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Original Article

Comparative study of UPLC–MS/MS and HPLC–MS/MS to determine procyanidins and alkaloids in cocoa samples

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ABSTRACT

This study develops a method using ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) to identify and quantify procyanidins, monomers to oligomers, and alkaloids, theobromine and caffeine, in cocoa samples. UPLC analyses were carried out using an Acquity HSS T3 column (100 mm × 2.1 mm i.d., 1.8 μm particle size) and gradient elution with water/acetic acid (99.8/0.2, v/v) and acetonitrile. This method was compared with an HPLC–MS/MS method in terms of speed, sensitivity, selectivity, peak efficiency, linearity, reproducibility, detection limits (LODs) and quantification limits (LOQs). Both methodologies were applied and compared to identify and quantify procyanidins and alkaloids from samples of cocoa nib. The results obtained showed that the UPLC–MS/MS methodology allow to determine procyanidins up to nonamers at low-concentration levels in a short analysis time, i.e. less than 12.5 min.

1. Introduction

Cocoa products are known to be rich in polyphenols, especially procyanidins, the concentrations of which vary depending on their origin (Counet et al., 2004; Niemenak et al., 2006), and processing conditions (Wollgast and Anklam, 2000; Summa et al., 2006). The procyanidins identified in cocoa can range in size from monomers to long-chain polymers, such as tannins, and are usually bound to carbohydrates, which constitute repeating subunits of high molecular weight polymers. Moreover, cocoa has been found to be beneficial for health because of its antioxidant (Adamson et al., 1999; Counet and Collin, 2003; Lee et al., 2003; Othman et al., 2007), anti-inflammatory (Ramiro et al., 2005) and anti-atherosclerotic activity (Vinson et al., 2006) in *in vitro* and also *in vivo* studies (Sies et al., 2005). Due to the possible beneficial implications of procyanidins for human health, there is interest in analyzing their content and the specific oligomers and polymers in cocoa.

Apart from procyanidins, cocoa samples are also known to contain alkaloids such as theobromine and caffeine. These compounds affect the flavor of cocoa products (Luna et al., 2002) and have received increasing attention because of their physiological effects (Buchelli et al., 2001).

The alkaloid compounds in cocoa samples have been analyzed by capillary electrophoresis (CE) (Gotti et al., 2006) and by high-performance liquid chromatography (HPLC) (Naik, 2001; Brunetto et al., 2007). The detection system most frequently employed in these studies was ultraviolet detection (UV). On the other hand, procyanidin monomers (catechin and epicatechin) and oligomers in cocoa samples have been mainly analyzed by HPLC in the normal phase (Adamson et al., 1999; Hammerstone et al., 1999; Lazarus et al., 1999; Natsume et al., 2000; Gu et al., 2002; Rabaneda et al., 2003) and in the reversed-phase (Natsume et al., 2000; Wollgast et al., 2001; Cooper et al., 2007). Apart from the normal and reversed HPLC modes, hydrophilic interaction chromatography (HILIC) has also been used to analyze procyanidins in cocoa samples and successful results were reported when the diol stationary phase was used (Kelm et al., 2006). Apart from HPLC, other techniques, such as thin-layer chromatography (TLC) (Porter et al., 1991) and CE (Gotti et al., 2006) have also been used.

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Several detection systems have been explored for characterizing procyanidins in cocoa samples by HPLC. Photodiode array (PDA) (Rigaud et al., 1993) and fluorescence (Adamson et al., 1999; Lazarus et al., 1999) have been shown to be efficient. However, the coupling of HPLC with mass spectrometry (MS) (Hammerstone et al., 1999; Natsume et al., 2000; Gu et al., 2002; Kelm et al., 2006) and tandem MS (MS/MS) (Wollgast et al., 2001; Rabaneda et al., 2003; Cooper et al., 2007) has been shown to be an effective tool for identifying procyanidins at low-concentration levels for the analysis of complex matrices, such as cocoa samples. Unfortunately, all these methods require a long analysis time to determine procyanidins by HPLC. This takes between 50 and 80 min per sample. In addition, when analyses are carried out in the normal phase, this requires the use of toxic chlorinated solvents that are considered an ecological hazard (Gu et al., 2002).

Recently, there have been improvements in the LC technique with the development of ultra-performance liquid chromatography (UPLC). This technique takes full advantage of chromatographic principles to perform separations using columns packed with smaller particles (1.7 μm) and/or at higher flow rates. This leads to a shorter analysis time, higher peak efficiency and higher resolution. The reversed-phase UPLC–MS/MS technique has been applied by Cooper et al. (2007) to quantify the major chocolate polyphenols: catechin, epicatechin, dimers B_2 and B_5, trimer C_1 and tetramer D. The application of this technique to the analysis of 68 chocolate samples showed that epicatechin concentrations can be used to predict the content of other polyphenols, especially B_2 and C_1, and the total polyphenol content in an analysis time of only 3 min.

