatment lasted less than 5 minutes. Once extruded, samples were dried until a final moisture content below 5 g/100 g was reaching using a forced ventilation stove at 100ºC and powdered by employing a coffee milling machine.

2.4. General functional characterization

2.4.1. Obtaining the polyphenolic extract

To extract the polyphenols present in samples, an extraction protocol was employed, based on a combination of the conditions described by Andres-Lacueva et al (2008), Arranz et al (2009) and

Hellström and Mattila (2008). In this method, 1g of cocoa powder was subjected to three extraction cycles: in the first two, cocoa was dissolved in 20 mL of a methanol and hydrochloric acid 16 mM mixture (50:50), and in the third one, cocoa was dissolved in 20 mL of a acetone and distillated water mixture (70:30). In each cycle, cocoa was sonicated for 15 minutes at room temperature in an ultrasound bath model Elmasonic S 40H (Elma, Singen, Germany). After treatment, samples were centrifuged at 13000 g, at 4ºC for 15 minutes. The supernatants of each step were kept in the dark before being combined and taken to a final volume of 60 mL. The polyphenolic extracts were kept at 4ºC until they were analyzed.

2.4.2. Total phenolic content

The total polyphenolic content was quantified following the method described by Todorovic et al. (2015) with some changes. For the assay, 50 µL of each polyphenolic extract were mixed with 0.45 mL of methanol/water (1:1) and 5 mL of Folin-Ciocalteu solution. Then 4 mL of Na_2CO_3 solution were added to the previous mixture, which was kept in the dark for 1 h. The absorbance of samples was measured at 750 nm. Samples were analyzed in triplicate. The results were expressed as g Gallic acid Equivalent/100 g cocoa powder.

2.4.3. Antioxidant activity

The determination of the antioxidant activity of cocoa samples was made by following the DPPH method described by Todorovic et al. (2015) with some changes. For the assay, 6 µL of each polyphenolic extract were mixed with 294 µL of methanol. Then 2.7 mL of the DPPH solution were added. Next samples were shaken and kept for 1 h in the dark before being measured at 517 nm. Samples were analyzed in triplicate. The results were expressed as g Trolox Equivalent/100 g of cocoa.

2.4.4. Determination of polyphenols

The quantification of catechin and epicatechin, their oligomers, and the other four polyphenols was done by HPLC following the external standard method, in which a calibration curve of the peak area against the compound concentration was built. The separation and quantification conditions were

selected from the method described by D'Souza et al (2017) with some modifications. A ZORBAX Eclipse Plus C18 column (2.1 x 100 mm) (Agilent Technologies, Waldbronn, Germany) was utilized. The employed mobile phases were: 0.05% aqueous formic acid (phase A) and acetonitrile with 0.05% of formic acid (phase B). The gradient was: 0-1 min, 8% phase B; 1-2.5 min, 8-12% phase B; 2.5-8 min, 12-16.5% phase B; 8-9 min, 16.5-17% phase B; 9-10 min, 17-17.5% phase B; 10-11 min, 17.5% phase B; 11-12 min, 17.5-18.5% phase B; 12-13 min, 18.5% phase B; 13-23 min, 18.5-95% phase B; 23-33 min, 95% phase B; 33-40 min, 95-8% phase B. The other chromatographic conditions were: UV detection at 280 nm, column temperature of 40ºC, injection volume of 2 µL and a flow rate of 0.4 mL/min. The HPLC equipment was an Agilent 1260 HPLC system (Agilent Technologies, Waldbronn, Germany). A typical chromatogram showing the retention times of each analyte is presented in Supplementary Figure 3. Samples were analyzed in duplicate.

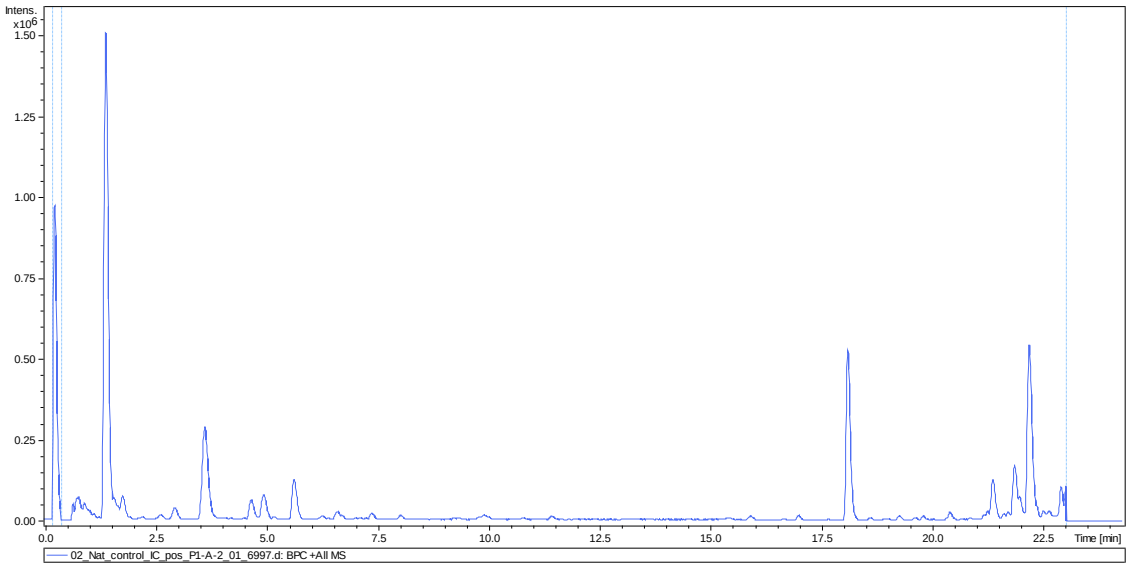

Figure S3. Fig 1. Representative chromatogram of a natural cocoa sample.

2.5. Comparison of commercial samples

To evaluate if the extrusion effects on the polyphenol profile were similar to those obtained by the commercial alkalization treatment, a set of commercial cocoa powders belonging to the different alkalization levels was employed: three natural, one dark natural, three light, two medium and two strongly alkalized cocoas. Samples were classified into different alkalization levels by following the classification by Miller: natural (pH 5-6), slight (pH 6-7.2), medium (pH 7.2-7.6) and strong alkalized (pH > 7.6) (Miller et al., 2008).

Once classified, the different cocoas were characterized following the same protocols as those described for the extruded samples (See Sections 2.4.2 to 2.4.4). Then the mean values were obtained for each alkalization level. These mean values were taken as a reference value and coded according to the alkalization levels of samples into dark natural (DN), light (L), medium (M) and strong alkalized (S).

For each alkalization level, the differences in the analyte contents between different samples (commercial references and extruded ones) were established by the analysis of variance (ANOVA) (95% confidence level of LSD; $p<0.05$), constructed using Statgraphics Centurion XV from Manugistics Inc. (Rockville, MD, USA).

3. Results

3.1. Model fitting

A response surface methodology was followed to study the evolution of antioxidant activity, total phenol content and the concentrations of 10 different polyphenols. In this work, two groups of response surfaces were built, one with K_2CO_3 and the other with NaOH, to model and analyze the effects of alkali concentration, water content and temperature on the functional features of cocoa. Table 2 shows the coefficients for each response variable that fitted the experimental data in the corresponding quadratic equation, along with their statistical significance.

