Distribution Patterns of Polyphenols and Alkaloids in Instant Coffee, Soft and Energy Drinks, and Tea

N. Manchón, L. Mateo-Vivaracho, M. d'Arrigo, A. García-Lafuente, E. Guillamon, Ana Villares Garicochea, M. A. Rostagno

Distribution Patterns of Polyphenols and Alkaloids in Instant Coffee, Soft and Energy Drinks, and Tea

Noelia MANCHÓN, Laura MATEO-VIVARACHO, Matilde D'ARRIGO, Ana GARCÍA-LAFUENTE, Eva GUILLAMÓN, Ana VILLARES and Mauricio Ariel ROSTAGNO

Food Quality Centre, National Institute for Agricultural Research and Experimentation (INIA), Soria, Spain

Abstract

Manchón N., Mateo-Vivaracho L., D'Arrigo M., García-Lafuente A., Guillamón E., Villares A., Rostagno M.A. (2013): **Distribution patterns of polyphenols and alkaloids in instant coffee, soft and energy drinks, and tea**. Czech J. Food Sci., **31**: 483–500.

A previously developed method of HPLC-DAD-Fl has been used for the determination of phytochemical profiles in different types of drinks: instant coffee, soft drinks, energy drinks, and different types of tea (green, white, black, and red tea). Using data on the concentrations of 20 main phytochemicals (phenolic acids, flavan-3-ols, flavonols, flavones, and alkaloids) it was possible to identify most of the sample types. Chlorogenic and caffeic acids and caffeine are the main target compounds in instant coffee; in soft and energy drinks, only caffeine was found. Tea has a more complex phytochemical composition. Unfermented tea is mainly composed of flavan-3-ols and alkaloids, with a high caffeine concentration. Black tea is composed of alkaloids and low levels of flavan-3-ols, which are affected by oxidative reactions during the fermentation. Flavonols are present in lower concentrations in all kinds of teas. The identified phytochemical distribution patterns were used to correctly differentiate instant coffee, soft drinks, energy drinks, unfermented tea and fermented tea (within fermented tea, black tea from red tea can also be differentiated).

Keywords: phytochemical profile; phenolic compounds; beverages; antioxidants; caffeine phenolic acids; flavan-3-ols; catechins; flavones; flavonols; alkaloids

Tea, mostly consumed as an infusion made by brewing leaves of the *Camellia sinensis* plant in water, is one of the most frequently consumed beverages worldwide, and has long been appreciated due to its pleasant taste and its potential health effects. Intake of tea has been suggested to be associated with a decreased risk of cardiovascular diseases and cancer (Gardner *et al.* 2007; Sharangi 2009). Several phytochemicals, such as polyphenols (phenolic acids, flavan-3-ols, flavonols, and flavones) and alkaloids (theobromine and caffeine) (Figure 1), are found in relatively high amounts in tea. Polyphenols have been the focus of intensive research due to their antioxidant properties and potential role in the chronic disease prevention (Mattila & Kumpulainen 2002; Garcia-Lafuente *et al.* 2009) and some of them (such as flavan-3-ols, also known as catechins) are found in tea leaves isolated or in combination with other phenolics, such as gallic acid. However, present polyphenols and their concentration will depend on several factors, including variety, growing environment, processing, and storage conditions (Astill *et al.* 2001; Peterson *et al.* 2004).

Tea can be classified according to the degree of fermentation during manufacturing. When *Camel-*

Supported by the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Projects No. AT 07-003 and No. RTA2009-00049-00-00.

lia sinensis leaves are dried using heat to avoid fermentation by the natural tea enzyme activities, unfermented tea, including green and white tea, is produced. If tea leaves are allowed to ferment before drying by heat treatment, dark tea, known as black tea, is produced. If the fermentation period is long (in the case of pu-erh and red tea), not only tea's natural enzymes act, but also other microorganisms are involved in the process. Oolong is a type of tea subjected to a partial fermentation process. Fermentation is an important operation since it can lead to changes in the phytochemical composition; it is known that during fermentation flavan-3-ols are oxidised, leading to significantly lower concentrations of this compound class in black tea. Due to its partial fermentation process, oolong tea usually has lower flavan-3-ols than green tea, but higher than black tea (Mattila & Kumpulainen 2002; Cabrera *et al*. 2006).