Thus, UPLC opens up new possibilities for improving the analytical methods for complex food samples that usually require high resolution and long analysis time. The aim of this study was to develop a rapid, reproducible and sensitive method to determine procyanidins (from monomers to oligomers) and alkaloids (caffeine and theobromine) by UPLC–MS/MS. The method developed was compared to HPLC–MS/MS method in the normal phase in terms of selectivity, sensitivity, analysis time, peak efficiency, operating costs, and quality parameters: linearity, reproducibility, detection limits (LODs) and quantification limits (LOQs). A further aim was to apply the developed methodologies and compare their potential for identifying and quantifying the procyanidins and the alkaloids in cocoa nibs.

2. Materials and methods

2.1. Chemicals and reagents

Standards of (−)-epicatechin, (+)-catechin, theobromine and caffeine were obtained from Sigma Aldrich (St. Louis, MO, USA). Procyanidin B_2 was purchased from Fluka Co. (Buchs, Switzerland). A stock standard solution of 2 mg/mL of (−)-epicatechin, (+)-catechin, procyanidin B_2 and caffeine was dissolved in acetonitrile. Meanwhile, theobromine was dissolved in acetone/MilliQ water/acetic acid (70/29.5/0.5, v/v/v). The standard working solution was prepared weekly by diluting the stock standard solutions in solvent extraction, acetone/water/acetic acid (70/29.5/0.5, v/v/v) and these were stored in dark-glass flasks at 4 °C. Dichloromethane, methanol, acetonitrile and acetone were HPLC grade (Scharlab, Barcelona, Spain); multisolvent grade acetic acid and hexane were purchased from Scharlab (Barcelona, Spain). Ultrapure water was obtained from a MilliQ water purification system (Millipore Corp., Bedford, MA, USA).

2.2. Chromatographic methods

The HPLC and UPLC analyses were performed using a Waters Acquity Ultra-Performance™ liquid chromatography system (Waters, Milford, MA, USA), equipped with a binary pump system (Waters, Milford, MA, USA).

The HPLC normal phase separations were carried out on a Phenomenex Luna silica column (25 cm × 4.60 mm, 5 μm) at 30 °C, equipped with a silica precolumn (5 μm, 1 cm × 4.6 mm) (Technokroma, Barcelona, Spain). The mobile phase was dichloromethane (eluent A), methanol (eluent B), and water/acetic acid (50/50, v/v) (eluent C). The elution gradient at 30 °C was of B into A with a constant 4% C at a flow rate of 1 mL/min as follows: elution starting with 14% B in A; 14–28.4% B in A, 0–30 min; 28.4–39.2% B in A, 30–45 min; 39.2–86% B in A, 45–50 min.

The UPLC analyses were performed using an Acquity HSS T3 column (100 mm × 2.1 mm i.d., 1.8 μm particle size) (Waters, Milford, MA, USA) with a binary phase at a flow rate of 0.4 mL/min. The eluent A was water/acetic acid (99.8/0.2, v/v) and B was acetonitrile. The elution gradient at 30 °C was: 0–10 min, 5–35% B; 10–10.10 min, 35–80% B; 10.10–11 min, 80% B isocratic; 11–11.10 min, 80–5% B; 11.10–12.50 min, 5% B isocratic.

2.3. MS/MS conditions

Both the HPLC and UPLC systems were coupled to a triple quadrupole detector (TQD) mass spectrometer (Waters, Milford, MA, USA) using an electrospray ionization (ESI) source Z-spray™. For all the standards and samples, the MS was simultaneously operated in negative and positive modes to analyze the procyandins and the alkaloids (caffeine and theobromine), respectively, in a single run. The data were acquired by selected reaction monitoring (SRM). The working conditions for the ionization source were as follows: capillary voltage, 3 kV; source temperature, 150 °C; cone gas flow rate, 80 L/h and desolvation gas flow rate, 800 L/h; desolvation temperature, 400 °C. Nitrogen (>99% purity) and argon (99% purity) were used as nebulizing and collision gases, respectively. The cone voltages and collision energies were optimized for each analyte by infusing 10 μg/mL of each standard, and two transitions were acquired, one for quantification and the other for confirmation purposes. These results are shown in Table 1. The dwell time established for each transition was 100 ms for HPLC and 30 ms for UPLC. Data acquisition was carried out by MassLynx v 4.1 software.

Table 1
Optimized SRM conditions for the analysis of catechin, epicatechin, dimer B_2, caffeine and theobromine by HPLC–MS/MS and UPLC–MS/MS.

Compound	[M−H]	SRM_1 (quantification)	Cone voltage (V)	Collision energy (eV)	SRM_2 (confirmation)	Cone voltage (V)	Collision energy (eV)
ESI (−)							
Catechin	$[M-H]^-$	289 > 245	45	10	289 > 205	45	15
Epicatechin	$[M-H]^-$	289 > 245	45	10	289 > 179	45	15
Dimer B_2	$[M-H]^-$	577 > 289	45	20	577 > 425	45	15
ESI (+)							
Caffeine	$[M-H]^+$	195 > 138	45	15	195 > 110	45	20
Theobromine	$[M-H]^+$	181 > 163	45	15	181 > 140	45	10

2.4. Quality parameters

The quality parameters of the two chromatographic methods, the HPLC–MS/MS and UPLC–MS/MS, were studied by using a serial dilution of the stock solution of the standards: catechin, epicatechin, dimer B_2, caffeine and theobromine. The parameters considered were linearity, calibration curve, reproducibility, LOD and LOQ.