An analysis of variance (ANOVA) of the models showed that most of the resulting equations had regression coefficients (R^2) above 0.8. This means that the models correctly fitting the difference responses. The lack of fit component was also calculated. As the values of this parameter for most models were not significant, save a few exceptions, the proposed models were suitable for describing the observed data.

In addition to R^2, the significance of the different coefficients was evaluated to identify which ones affected the different response parameters. Of all the variables, alkali concentration was generally that which most affected the concentration of the evaluated polyphenols (p-value < 0.05), either alone or in combination with other variables. With antioxidant activity and total phenol content, contents were affected by different variables according to the employed alkali.

3.2 Effects of the extrusion treatment variables on antioxidant activity and total phenolic content

In this section, the effects of temperature and alkali type and concentration on the functional characteristics of the developed powders were evaluated (Figure 1).

In general, extrusion reduced antioxidant activity and total phenolic content (Table 3). In the untreated cocoa, the antioxidant capacity and total phenolic content values were 4.7±0.2 and 4.4±0.3 g/100g, respectively. These values are lowered with 3 g/100 g of cocoa after applying extrusion under very soft conditions (0.28% alkali, 20% water content, 63ºC), which means that at very low temperature

and low alkali concentrations, extrusion negatively impacts antioxidant capacity and total phenolic content. However, as alkali content and temperature increased, unexpectedly the values of both parameters proportionally increased. These increases became more evident in the cocoas treated with NaOH (92% and 46% of antioxidant capacity and total phenolic content in relation to the same sample treated under the softest conditions, respectively) than in those treated with K_2CO_3 (76% and 17%). The antioxidant capacity and total phenolic content values in the best obtained scenario (6.7% NaOH and 162ºC) would ensure that it is possible to restore part of the lost antioxidant activity and total phenol content of natural cocoa by selecting suitable extrusion variables. The increase in both parameters could be due to the formation, or the release from the non-extractable matrix, of catechin and other polyphenols as a result of alkali treatment at high temperature (Gültekin-Özgüven et al., 2016; Hurst et al., 2011; Lacueva et al., 2008; Jolić et al., 2011; Rodríguez, Pérez and Guzmán, 2009). These results provide a possibility to obtain alkalized cocoas with barely any alteration to their functional properties, not even after being processed under the strongest conditions.

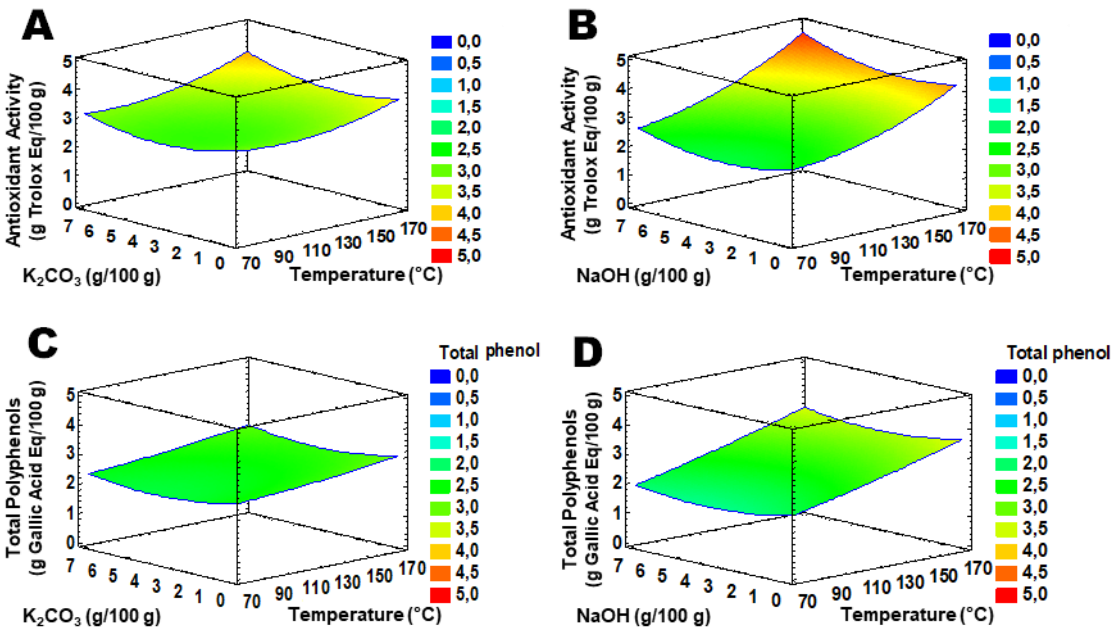

Figure 1. Effects of temperature and concentration of the alkalis K_2CO_3 (A, C) and NaOH (B, D) on the antioxidant capacity (A, B) and total phenolic content (C, D) of cocoa powders processed with 20% of water.

3.3. Effect of the variables of extrusion treatment on catechin and epicatechin and their oligomers

Apart from the overview provided by total phenolic content and antioxidant activity, the concentrations of catechin and epicatechin, the two main cocoa polyphenols, and their oligomers, were analyzed. As seen in Table 3, the natural cocoa powder contained the six analyzed compounds. The main one, as with other works (Quelal-Vásconez, 2020), was epicatechin with 70±3 mg/100 g. After alkalization, the values of all the different compounds lowered, in which case the tetrameter A1 contents went below the detection limit under all the assayed conditions. This loss of polyphenols due to extrusion processes has also been reported in other food matrices, such as bean/corn mixtures, Kañiwa (*Chenopodium pallidicaule*) or pineapple fruit leather (Delgado-Licon et al., 2009; Repo-Carrasco-Valencia et al., 2009; Sharma et al., 2016).

After studying the effect of the different treatment variables on distinct analytes content, Figure 2A shows the effects of temperature and K_2CO_3 concentration on catechin and epicatechin and their oligomers. In general, they all significantly reduced as the alkali concentration rose, and temperature was a non significant parameter. In addition, the compounds shared the same degradation patterns, which was expected as all the compounds shown in Figure 2 were catechin, epicatechin, or combinations of both.

As an example of the degradations induced by an increased K_2CO_3 concentration, catechin and epicatechin contents lowered from 36±1 and 70±3 mg/100 g of untreated cocoa to 37.0 or 56.2 and to 27.6 and 48.2 mg/100 g in the cocoas treated with 0.28% or 6% of K_2CO_3, respectively. With NaOH, values lowered to 58% and 80% for catechin and epicatechin, respectively, when samples were treated with the strongest processing variables. These results agree with other authors in line with two facts: (1) cocoa alkalization leads to general polyphenols degradation (Gültekin-Özgüven et al., 2016; Miller et al., 2008; Gu et al., 2006; Jolić et al., 2011; Zhu et al., 2002); (2) (-)-epicatechin is more sensitive to alkalization than (+)-catechin (Gültekin-Özgüven et al., 2016; Andres-LaCueva et al., 2008).