The great variability in the composition and concentration of phytochemicals present in tea is one of the main challenges to be faced by intervention and epidemiological studies. Correct interpretation of the physiological and pharmacological effects of tea requires detailed background information about its phytochemical composition, with special emphasis on potentially bioactive compounds. One possible solution is to create databases which include data amounts about flavonoids, alkaloids, and other bioactive compounds for different tea varieties and different brewing techniques for applying to dietary assessment studies.

But not only tea is a source of polyphenols. Coffee, an infusion of ground roasted coffee beans, is another of the most popular beverages in the world, appreciated for its characteristic taste and aroma. Recently, the interest in coffee has increased due to its potential beneficial effects on human health, which has been suggested to be related to a complex chemical mixture including phenolic acids and alkaloids (Dorea & Da Costa 2005). Maybe the most important compound in coffee for the population is caffeine, but it is not the unique one. There are several studies in which the most important components are, apart from caffeine, hydroxycinnamic acids, such as chlorogenic and caffeic acids (Fujioka & Shibamoto 2008; Duarte *et al*. 2010; Meinhart *et al.* 2010; Santini *et al*. 2011).

Apart from that, caffeine is also found in other products that have gained popularity in the last years, like soft and energy drinks (Barone & Roberts 1996; Daghbouche *et al*. 1997; Frary *et al*. 2005), and its amount is higher than that found in coffee and tea beverages.

From the consumer's point of view, information about the phytochemical composition may be used to differentiate quality products from conventional ones, and serve as geographic origin tracer (Fernández *et al*. 2002; Owuor & Obanda 2007). Furthermore, phytochemical content and profile influence organoleptic properties of infusion, and may affect consumer preferences (Drewnowski & Gomez-Carneros 2000; Obanda *et al*. 2001).

In this context, the objectives of this work were to study the distribution of phenolic compounds and alkaloids in tea, coffee, soft and energy drinks available in the Spanish market, and to identify distribution patterns that may provide a more precise differentiation among different types of tea.

MATERIAL AND METHODS

Chemicals and reagents. HPLC grade methanol and acetonitrile were obtained from VWR-International (Darmstadt, Germany) while phosphoric acid from Merck (Darmstadt, Germany). Ultra-pure water was supplied by a Milli-Q Advantage A10 water purifier system from Millipore (Bedford, USA). Standards of caffeine (Caf), theobromine (TheB), gallic acid (GalAc), protocatechuic acid (PrtAc), chlorogenic acid (ChlAc), caffeic acid (CafAc), *p*-coumaric acid (CouAc), (–)-gallocatechin gallate (GCG), (–)-epicatechin (EC), and (–)-catechin (CAT) were purchased from Sigma Chemical Co. (St. Louis, USA). (–)-Epigallocatechin (EGC), (–)-gallocatechin (GC), (–)-epigallocatechin gallate (EGCG), (–)-epicatechingallate (ECG), myricetin-3-*O*-rhamnoside (MyrR), quercetin-3-*O*-rutinoside (QueR), quercetin-3-*O*-glucopyranoside (QueG), kaempferol-3-*O*-rutinoside (KpfR), kaempferol-3-*O*-glucoside (KpfG), and luteolin-7-*O*-glucoside (LutG) standards were supplied by Extrasynthese (Genay Cedex, France). Their chemical structures are shown in Figure 1. Reference standards of all compounds were HPLC grade. Stock solutions were prepared in 80% aqueous methanol and stored at –20°C.

Samples. Seventeen samples of instant coffee, 18 of soft and energy drinks and 44 samples of commercial tea were purchased from local supermarkets, and stored at room temperature until submitted to sample preparation. Samples included different kinds of coffee (C): regular coffee 12 sam-