The calibration curves (based on the integrated peak area) were calculated using five points at different concentrations (from 0.002 to 50 μg/mL) and each standard solution was injected three times. The reproducibility, expressed by relative standard deviations (RSDs) of the concentration, was studied at two concentrations, 1 and 10 μg/mL, on three different days with one injection each day. The LODs and LOQs were calculated using a signal-to-noise-criterion of 3 and 10, respectively.

2.5. Cocoa phenol extraction and analysis

The cocoa nib samples corresponded to the Forastero variety from Ghana (West Africa). The cocoa nibs are obtained from roasted cocoa beans separated from their husks and broken into small bits. They are the essence of chocolate.

The extraction of procyanidins from the cocoa nibs was based on the work by Hammerstone et al. (1999), slightly modified. The cocoa nibs were ground in a laboratory mill for 30 s before phenol extraction to obtain a homogenous material. In order to achieve maximum extraction efficiency during the sample preparation, 15 g of this sample was firstly defatted four times with 125 mL of hexane for 20 min in an orbital shaker (Selecta, Barcelona, Spain) at 200 rpm and subsequently centrifuged for 15 min at 15,000 × *g*. The defatted cocoa sample was then extracted four times with solvent extraction (acetone/MilliQ water/acetic acid, 70/29.5/0.5, v/v/v) at a ratio of 1:5. After the addition of extraction solvent, the tubes were vortexed for 3 min and centrifuged. The supernatants from the centrifuged tubes were combined and filtered through glass wool. The organic solvent was removed by rotary evaporation (Büchi, Labortechnick AG, Switzerland) under partial vacuum at 30 °C. Finally, the water extract was freeze-dried in Lyobeta 15 equipment (Ima-Telstar, Spain) to obtain the cocoa phenolic extracts and these were stored in inert atmosphere with nitrogen before the chromatographic analysis.

The lyophilized phenolic extracts were dissolved in extraction solvent (10 mg/mL) and filtered through 0.45 and 0.22 μm nylon filters prior to chromatographic analysis by HPLC and UPLC, respectively. The sample volume injected was 10 μL for HPLC and 2.5 μL for UPLC.

The quantification was performed by using external calibration. The dimers were quantified by using the calibration curve of procyanidin B_2, and the oligomer procyanidins were tentatively quantified by using the calibration curve of the monomer catechin. The results of the quantification of the catechin, epicatechin and dimer B_2 were expressed as μg of the corresponding standard per gram of phenolic extract. The results were expressed as mg of catechin per gram of phenolic extract. The results for the alkaloids were expressed as mg of the corresponding standard per gram of phenolic extract.

2.6. Statistical analysis

The data from the comparative quantification of procyanidins and alkaloids in phenolic cocoa extracts by HPLC–MS/MS and UPLC–MS/MS were expressed as mean ± standard deviation. The data were analyzed by a one-way ANOVA test to assess differences between quantification by HPLC and UPLC. A significant difference was considered at level of $p < 0.05$. All of the statistical analyses were carried out using STATGRAPHICS Plus 5.1.

3. Results and discussion

3.1. Comparative analysis by HPLC and UPLC of the procyanidins and the alkaloids in standard solutions

During the first part of the study, the analysis times using HPLC and UPLC were compared. The initial analyses were carried out with HPLC in the normal phase as the most widely used method for analyzing the cocoa phenols in the literature (Adamson et al., 1999; Hammerstone et al., 1999; Lazarus et al., 1999; Natsume et al., 2000) using MS/MS detection. A silica column was used as the stationary phase and dichloromethane, methanol, water and acetic acid as the mobile phase. Fig. 1A shows the extracted ion chromatograms for the analysis of the five compounds studied in standard solutions by HPLC–MS/MS in the normal phase. The concentration of all the analytes was 10 μg/mL. These were eluted