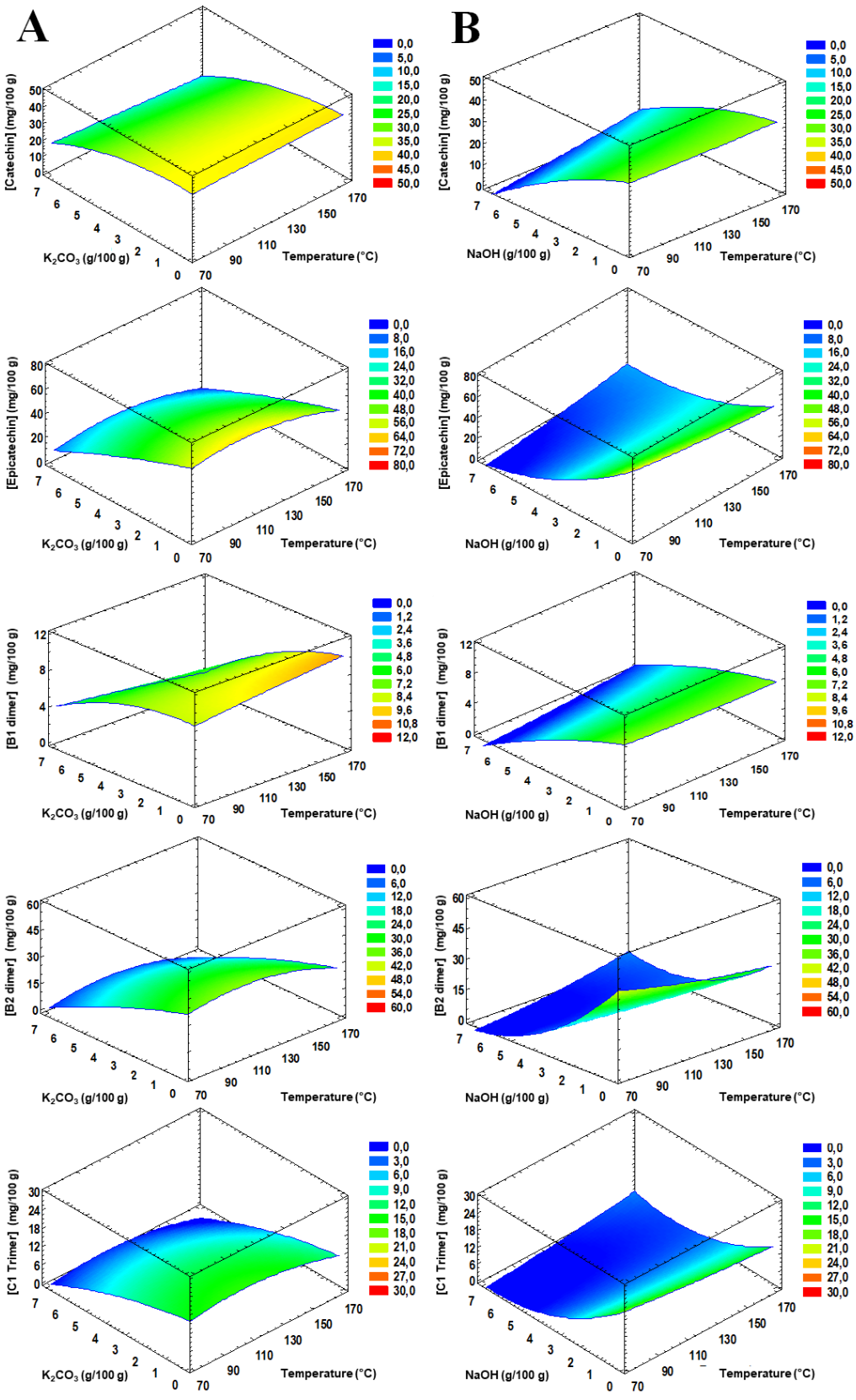

Figure 2. Effects of temperature and concentration of the alkalis K_2CO_3 (A) and NaOH (B) on contents of catechin, epicatechin and their oligomers of cocoa powders processed with 20% of water.

Moreover, it can be stated that loss of the analyzed flavanols was greater with NaOH for all the compounds. The ability of NaOH to reach higher degradations compared to K_2CO_3 is based on its capacity to produce more marked increase in pH. During alkalization, the generation of an alkaline medium enhances several chemical processes, such as the monomerization of polymers, the oxidation and chemical rearrangement of catechin and epicatechin (Gültekin-Özgüven et al., 2016; Hurst et al., 2011; Lacueva et al., 2008; Jolić et al., 2011), and other reactions such as their non enzymatic glycosylation and their interaction with Maillard reaction products (Stark and Hofmaan., 2006; Totlani and Peterson, 2005, 2007; Zhang et al., 2014). Reaching higher pH values can promote all these reactions and lead to more marked reductions in polyphenol content, which is what happens with NaOH *versus* K_2CO_3.

Apart from alkali concentration, other variables had an effect on the concentration of dimer B2 and trimer C1. Both compounds shared the same behavior. At low NaOH concentrations, their concentrations lowered as water content increased. By way of example, in the samples treated at 63ºC with 0.28% of NaOH, dimer B2 lowered from 38.3 to 28.7 mg/100 g as water content increased from 20% to 30%. Both compounds increased when raising the temperature at high alkali concentrations. For example, in the samples treated with 30% water content and 6.7% alkali, the dimer B2 content changed from 2.9 to 7.3 mg/100 g as temperature increased from 63ºC to 162ºC.

All the degradations observed in this section contrasted with the evolution reported for antioxidant activity and total phenol content (Figure 2). These two features either increased or maintained at high temperatures and alkali concentrations, while the concentrations of catechin and epicatechin, and their oligomers, significantly lowered under those strong conditions (Figure 3). Therefore, it is important to point that although the concentrations of the two main polyphenols in cocoa lowered, others were released and formed as a result of alkalization treatment (see Section 3.4). On the one hand, several researchers have reported two fractions of polyphenols in food matrices: the free and normally analyzed ones, and the non extractable fraction, which is formed by polyphenols linked with other molecules or cellular structures. The non extractable group has been found to be higher than the

extractable one in different matrices, and is released by some treatments, such as using NaOH during alkalization (Gonzales et al., 2015; Domínguez-Rodríguez et al., 2017). If we take this into account, extrusion combined with the employed alkalis might be able to release them. On the other hand, the formation of new compounds could also be responsible for maintaining antioxidant activity and total phenol content. For example, several authors have reported that (-)-catechin forms at the same time that (-)-epicatechin and (+)-catechin are degraded by cocoa alkalization (Gültekin-Özgüven et al., 2016; Hurst et al., 2011; Kofink et al., 2007; Ortega et al, 2008). The increase in this compound, and in ones, might be responsible for the observed maintenance of the above-mentioned features.