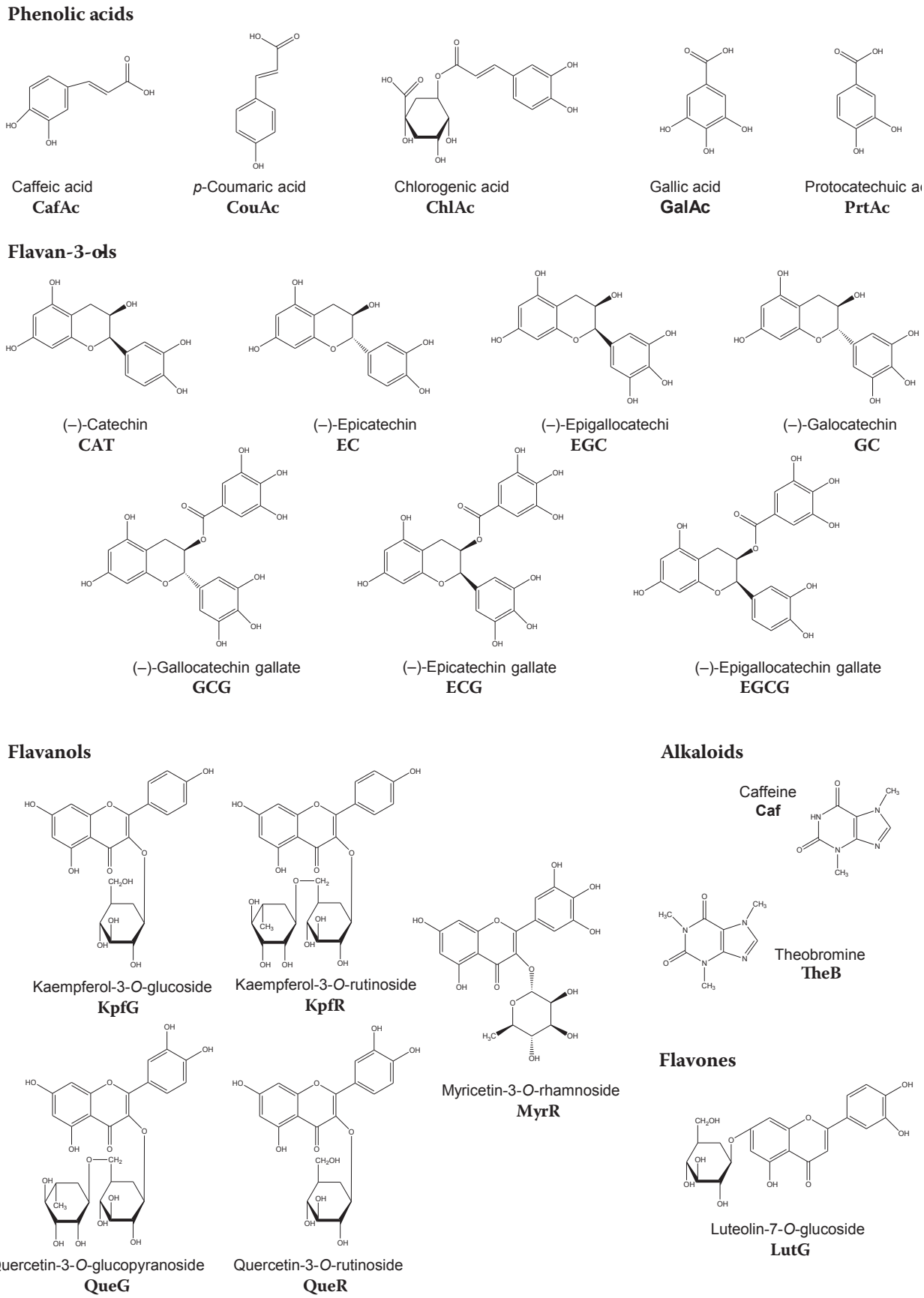

Figure 1. Chemical structure of analysed phenolic acids, flavan-3-ols, flavones, flavonols, and alkaloids