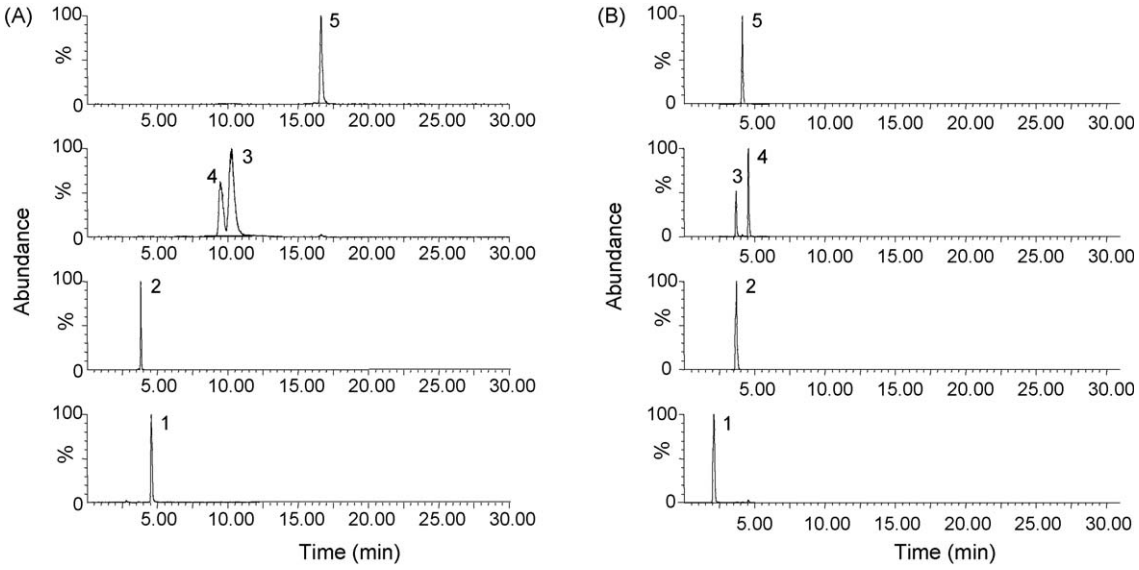

Fig. 1. Extracted ion chromatograms for the analysis of the phenolic compounds by HPLC–MS/MS (A) and UPLC–MS/MS (B) in the standard solution. The concentration of the analytes was 10 μg/mL. The peak designation was (1) theobromine, (2) caffeine, (3) catechin, (4) epicatechin, and (5) dimer B2.

in order of increasing polarity and increasing size. Thus, caffeine (peak 2) eluted before 5 min, whereas dimer B_2 (peak 5) showed the longest retention time at 17 min.

UPLC–MS/MS was applied with the aim of decreasing the analysis time. To date, silica columns for UPLC in normal phase have still to be developed, and therefore the studied compounds were resolved in the reversed or "aqueous normal" phase. Three columns were investigated for this purpose. These were the BEH C_{18}, the BEH HILIC and the new high strength silica (HSS) T3 (Waters, Milford, MA, USA). The first, the BEH C_{18}, is the universal column choice for most UPLC separations. The second, the BEH HILIC, uses a polar stationary phase and the mobile phase contains high concentrations of apolar solvents and low concentrations of polar aqueous solvents, similar to those in the reversed-phase (Waters Cromatografia S.A.). This column is designed to retain and separate very basic polar compounds. Successful results were reported for diol and HILIC columns for the analysis of procyanidins in cocoa (Kelm et al., 2006) and apple (Yagagida et al., 2007) samples. Finally, the HSS T3 column, 100% silica particle, is used to retain and separate smaller, more water-soluble polar organic compounds than the BEH C_{18} and HILIC columns. As a result of the application of the three columns in this study to the analysis of the standard solutions of procyanidins and alkaloids (data not shown), the HSS T3 column was chosen to provide higher retention for the polar analytes, such as oligomers.

The mobile phase was 0.2% acetic acid as eluent A, acid acetic was used to improve the peak shape, and acetonitrile as eluent B. Acetonitrile was selected since, like dichloromethane, it is a non-polar aprotic solvent. Both solvents have shown similar relative properties for hydrogen-bonding and dipole interaction (Snyder, 1978). Next, by using a calculator software tool (version 1.1.1), the gradient HPLC method was converted to the gradient UPLC method. Fig. 1B shows the extracted ion chromatograms for the analyses of five compounds in standard solutions studied by UPLC–MS/MS. The concentration for all the analytes was 10 μg/mL. In this case, the analytes were eluted according to their apolarity and increasing size.

By comparing the chromatograms obtained from analyzing the compounds by HPLC–MS/MS (Fig. 1A) and UPLC–MS/MS (Fig. 1B) a shorter analysis time was observed for the latter. For instance, the retention time of catechin was 10.99 min in HPLC, but only 3.56 min in UPLC.

The studied standards compounds (Table 2) were eluted in less than 5 min by the application of UPLC method. However, the elution gradient of this method was carried out for 12.5 min because the aim of this work was also focused on the determination and characterization of the oligomers in cocoa samples.

Apart from the analysis time, other differences between the two methodologies were observed. A different elution order of the analytes was shown as a consequence of the different partitioning of the analytes between the two stationary and mobile phases. For instance, caffeine eluted before theobromine in HPLC–MS/MS (normal phase) (Fig. 1A), while it eluted after theobromine in UPLC–MS/MS (reversed-phase) (Fig. 1B). The dimer B_2 eluted after the monomer epicatechin in HPLC–MS/MS, and this compound eluted between the monomers catechin and epicatechin in UPLC–MS/MS. The elution order of the monomers catechin and epicatechin was also different in the two methodologies.