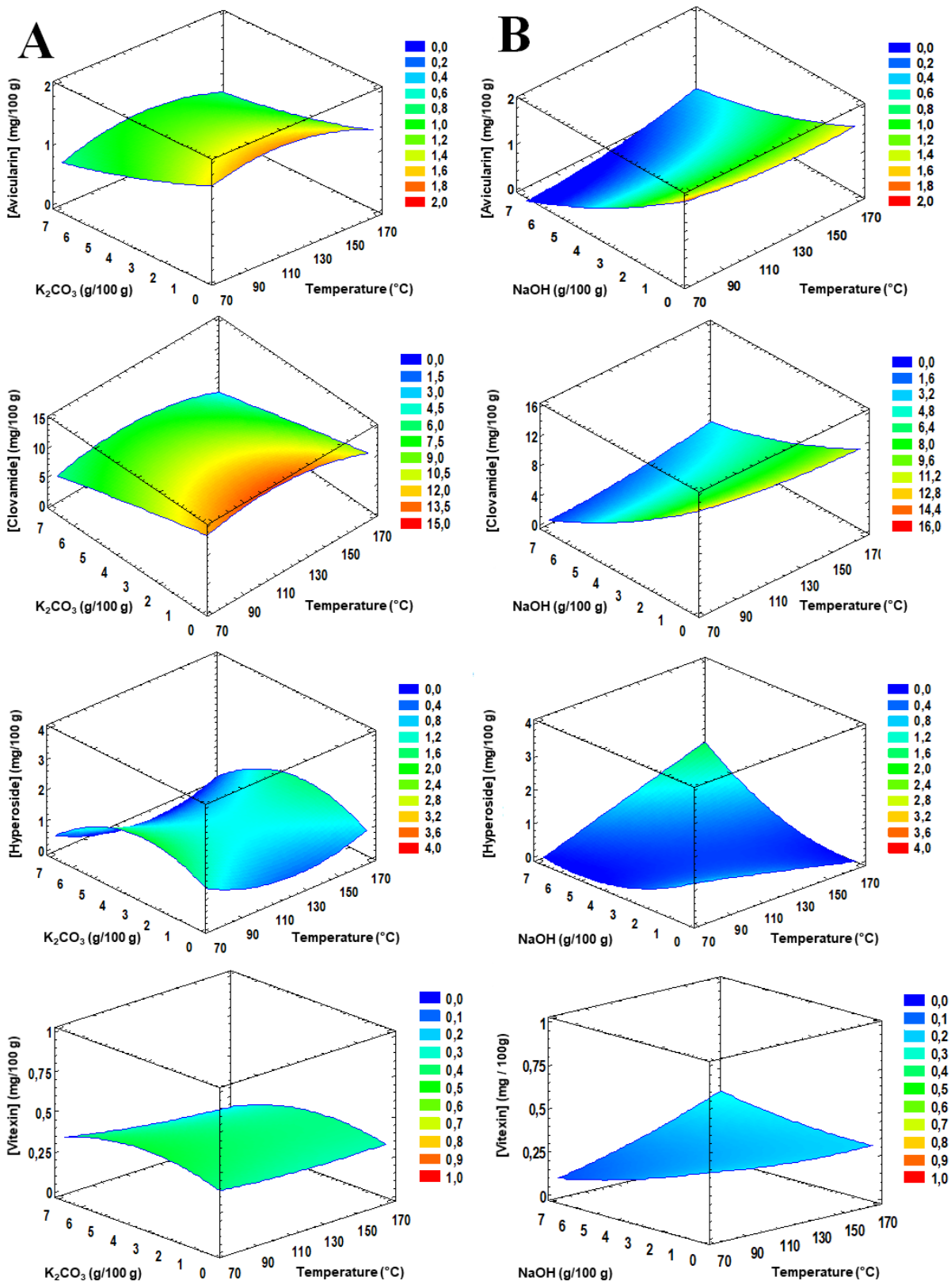

Figure 3. Effects of temperature and concentration of the alkalis K_2CO_3 (A) and NaOH (B) on contents of avicularin, clovamide, hyperoside and vitexin of cocoa powders processed with 20% of water.

3.4. Effect of the variables of the extrusion treatment on other polyphenols

In addition to flavanols, the effects of treatment on the polyphenols of other families (clovamide, hyperoxide, vitexin, avicularin) were evaluated. These compounds were selected for their different functional effects and were divided into two groups according to their shared behaviors: one composed ofavicularin and clovamide, and a second one with vitexin and hyperoside. Figure 3 shows the evolution of the above compounds as an effect of extrusion alkalization treatment.

In relation to the first group, it has to be stated that avicularin (or quercetin-3-α-L-arabinofuranoside) is a plant flavonoid and a quercetin derivative that has been reported to have anti-inflammatory, anti-allergic, antioxidant, anti-tumor and hepatoprotective effects (Vo et al., 2012), and clovamide (or N-caffeoyl-L-dihydroxyphenyl-alanine) is a polyphenol-amino acid conjugate that has been reported to have anti-inflammatory, antioxidant, neuroprotective and anti-Alzheimer's disease effects (Bouchez et al., 2019).

The concentrations of both compounds were generally higher in the extruded cocoas than in the untreated one. As shown in Table 3, in the untreated cocoas, the concentrations of avicularin and clovamide were 1.3±0.1 and 9.9±0.9 mg/ 100 g, while the mean concentration values of these compounds were ca. 1.3 and 13 mg/100 g for both alkalis, respectively, in the soft extruded cocoas (0.28% alkali, 20% water content, 63ºC). This reveals that extrusion with small amounts of alkali positively impacts cocoa functionality and may explain how antioxidant activity remains even after the degradation of some families of polyphenols.

However, as the alkali concentration rose, the concentrations of avicularin and clovamide significantly dropped. For example, in the cocoas treated with 20% water content at 63ºC, avincularin went from 1.6 to 0.8 mg/100 g and clovamide from 12.6 to 6.7 mg/100 g as the K_2CO_3 concentration increased from 0.28% to 6%. This behavior was similar to that observed with catechin and its oligomers. In addition, the treatment with NaOH was more aggressive and led to more marked reductions than that one with K_2CO_3.

In the second group of compounds, vitexin (or apigenin-8-C-glucoside) is a c-glycosilated flavone with many pharmacological activities (anti-cancer, anti-Alzheimer's disease, anti-hypertensive, anti-spasmodic, anti-depressant, antioxidant, anxiolytic effects, anti-inflammatory and anti-nociceptive activities, among others) (He et al., 2016). Hyperoside (hyperin or quercetin-3-O-galactoside) is a type of flavonoid that has been documented to have anti-inflammatory, anti-nociceptive, cardioprotective, hepatoprotective and gastrimucosal-protective effects (Verma et al., 2013).

As seen in Table 3, the contents of both molecules reduced for both soft and strong treatment conditions. In the untreated cocoas, the concentrations of hyperoside and vitexin were 4.4±0.1 and 0.7±0.1 mg/100 g after extrusion under soft conditions (0.28% alkali, 20% water content and 63ºC), the mean hyperoside concentration was 1.2 (K_2CO_3) or 0.9 (NaOH), and vitexin concentration was 0.4 mg/100 g in both cases. Degradation behavior differed depending on the employed alkali. Whereas the concentrations of both compounds increased at medium alkali concentrations (3.5%) (Figure 6A) in the samples treated with K_2CO_3, the concentrations of both analytes increased as temperature and alkali concentration rose in the cocoas treated with NaOH (Figure 6B). The observed shared behavior of these molecules suggests a common synthetic pathway because their surface responses displayed a similar trend which, at the same time, was totally different from that exhibited by the other polyphenols herein studied.