Table 1. Manufacturer information and identification codes of samples

Code	Sample	Brand	Manufacture's description
GT1	Green tea	1	Java green tea
GT2	Green tea	2	–
GT3	Green tea and mint	3	70% green tea; 30% mint
GT4	Green tea	4	98.2% green tea; 1.1% natural flavour; 0.4% orange blossom
GT5	Green tea and lemon peel	5	75% green tea; 11% lemon peel; lemongrass, lemon aroma
GT6	Green tea	6	–
GT7	Green tea	7	–
GT8	Green tea and mint	8	green tea and mint (% not specified)
GT9	Green tea	3	–
GT10	Green tea	9	–
GT11	Green tea	10	Grüner tea
GT12	Beauty Antioxidant	5	61% tea; green tea and vanilla aroma
WT1	White tea	8	–
WT2	Tea blend (White)	6	35% white tea; green tea; red tea and aroma
WT3	White tea antioxidant	5	93% tea; 0.5% orange blossom, jasmine aroma
WT4	Tea blend	2	35% white tea; 30% green tea; 30% red tea; 5% plum aroma
BT1	Black tea	2	black tea blend (Ceylon and Assam)
BT2	Black tea	11	black tea blend (Assam, African and Ceylon)
BT3	Black tea	7	–
BT4	Black tea	4	–
BT5	Black tea	12	black tea blend
BT6	Earl Grey tea	1	black tea and bergamot oil
BT7	Prince of Wales tea	1	black tea blend
BT8	Darjeeling tea	1	Darjeeling tea
BT9	English breakfast tea	1	black tea blend
BT10	Lady grey tea	1	black tea, flavoured bergamot oil, lemon, orange peel
BT11	English afternoon tea	13	black tea blend
BT12	Black tea breakfast	2	–
BT13	Earl grey tea	2	98% black tea; 2% bergamot orange
BT14	Lemon tea	2	–
BT15	Black tea and blackcurrant	1	tea; 4% black currant flavouring; 1% black currant pieces
BT16	Black tea and cherry	1	tea; 6% cherry flavouring; 1% cherry pieces
BT17	Black tea and lemon	1	tea; 12.5% lemon flavouring; 1% lemon pieces
BT18	Black tea and red fruits	1	tea; 8% flavouring; 1% cherry pieces; 1% redcurrant pieces; 1% raspberry pieces; 1% strawberry pieces
BT19	Black tea and mango, orange and passion fruit	1	tea; 8% orange flavouring; 6% mango flavouring; 3% passion fruit flavouring; 1% mango pieces; 1% passion fruit pieces; 1% orange pieces
BT20	Black tea	3	Ceylan tea
BT21	Black tea decaffeinated	2	–
RT1	Red tea	8	–
RT2	Red tea	2	–
RT3	Red tea and lemon	1	Pu-erh; fermented green tea; 10% lemon flavouring
RT4	Red tea	4	53.8% black tea; 35.3% rooibos; 6.4% hibiscus; 4.3% flavour
RT5	Red tea	7	–
RT6	Red tea antioxidant	5	84% tea; 14% anise; plum aroma
RT7	Red tea	6	Pu-erh
C1	Coffee	14	regular coffee
C2	Coffee	14	regular coffee
C3	Coffee	14	regular coffee; green blend
C4	Coffee	14	regular coffee; espresso; arabica
C5	Coffee	14	regular coffee; latin america

Table 1 to be continued

Code	Sample	Brand	Manufacture's description
C6	Coffee	14	regular coffee; colombia
C7	Coffee	15	regular coffee; colombia
C8	Coffee	15	regular coffee; natural
C9	Coffee	15	regular coffee; espresso
C10	Coffee	16	regular coffee
C11	Coffee	17	regular coffee
C12	Coffee	18	regular coffee; creme
C13	Coffee	14	decaffeinated coffee
C14	Coffee	15	decaffeinated coffee
C15	Coffee	19	decaffeinated coffee
C16	Coffee	16	decaffeinated coffee
C17	Coffee	17	decaffeinated coffee
SD1	Soft Drink	20	soft drink; cola; decaffeinated
SD2	Soft Drink	20	soft drink; cola light; decaffeinated
SD3	Soft Drink	20	soft drink; cola; sugar free
SD4	Soft Drink	20	soft drink; cola
SD5	Soft Drink	20	soft drink; cola light
SD6	Soft Drink	21	soft drink; cola; lemon
SD7	Soft Drink	21	soft drink; cola
SD8	Soft Drink	21	soft drink; cola light
SD9	Soft Drink	21	soft drink; cola light; lime
SD10	Soft Drink	21	soft drink; cola; sugar free
SD11	Soft Drink	22	soft drink; cola
EN1	Energy Drink	20	energy drink; with fruit
EN2	Energy Drink	20	energy drink
EN3	Energy Drink	23	energy drink
EN4	Energy Drink	24	energy drink
EN5	Energy Drink	24	energy drink; sugar free
EN6	Energy Drink	25	energy drink
EN7	Energy Drink	26	energy drink

ples and decaffeinated coffee 5; soft drinks (SD) 11; energy drinks (ED) 7; fermented tea (black tea (BT) 21 and red tea (RT) 7) and unfermented tea (green tea (GT) 12 and white tea (WT) 4). Details about their characteristics and identification codes are reported in Table 1.