Another difference between the chromatograms is the resolution of the monomers catechin and epicatechin. In the UPLC methodology (reversed-phase), the two monomers were baseline-separated but, in contrast, these compounds were not completely separated in the HPLC methodology (normal phase). Other difference between the two methodologies is the coelution of catechin and caffeine in UPLC–MS/MS, which was not problematic because these compounds have different molecular weight and these are ionized in different modes, catechin in the negative mode and caffeine in the positive mode.

3.2. Quality parameters

The quality parameters of the two methodologies, HPLC–MS/MS and UPLC–MS/MS, for the analysis of the phenolic compounds in standard solutions are shown in Table 2. The linear response of procyanidins was in the concentration range of 0.01–50 μg/mL and 0.01–40 μg/mL for HPLC–MS/MS and UPLC–MS/MS, respectively. The linear response of the alkaloids was in the concentration range of 0.002–50 μg/mL and 0.002–20 μg/mL for HPLC–MS/MS and UPLC–MS/MS, respectively. The correlation coefficients (R) of the calibration curves were higher than 0.996 for all the compounds in both methods.

The reproducibility, expressed by relative standard deviations (RSDs) of the concentration, was studied at two concentrations, 1 and 10 μg/mL, on three different days. As can also be seen in Table 2, the RSDs rose as the level of concentration decreased, and the RSDs in the UPLC–MS/MS were lower than the RSDs in the HPLC–MS/MS for both concentrations studied, except for the epicatechin standard. For instance, these values were below 3.2% and 1.9% for the analysis of the compounds studied by HPLC–MS/MS and UPLC–MS/MS, respectively, at a concentration level of 10 μg/mL.

The LODs for the procyanidin analysis were in the 0.009–0.02 μg/mL range for HPLC–MS/MS and 0.007–0.01 μg/mL for UPLC–MS/MS. In reference to the analysis of alkaloids, the LODs were similar, being between 0.001 and 0.03 μg/mL for the analysis of caffeine and theobromine. The LOQs for the analysis of procyanidins were in the 0.02–0.2 μg/mL range for HPLC–MS/MS and 0.01–0.09 μg/mL for UPLC–MS/MS, respectively. The LOQs

Table 2
Retention time, linearity, calibration curves, reproducibility, LODs and LOQs for the analysis of the studied compounds by HPLC–MS/MS and UPLC–MS/MS in standard solutions.

Methodology	Compound	RT (min)	Linearity (μg/mL)	Calibration curve[a]	RSD% (n = 3)		LOD (μg/mL)	LOQ (μg/mL)
					10 μg/mL	1 μg/mL		
HPLC–MS/MS	Catechin	10.99	0.05–50	$y = 4438.7x + 3149.3$	3.0	3.6	0.02	0.08
	Epicatechin	10.27	0.1–50	$y = 4713.1x + 6233.1$	0.6	1.8	0.05	0.2
	Dimer B_2	16.54	0.01–20	$y = 8204.5x - 895.5$	3.7	4.7	0.009	0.02
	Caffeine	3.77	0.002–50	$y = 48502x - 12900$	2.8	3.5	0.001	0.003
	Theobromine	4.56	0.05–50	$y = 20823x - 2974.5$	3.2	4.3	0.03	0.1
UPLC–MS/MS	Catechin	3.68	0.02–40	$y = 1129.5x + 336.4$	1.9	2.6	0.01	0.04
	Epicatechin	4.56	0.05–20	$y = 1871.8x + 404.7$	1.5	1.9	0.03	0.09
	Dimer B_2	4.12	0.01–20	$y = 2763.2x - 217.0$	1.4	3.0	0.007	0.01
	Caffeine	3.69	0.002–20	$y = 39062x + 6351.7$	1.9	3.0	0.001	0.003
	Theobromine	2.13	0.05–20	$y = 6592.5x + 756.0$	0.7	2.2	0.03	0.1

[a] *Calibration curve*: $y = mx + b$ where y is the integrated peak area and x is the concentration in μg/mL. RT: retention time.

Table 3
Optimized SRM conditions for the analysis of procyanidin oligomers by HPLC–MS/MS and UPLC–MS/MS.

Compound	[M−H]	SRM_1 (quantification)	Cone voltage (V)	Collision energy (eV)	SRM_2 (confirmation)	Cone voltage (V)	Collision energy (eV)
ESI (−)							
Trimer	$[M-H]^-$	865 > 577	60	20	865 > 695	60	25
Tetramer	$[M-H]^-$	1153 > 865	70	20	1153 > 575	70	30
Pentamer	$[M-H]^-$	1441 > 1028	80	25	1441 > 1151	80	30
Hexamer	$[M-H]^-$	1729 > 1153	80	30	1729 > 863	80	30
Heptamer	$[M-2H]^{-2/2}$	1008 > 865	60	20	1008 > 575	60	20
Octamer	$[M-2H]^{-2/2}$	1152 > 875	65	25	1152 > 983	65	25
Nonamer	$[M-2H]^{-2/2}$	1296 > 577	50	30	1296 > 1152	50	30

were also similar, being between 0.003 and 0.1 μg/mL for the analysis of caffeine and theobromine. The LODs and LOQs were slightly lower with the UPLC–MS/MS methodology than the HPLC–MS/MS for the procyanidin standards (catechin, epicatechin and dimer B_2). Although the HPLC–MS/MS methodology was less sensitive for catechin, epicatechin and dimer B_2, the calibration curves showed higher slopes than with UPLC–MS/MS.