As for the effect of the different processing variables on vitexin and hyperoside contents, alkali concentration and type were the main variables that negatively affected the concentration of the various polyphenols. Water content and temperature were also important, but to a lesser extent. Of all the compounds, hyperoside and vitexin increased due to alkalization treatment. This suggests that the release or formation of other polyphenols like these two could take place after the observed maintenance and increase in antioxidant activity and total phenol content. These results render an in-depth analysis of the polyphenol profile necessary to identify those compounds whose concentration increased and to understand the real functional importance of alkalized cocoa.

3.5. Comparison to commercial samples

3.5.1. Functional comparison

After evaluating how the different variables of extrusion alkalization affected the functional features of cocoa, the produced samples were compared to a set of commercial powders to study the suitability of the new alkalizing method.

The darkest cocoas belonging to each alkalization level were selected for the comparison study. The results are shown in Figures 4, 5 and 6.

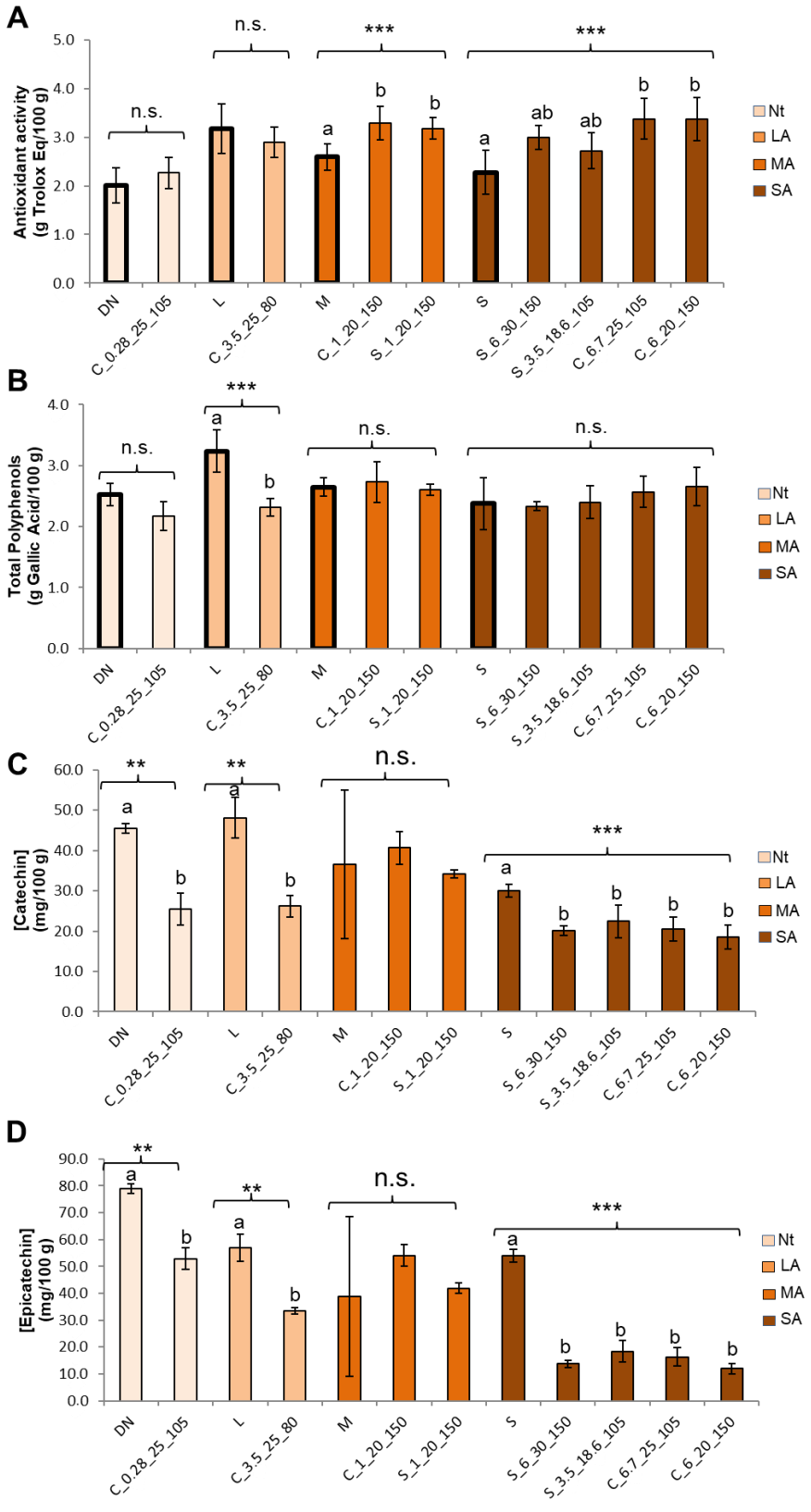

Figure 4. Comparison of the selected samples of natural (Nt), lightly (LA), moderately (MA) and strongly alkalized (SA) cocoas with commercial ones in terms of the antioxidant activity (A) and total phenol (B), catechin (C) and epicatechin(D) content. Non-significant (n.s.), * (0.01<p-value<0.05), ** (0.001<p-value<0.01) and *** (p-value<0.001) Sample codification refers to the type of alkali.