Sample preparation. The protocol used for the extraction of target analytes from the solid samples was based on Rostagno *et al.* (2003, 2009). Briefly, the ultrasound-assisted extraction method used involved the three sequential extraction of 0.5 g of sample, with 15 ml of 50, 75, and 100% methanol, each of them at 60°C for 20 minutes. After each extraction step, the sample was centrifuged at 10°C for 10 min at 4000 rpm in a Universal 320R centrifuge (Andreas Hettich, Tuttlingen, Germany), the supernatant was collected and the solid was submitted to the following extraction step. Extractions were carried out on a multi-frequency ultrasonic bath (Transsonic TH-I-55; Elma Hans Schmidbauer, Singen, Germany) operating at 25 kHz at 100% intensity output. Supernatants were combined and brought up to 100 ml with water and an aliquot was filtered through 0.2 µm nylon syringe filter (VWR-International, Darmstadt, Germany) before the HPLC analysis.

Liquid samples (soft and energy drinks) were degassed by ultrasounds for 10 min, filtered through 0.2 µm nylon syringe filter and injected directly in the HPLC system.

High-performance liquid chromatography. The HPLC analyses were carried out on a Waters system (Waters Corp., Milford, USA) consisting of separation module (Waters 2695) with integrated column heater and auto-sampler, and a photodiode array detector (Waters 2998) coupled online with a multi-wavelength fluorescence detector (Waters 2475). Separation of compounds was achieved

using a fast method developed by Rostagno *et al*. (2011), which uses a fused-core type column (Kinetex™ C18, 2.6 µm, 100 Å, 100 × 4.6 mm; Phenomenex, Torrance, USA) operating at 55°C. Water with 1% phosphoric acid (A) and acetonitrile with 1% phosphoric acid (B) were used as mobile phases. Flow rate was 2.2 ml/min, and mobile phase composition was modified using the following gradient: 0 min – 5% B, 0.5 min – 10% B, 2.0 min – 12.5% B, 3.0 min – 15% B, 4.0 min – 80% B, 5.0 min – 100% B, 6.0 min – 100% B, 7.0 min – 5% B. Equilibration time between runs was 3 minutes. Injection volume was 10 µl. UV absorbance was monitored from 200 to 400 nm and fluorescence detection was carried out using 280 and 310 nm as excitation and emission wavelengths, respectively. Different wavelengths were used to retrieve peak areas (260, 270, 280, and 320 nm) in order to maximise the generated signal and to reduce detection and quantitation limits. The software for control and data acquisition was Empower 2 Version 6.10.01.00 (Waters Corp., Milford, USA). Identification of each compound was achieved by comparison of retention times and UV spectra of separated compounds as well as by co-elution with pure standards. Data on the concentration of all compounds presented a variability lower than 5%.

Table 2. Detection (LOD) and quantification (LOQ) limits for each analyte (in µg/l)

	LOD	LOQ		LOD	LOQ
Caf	4.2	14	QueG	14	47
TheB	4.2	14	LutG	24	78
GC	211	704	KpfR	28	92
EGC	109	365	KpfG	174	579
CAT	0.5	1.7	GalAc	102	339
EC	0.5	1.3	PrtAc	7.7	26
EGCG	23	76	ChlAc	13	44
GCG	8.7	29	CafAc	5.3	18
ECG	14	45	CouAc	1.9	6.3
MyrR	6.1	20	CAT Fluo	0.5	1.7
QueR	12	41	EC Fluo	0.5	1.5