The LOD and LOQ values obtained by UPLC–MS/MS in this study, which ranged between 0.03 and 0.1 μg/mL, were similar to, or lower than, those reported in the literature for the analysis of these compounds by CE-UV (Gotti et al., 2006), HPLC–MS (Adamson et al., 1999), and HPLC-UV (Brunetto et al., 2007).

3.3. Application of the chromatographic methods

The HPLC–MS/MS and UPLC–MS/MS chromatographic methods were applied to quantify procyanidins and alkaloids in samples of

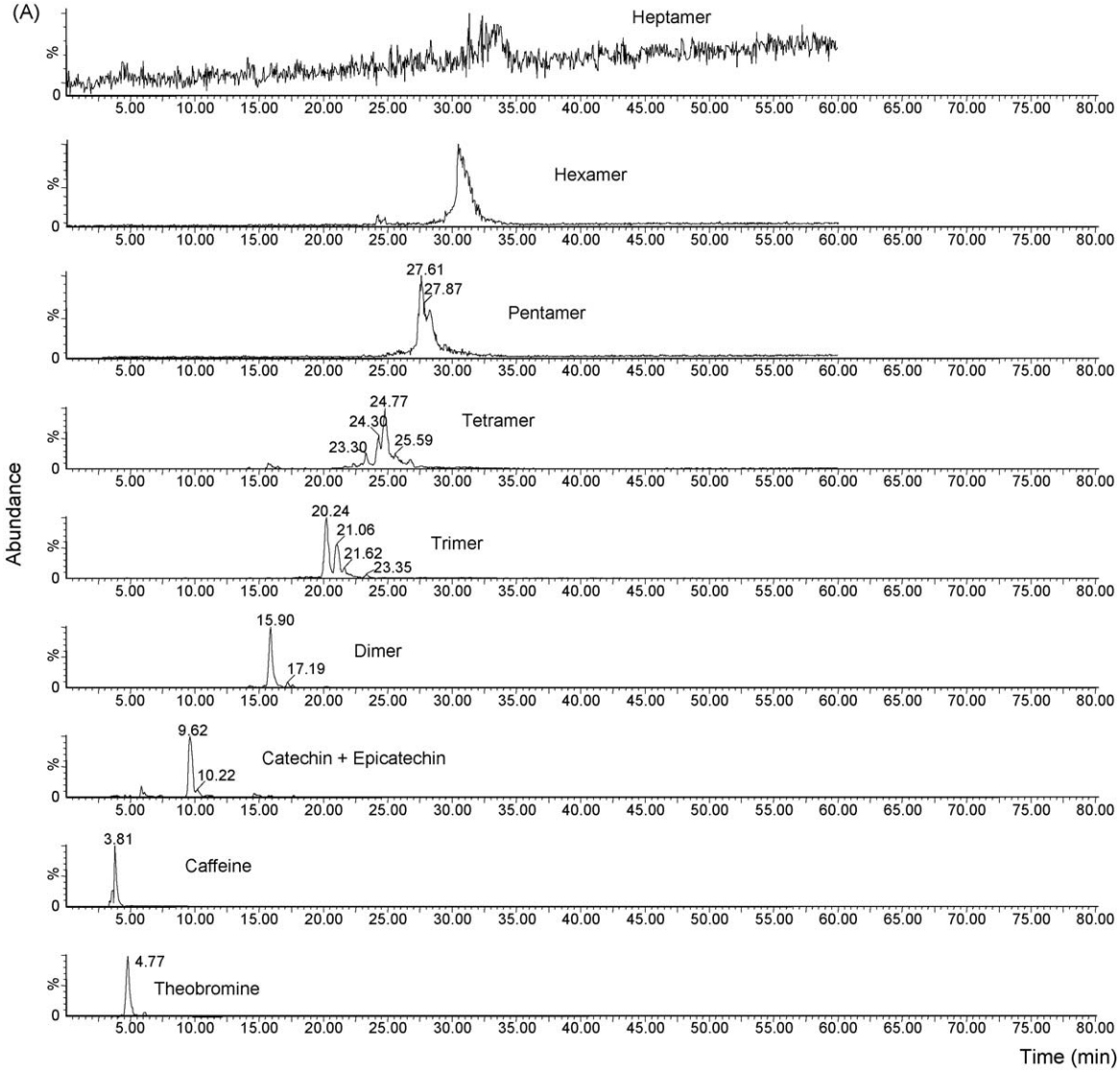

Fig. 2. Extracted ion chromatograms for the analysis of procyanidins and the alkaloids by HPLC/MS-MS (A) and UPLC–MS/MS (B) in the phenolic extract of cocoa nib at a concentration of 10 mg/mL.