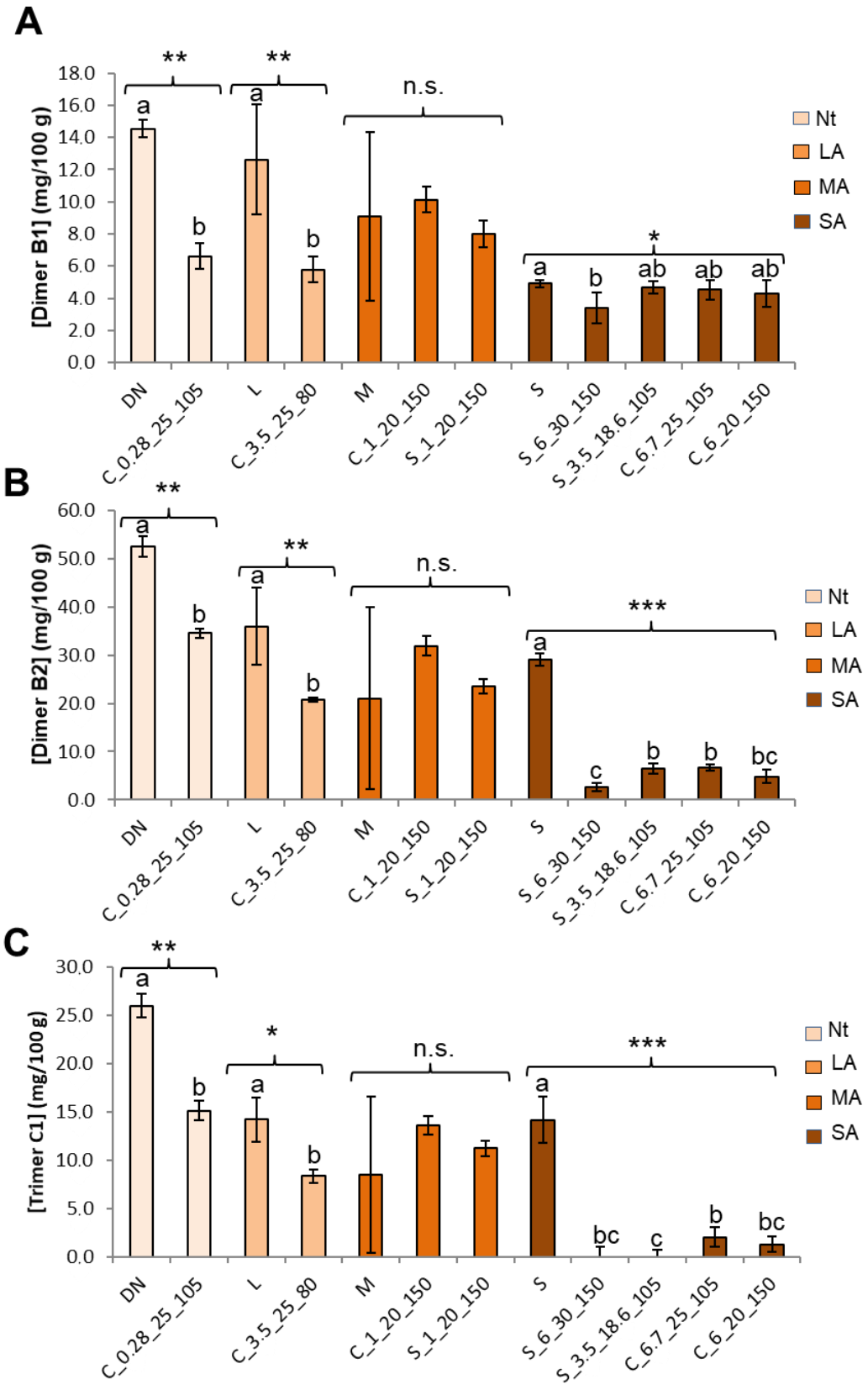

Figure 5. Comparison of the selected samples of natural (Nt), lightly (LA), moderately (MA) and strongly alkalized (SA) cocoas with commercial ones in terms of procyanidins dimer B1 (A), dimer B2 (B) and trimer (C) content. Non-significant (n.s.), * (0.01<p-value<0.05), ** (0.001<p-value<0.01) and *** (p-value<0.001)

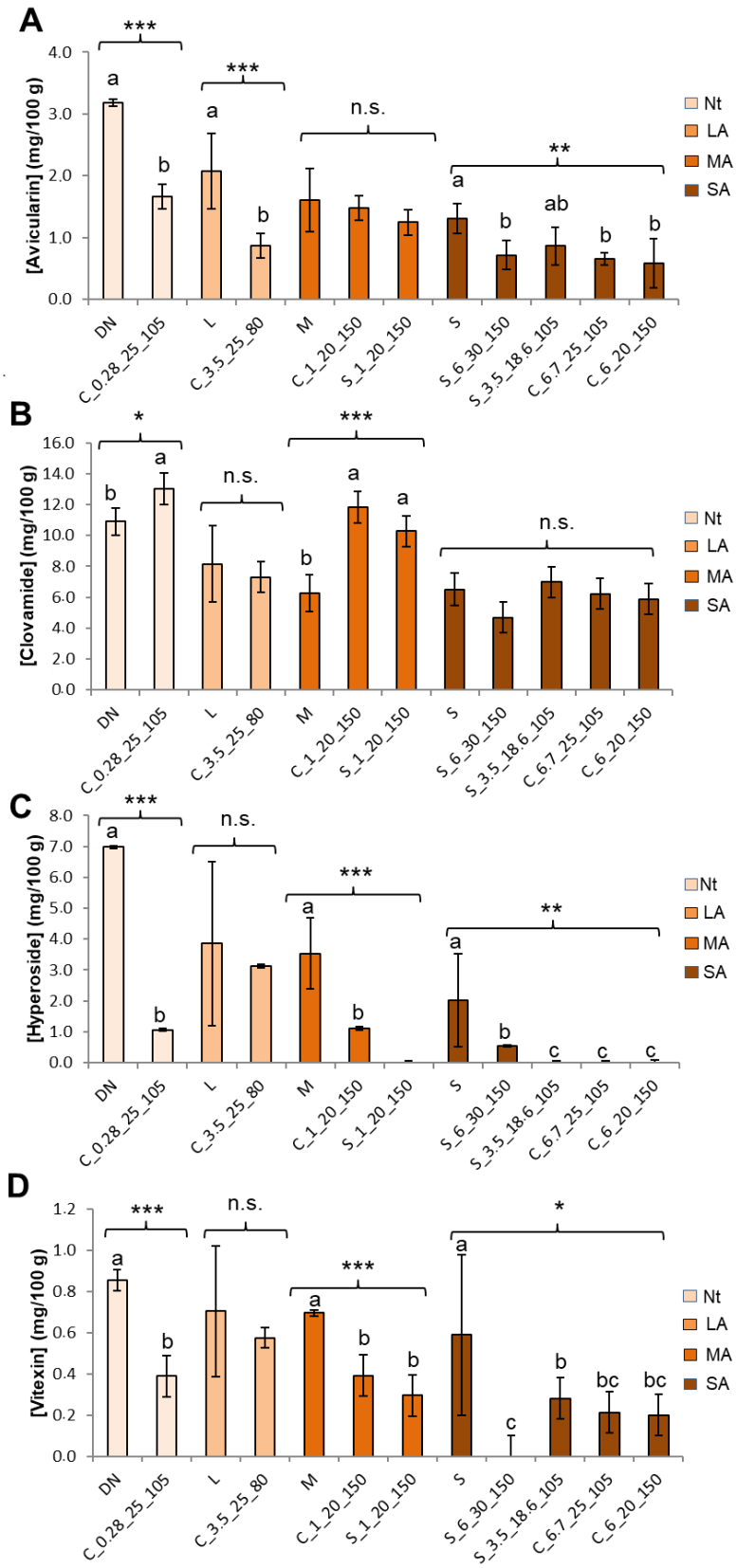

Figure 6. Comparison of the selected samples of natural (Nt), lightly (LA), moderately (MA) and strongly alkalized (SA) cocoas with commercial ones in terms of avicularin (A), clovamide (B), hyperoside (C) and vitexin (D) content. Non-significant (n.s.), * (0.01<p-value<0.05), ** (0.001<p-value<0.01) and *** (p-value<0.001)

No difference in antioxidant activity (Figure 4A) was found at the natural and slight alkalization levels between the extruded and traditionally produced cocoas, while the extruded samples displayed greater antioxidant activity at the medium and strong levels.

No significant difference was found for total polyphenol content (Figure 4B) between the samples belonging to the different alkalization levels, except for the slightly alkalized cocoas. This indicates that despite extrusion being reported to bring about major losses in total phenol content (Sharma et al., 2016), similar losses were generated to the conventional alkalization method.

At almost all the alkalization levels for catechin and epicatechin (Figure 4C and D), the extruded samples had lower catechin and epicatechin concentrations than the conventionally alkalized powders, which means that extrusion is a more aggressive technique. Furthermore, when the concentration of the oligomers of catechin and epicatechin were studied, they lowered as the alkalization level increased, and their concentrations were generally lower than those exhibited by commercial cocoas (Figure 5).

Finally, the evolution of the other four analyzed polyphenols (avicularin, clovamide, hyperoside, vitexin) was studied. The results are shown in Figure 6. Wide variability was observed in some commercial samples, but we ought to remember that these values were obtained by averaging the different traditionally produced cocoas belonging to that alkalization group.

In general, the concentration of three polyphenols (avicularin, hyperoside, vitexin) were lower (or similar) in the extruded samples than in the traditionally alkalized ones. Clovamide (Figure 6B) was the only polyphenol whose concentration was higher in the extruded cocoas than in the commercial ones. This molecule is an example of a polyphenol that increases through extrusion, which would explain the higher antioxidant activity and similar total phenol content observed between the extruded and commercial cocoas despite the general reduction in the concentration of catechin and its oligomers (Figure 4A and B).

4. Conclusions

The present work analyzed and characterized the effects of extrusion alkalization on the functional features of cocoa.

Of all the evaluated variables of the extrusion alkalization method, alkali type and concentration were those that led mainly to a reduction in the concentration of all the studied polyphenols. Both antioxidant activity and total phenol content remained mostly unchanged, and even increased after alkalization. This could be related to the release and formation of new polyphenols, such as hyperoside and vitexin.

In the comparison between extrusion and alkalization treatment, extrusion improved the functional characteristics of cocoa. However, its fast speed, continuous treatment and lower energy use make this alkalization method an interesting one to replace traditional treatments.

5. Funding

This work was funded by the Spanish Government and European Regional Development Fund (Project RTC-2016-5241-2).

6. Acknowledgments

Damián Valverde would like to thank the Regional Ministry of Education, Culture and Sports of Generalitat Valenciana for his PhD grant. We also thank Helen Warburton for editing the English style.

7. References

Andres-Lacueva, C., Monagas, M., Khan, N., Izquierdo-Pulido, M., Urpi-Sarda, M., Permanyer, J., & Lamuela-Raventós, R. M. (2008). Flavanol and Flavonol Contents of Cocoa Powder Products: Influence of the Manufacturing Process. Journal of Agricultural and Food Chemistry, 56, 3111-3117. https://doi.org/10.1021/jf0728754

427 Aprotosoaie, A. C., Luca, S. V., & Miron, A. (2015). Flavor chemistry of cocoa and cocoa products:
428 an overview. Comprehensive Reviews in Food Science and Food Safety, 15 (1), 73-91.
429 https://doi.org/10.1111/1541-4337.12180
430 Arranz, S., Saura-Calixto, F., Shaha, S., & Kroon, P. A. (2009). High Contents of Nonextractable
431 Polyphenols in Fruits Suggest That Polyphenol Contents of Plant Foods Have Been Underestimated.
432 Journal of Agricultural and Food Chemistry, 57 (16), 7298-7303. https://doi.org/10.1021/jf9016652
433 Bouchez, P., Benites, V. T., Baidoo, E. E. K., Mortimer, J. C. , Sullivan, M. L., Scheller, H. V., &
434 Eudes, A. (2019). Production of clovamide and its analogues in Saccharomyces cerevisiae and
435 Lactococcus lactis. Letters in Applied Microbiology, 69 (3), 181-189.
436 https://doi.org/10.1111/lam.13190
437 Brandon, M. J., & Terink, J. L. (1981). Alkalized cocoa powders and foodstuffs containing such
438 powders. US4435436A.
439 Chalin, M. L. (1974). Method of dutching cocoa. US3868469A.
440 Delgado-Licon, E., Ayala, A. L. M., Rocha-Guzman, N. E., Gallegos-Infante, J. A., Atienzo-Lazos,
441 M., Drzewiecki, J., Martínez-Sánchez ,C. E. & Gorinstein, S. (2009). Influence of extrusion on the
442 bioactive compounds and the antioxidant capacity of the bean/corn mixtures. *International Journal*
443 *of Food Sciences and Nutrition*, *60*(6), 522-532. https://doi.org/10.1080/09637480801987666
444 D'Souza, R. N., Grimbs, S., Behrends, B., Bernaert, H., Ullrich, M. S., & Kuhnert, N. (2017). Origin-
445 based polyphenolic fingerprinting of *Theobroma cacao* in unfermented and fermented beans. Food
446 Research International, 99, 550-559. https://doi.org/10.1016/j.foodres.2017.06.007
447 De Zaan cocoa, 2006. Cocoa & Chocolate manual. ADM Cocoa, Switzerland.
448 Del Rio, D., Costa, L. G., Lean, M. E. J., Crozier, A. (2010). Polyphenols and health: What
449 compounds are involved? Nutrition, metabolism and cardiovascular diseases, 20 (1), 1-6.
450 https://doi.org/10.1016/j.numecd.2009.05.015

Domínguez-Rodríguez, G., Marina, M. L., & Plaza, M. (2017). Strategies for the extraction and analysis of non-extractable polyphenols from plants. Journal of Chromatography A, 1514, 1-15. https://doi.org/10.1016/j.chroma.2017.07.066

El Gharras, H. (2009). Polyphenols: food sources, properties and applications – a review. International Journal of Food Science & Technology, 44 (12), 2512-2518. https://doi.org/10.1111/j.1365-2621.2009.02077.x

Ellis, L. D. (1990). Process for making dark cocoa. US5114730A.

Gonzales, G. B., Raes, K., Vanhoutte, H., Coelus, S., Smagghe, G., & Camp J. V. (2015). Liquid chromatography–mass spectrometry coupled with multivariate analysis for the characterization and discrimination of extractable and nonextractable polyphenols and glucosinolates from red cabbage and Brussels sprout waste streams. Journal of Chromatography A, 1402, 60-70. https://doi.org/10.1016/j.chroma.2015.05.009

Gu, L., House, S. E., Wu, X., Ou, B., & Prior, R. L. (2006). Procyanidin and catechin contents and anxiotidant capacity of cocoa and chocolate products. Journal of Agricultural and Food Chemistry, 54 (11), 4057-4061. https://doi.org/10.1021/jf060360r

Gültekin-Özgüven, M., Berktaş, I., & Özçelik, B. (2016). Change in stability of procyanidins, antioxidant capacity and in-vitro bioaccessibility during processing of cocoa powder from cocoa beans. LWT - Food Science and Technology, 72, 559-565. https://doi.org/10.1016/j.lwt.2016.04.065

He, M., Min, J. W., Kong, W. L., He, X. H., Li, J. X., Peng, B. W. (2016). A review on the pharmacological effects of vitexin and isovitexin. Fitoterapia, 115, 74-85. https://doi.org/10.1016/j.fitote.2016.09.011

Hellström, J. K., & Mattila, P. H. (2008). HPLC Determination of Extractable and Unextractable Proanthocyanidins in Plant Materials. Journal of Agricultural and Food Chemistry, 56 (17), 7617-7624. https://doi.org/10.1021/jf801336s