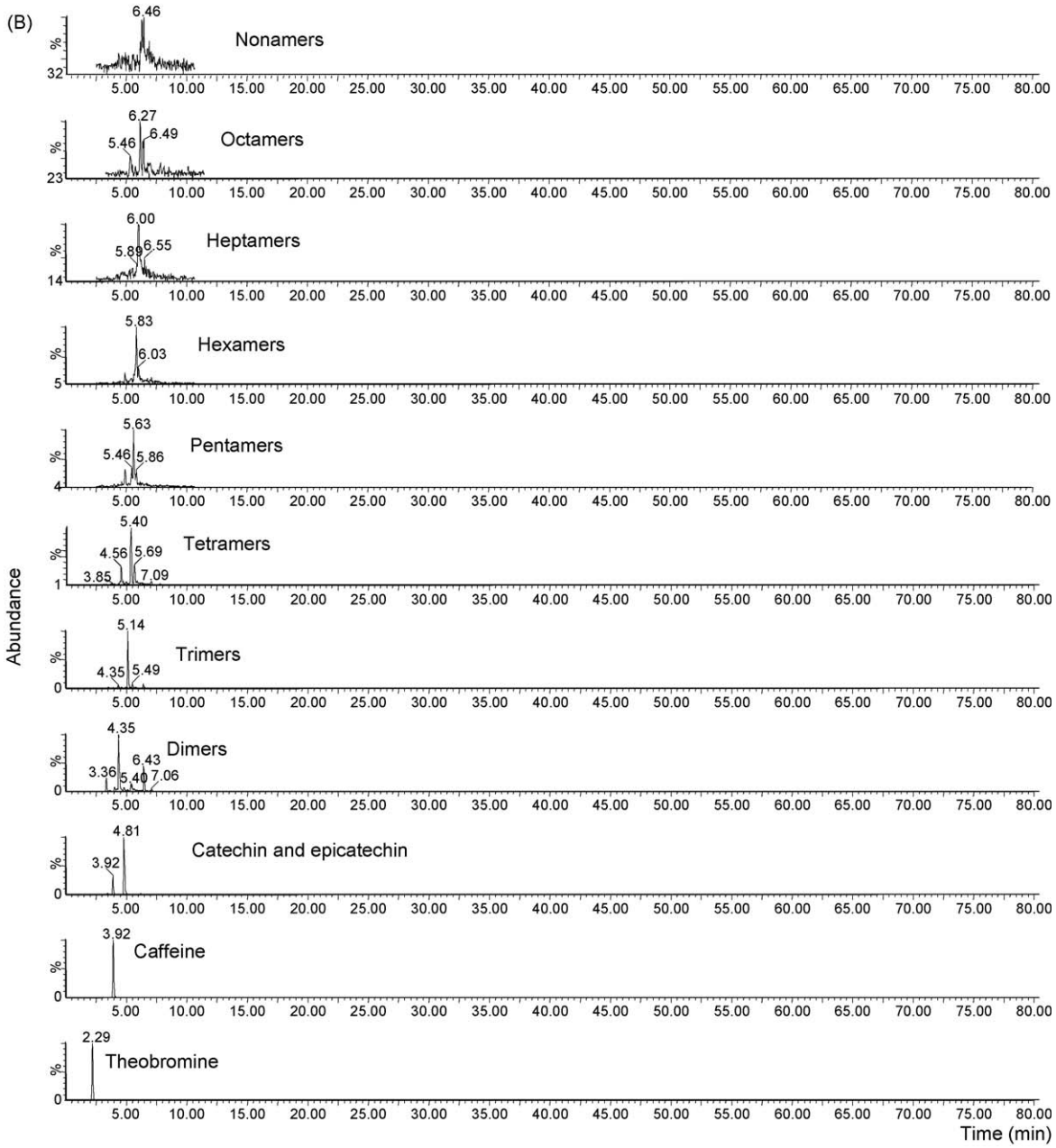

Fig. 2. (*Continued*).

cocoa nibs. The phenolic extracts were prepared as described in Section 2.5.

Before the analysis of the phenolic extract, the matrix effect was evaluated. Matrix effects result from coeluting matrix components that compete for ionization capacity, and this effect is observed by an decrease or increase in the analyte signal in a matrix extract with the same analyte present in the organic solvent, respectively (Choi et al., 2001). In our study, signal suppression was evaluated by comparing the detector response of the compounds studied spiked in organic solvent (solvent extraction) with those from the cocoa extract, at two different concentrations, 20 and 0.2 μg/mL. Either a positive or negative effect was observed, which is an increase or decrease in the detector response, respectively, lower than 17% (data not shown). This effect could be considered small and probably has no a significant effect on signal suppression.

Apart from quantifying the monomers and dimers of procyanidins, attention was also paid to the oligomers with a high degree of polymerization. For this purpose, the SRM conditions, cone voltage and collision energy of these compounds should be investigated. However, there are no commercial standards of polymeric procyanidins available. With the aim of overcoming this, the MS/MS parameters were optimized by analyzing a phenolic extract of the cocoa nibs at a concentration of 10 mg/mL by UPLC–MS/MS. The results are shown in Table 3 and these were the same as those obtained by HPLC–MS/MS.