475 Huang, Y., & Barringer, S. A. (2010). Alkylpyrazines and other volatiles in cocoa liquors at pH 5 to
476 8, by Selected Ion Flow Tube-Mass Spectrometry (SIFT-MS). Journal of Food Science, 75 (1), 121–
477 127. https://doi.org/10.1111/j.1750-3841.2009.01455.x
478 Hurst, W. J., Krake, S. H., Bergmeier, S. C., Payne, M. J., Miller, K. B., & Stuart, D. A. (2011).
479 Impact of fermentation, drying, roasting and Dutch processing on flavan-3-ol stereochemistry in
480 cacao beans and cocoa ingredients. Chemistry Central Journal, 5 (53). https://doi.org/10.1186/1752-
481 153X-5-53
482 Jolić, M. A., Redovniković, I. R., Marković, k., Šipušić, D. I., & Delonga, K. (2011). Changes of
483 phenolic compounds and antioxidant capacity in cocoa beans processing. International Journal of
484 Food Science & Technology, 46 (9), 1793-1800. https://doi.org/10.1111/j.1365-2621.2011.02670.x
485 Kofink, M., Papagiannopoulos, M., & Galensa, R. (2007). (-)-Catechin in cocoa and chocolate:
486 occurrence and analysis of an atypical flavan-3-ol enantiomer. Molecules, 12 (7), 1274-1288.
487 https://doi.org/10.3390/12071274
488 Kopp, G. M., Hennen, J. C., Seyller, M., & Brandstetter, B. (2009). Process for producing high
489 flavour cocoa. EP2241190B1.
490 Li, Y., Feng, Y., Zhu, S., Luo, C., Ma, J., & Zhong, F. (2012). The effect of alkalization on the
491 bioactive and flavor related components in commercial cocoa powder. Journal of Food Composition
492 and Analysis, 25 (1), 17-23. https://doi.org/10.1016/j.jfca.2011.04.010
493 Martín, M. A., & Ramos, S. (2017). Health beneficial effects of cocoa phenolic compounds: a mini-
494 review. Current Opinion in Food Science, 14, 20-25. https://doi.org/10.1016/j.cofs.2016.12.002
495 Martín, M. A., Goya, L., & Ramos, S. (2013). Potential for preventive effects of cocoa and cocoa
496 polyphenols in cancer. Food and Chemical Toxicology, 56, 336-351.
497 https://doi.org/10.1016/j.fct.2013.02.020
498 Miller, K. B., Hurst, W. F., Payne, M. J., Stuart, D. A., Apgar, J., Sweigart, D. S., & Ou, B. (2008).
499 Impact of alkalization on the antioxidant and flavanol content of commercial cocoa powders. Journal
500 of Agricultural and Food Chemistry, 56 (18), 8527-8533. https://doi.org/10.1021/jf801670p

501 Ortega, N., Romero, M. P., Macià, A., Reguant, J., Anglès, N., Morelló, J. R., & Motilva, M. J.
502 (2008). Obtention and characterization of phenolic extracts from different cocoa sources. Journal of
503 Agricultural and Food Chemistry, 56 (20), 9621-9627. https://doi.org/10.1021/jf8014415
504 Quelal-Vásconez, M. A., Lerma-García, M. J., Pérez-Esteve, É., Arnau-Bonachera, A., Barat, J. M.,
505 & Talens, P. (2020). Changes in methylxanthines and flavanols during cocoa powder processing and
506 their quantification by near-infrared spectroscopy. *LWT*, *117*, 108598.
507 https://doi.org/10.1016/j.lwt.2019.108598
508 Sharma, P., Ramchiary, M., Samyor, D., & Das, A. B. (2016). Study of the phytochemical properties
509 of pineapple fruit leather processed by extrusion cooking. Food Science and Technology, 72, 534-
510 543. https://doi.org/10.1016/j.lwt.2016.05.001
511 Stark, T., & Hofmaan, T. (2006). Application of a molecular sensory science approach to alkalized
512 cocoa (Theobroma cacao): structure determination and sensory activity of nonenzymatically C-
513 glycosylated flavan-3-ols. Journal of Agricultural and Food Chemistry, 54 (25), 9510-9521.
514 https://doi.org/10.1021/jf062403
515 Todorovic, V., Redovnikovic, I. R., Todorovic, Z., Jankovic, G., Dodevska, M., & Sobajic, S. (2015).
516 Polyphenols, methylxanthines, and antioxidant capacity of chocolates produced in Serbia. Journal of
517 Food Composition and Analysis, 41, 137-143. https://doi.org/10.1016/j.jfca.2015.01.018
518 Totlani, V. M., & Peterson, D. G. (2007) Influence of epicatechin reactions on the mechanisms of
519 Maillard product formation in low moisture model systems. Journal of Agricultural and Food
520 Chemistry, 55 (2), 414−420. https://doi.org/10.1021/jf0617521
521 Verma, N., Amresh, G., Sahu, P. K., Mishra, N., Rao, C. V., & Singh, A. P. (2013). Pharmacological
522 evaluation of hyperin for antihyperglycemic activity and effect on lipid profile in diabetic rats. Indian
523 Journal of Experimental Biology, 51, 65-72.
524 Repo-Carrasco-Valencia, R., de La Cruz, A. A., Alvarez, J. C. I., & Kallio, H. (2009). Chemical and
525 functional characterization of kaniwa (Chenopodium pallidicaule) grain, extrudate and bran. *Plant*
526 *Foods for Human Nutrition*, *64*(2), 94-101. https://doi.org/10.1007/s11130-009-0109-0

527 Vo, V. A., Lee, J. W., Chang, J. E., Kim, J. Y., Kim, N. H., Lee, H. J., Kim, S. S., Chun, W. & Kwon,
528 Y. S. (2012). Avicularin inhibits lipopolysaccharide-induced inflammatory response by suppressing
529 ERK phosphorylation in RAW 264.7 macrophages. Biomolecules & Therapeutics, 20 (6), 532-537.
530 https://dx.doi.org/10.4062%2Fbiomolther.2012.20.6.532
531 Wiant, M. J., Lynch, W.R., & LeFreniere, R. C. (1989). Method for producing deep red and black
532 cocoa. US5009917A.
533 Wissgott, U. (1985). Method of alkalization of cocoa in the aqueous phase. CH663324A5
534 Zhang, B., Liu, G., Ying, D., Sanguansri, L., & Augustin, M. A. (2017). Effect of extrusion conditions
535 on the physico-chemical properties and *in vitro* protein digestibility of canola meal. Food Research
536 International, 100, 658-664. https://doi.org/10.1016/j.foodres.2017.07.060
537 Zhu, Q. Y., Holt, R. R., Lazarus, S. A., Ensunsa, J .L., Hammerstone, J. F., Schmitz, H. H., & Keen,
538 C. L. (2002). Stability of the flvan-3-ols epicatechin and catechin and related dimeric procyanidins
539 derived from cocoa. Journal of Agricultural and Food Chemistry, 50 (6), 1700-1705.
540 https://doi.org/10.1021/jf011228o