Fig. 2A and B shows the extracted ion chromatograms for the analysis of procyanidins (from monomers to oligomers) and alkaloids in cocoa nib phenolic extracts by HPLC–MS/MS and UPLC–MS/MS, respectively. As can be seen, it is possible to identify procyanidins from monomers to nonamers by UPLC–MS/MS (Fig. 2B). In contrast, when the same sample was analyzed by HPLC–MS/MS, this method was only able to identify up to hexamers. This fact could be explained by the higher peak efficiency of the UPLC–MS/MS methodology. Additionally, the

Table 4
Comparative quantification by HPLC–MS/MS and UPLC–MS/MS of procyanidins, expressed as mg catechin per g of phenolic extract, and alkaloids, expressed as mg theobromine or caffeine per g of phenolic extract, respectively.

	HPLC–MS/MS[a]	UPLC–MS/MS
Procyanidin[*]		
Catechin	5.80 ± 0.01a	1.90 ± 0.02b
Epicatechin		4.02 ± 0.07b
Dimer	6.51 ± 0.22a	8.51 ± 0.40b
Trimer	3.10 ± 0.21a	3.90 ± 0.04b
Tetramer	0.60 ± 0.10a	0.72 ± 0.08b
Pentamer	0.07 ± 0.02a	0.09 ± 0.00b
Hexamer	0.06 ± 0.01a	0.08 ± 0.00b
Heptamer	n.d.	0.03 ± 0.00
Octamer	n.d.	0.03 ± 0.00
Nonamer	n.d.	0.01 ± 0.00
Total procyanidins	16.21 ± 0.36a	19.31 ± 0.23b
Alcaloid[*]		
Theobromine	19.41 ± 0.52a	18.50 ± 0.38a
Caffeine	0.09 ± 0.09a	0.08 ± 0.07a

n.d.: not detected.
[a] The quantification of catechin and epicatechin by HPLC–MS/MS is expressed by the sum of both compounds.
[*] Different letters in the same row indicate significative differences ($p < 0.05$) between HPLC and UPLC quantification.

peak broadness of certain oligomers was solely induced by the number of isomers, which increased exponentially with the degree of polymerization.

The analysis time was another remarkable difference between the two chromatographic methods. The total analysis time by HPLC for the complete elution of the procyanidin oligomers with a high degree of polymerization, cleaning the column with 100% of methanol, and reequilibrating the column to return to the initial analysis conditions was 80 min. The long analysis time is an important limitation when a high number of samples have to be analyzed. Thus, the UPLC methodology reduced the run time by a factor of around seven in comparison with the HPLC methodology, precisely 12.50 min as against 80 min (Fig. 2A and B).

The last part of the study was focused on the comparative quantification of procyanidins and alkaloids by the two chromatographic methods applied to cocoa nib phenolic extract (Table 4). The average concentrations of monomers (catechin and epicatechin) and the alkaloids were expressed as mg of the respective standard per g of phenolic extract. The several identified dimers were expressed as mg of dimer B_2 per g of phenolic extract. The oligomers were tentatively quantified using the calibration curve of catechin. The main differences in the quantification by the two chromatographic methods were based on the possibility for individual quantification of catechin and epicatechin by UPLC, which was not possible by HPLC as a consequence of the coelution of these compounds. In addition, UPLC allowed the detection and quantification of oligomers from trimer to nonamers, whereas with HPLC, it was only possible to quantify up to hexamers. Thus, when the extract was analyzed by UPLC, the concentration of procynidins was significant higher ($p < 0.05$) that when the analysis was done by HPLC. As expected base on data from Table 2, there were no significant differences between the two chromatographic methods when quantifying alkaloids.

4. Conclusion

To sum up, the current study develops a rapid and sensitive method for analyzing procyanidins and alkaloids in cocoa nibs based on UPLC–MS/MS. By determining procyanidins (from monomers to nonamers) and alkaloids, theobromine and caffeine in less than 12.5 min, UPLC chromatography can play a major role in improving productivity for the routine analysis of compounds in the field of trace analysis. In addition to simplicity and speed, the higher separation efficiency of the LC system allows catechin and epicatechin to be quantified separately, whereas the chromatographic coelution of these two compounds by HPLC normal phase analysis required their quantification as catechin + epicatechin.

Additionally, the efficiency of the peaks by UPLC allowed the procyanidin nonamers to be identified in contrast with the hexamers by HPLC–MS/MS. In addition, the UPLC methodology allowed a reduction of the run time by a factor of around seven in comparison with the HPLC methodology, thus reducing the total analysis time for a cocoa phenolic extract from 80 min by HPLC to 12.50 min by UPLC, including cleaning the column with 100% of methanol, and reequilibrating the column to return to the initial conditions. Therefore, the UPLC system represents a faster and more cost-effective method for analyzing the cocoa phenols than the HPLC system.

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