Caf – caffeine; TheB – theobromine; GC – (–)-gallocatechin; EGC – (–)-epigallocatechin; CAT – (–)-catechin; EC – (–)-epicatechin; EGCG – (–)-epigallocatechin gallate; GCG – (–)-gallocatechin gallate; ECG – (–)-epicatechingallate; MyrR – myricetin-3-*O*-rhamnoside; QueR – quercetin-3-*O*-rutinoside; QueG – quercetin-3-*O*-glucopyranoside; LutG – luteolin-7-*O*-glucoside; KpfR – kaempferol-3-*O*-rutinoside; KpfG – kaempferol-3-*O*-glucoside; GalAc – gallic acid; PrtAc – protocatechuic acid; ChlAc – chlorogenic acid; CafAc – caffeic acid; CouAc – *p*-coumaric acid; CAT Fluo – catechin detected by fluorescence; EC Fluo – epicatechin detected by fluorescence

Data analysis. The 7-point calibration curves for each compound were prepared by plotting the concentration against the area. Detection and quantitation limits were determined by considering the value 3 or 10 times, respectively, the deviation of background noise obtained from blank samples (n = 10) dividing by the slope of the calibration curve; these values for each compound were shown in Rostagno *et al*. (2011) and have been included in Table 2. To study the distribution of analysed samples we used chemometric calculations, such as Principal Component Analysis (PCA), carried out with the Unscrambler 7.5 (Camo Asa, Oslo, Norway).

RESULTS AND DISCUSSION

Distribution patterns of beverages

Concentrations of individual phenolic acids, flavan-3-ols, flavonols, flavones and alkaloids found in the analysed samples are presented in Tables 3–5.

Tea composition is the most complex one and will be discussed in next sections. In the case of coffee, caffeine, caffeic acid, and chlorogenic acid are the main compounds (as shown in chromatograms of Figure 2) while in soft and energy drinks only caffeine has been taken into account.

In the majority of regular coffees, caffeine is the compound present at a higher concentration [between 23.9 (C12) and 56.3 mg/g (C1)]. Only in one regular coffee, which was a blend of roasted and green seeds, chlorogenic acid was the compound present at a higher concentration (42.7 mg/g, C9), which is in accordance with its profile of organic acids. In general, chlorogenic acid in coffees ranged between 3.70 (C14) and 42.7 mg/g (C9) and caffeic acid was the minor compound detected [0.21 (C14) and 2.42 mg/g (C3)].

In soft drinks, caffeine was the only compound detected, with concentrations ranging from 30.7 (SD6) to 123 mg/l (SD1) for regular soft drinks, and from 0.09 (SD11) to 0.18 (SD10) mg/l for decaffeinated soft drinks; higher concentrations were found in light soft drinks and energy drinks presented the highest caffeine contents (69.0–349 mg/l).

To notice the general distribution patterns of all beverages, PCA taking into account concentra-

Table 3. Concentration of analysed compounds in coffee (C), soft (SD) and energy drinks (EN) /in mg/g)

Samples	Caf	ChlAc	CafAc	Samples	Caf	Samples	Caf	ChlAc	CafAc	Samples	Caf
C1	56.3	14.8	1.06	SD1	123	C10	28.4	16.2	0.57	SD10	0.18
RSD	0.59	0.16	0.07	RSD	0.39	RSD	0.06	0.20	0.17	RSD	1.32
C2	50.9	16.9	1.30	SD2	92.3	C11	27.3	9.16	0.38	SD11	0.09
RSD	0.12	0.14	0.09	RSD	0.60	RSD	0.07	0.20	0.22	RSD	4.46
C3	48.8	19.8	2.42	SD3	98.4	C12	23.9	28.0	0.67	EN1	313
RSD	0.43	0.01	0.21	RSD	0.84	RSD	0.05	0.14	0.10	RSD	2.36
C4	47.5	20.9	1.08	SD4	30.8	C13	2.26	12.5	1.30	EN2	323
RSD	0.04	0.03	0.11	RSD	0.91	RSD	0.15	0.10	0.06	RSD	1.55
C5	42.4	9.71	1.15	SD5	107	C14	1.74	3.70	0.21	EN3	339
RSD	0.18	0.07	0.16	RSD	0.53	RSD	0.24	0.07	0.39	RSD	1.24
C6	40.9	13.6	1.29	SD6	30.7	C15	1.71	11.0	0.82	EN4	227
RSD	0.82	0.18	0.11	RSD	0.35	RSD	0.07	0.23	0.19	RSD	2.71
C7	35.7	19.3	1.18	SD7	111	C16	1.75	18.21	1.46	EN5	69.0
RSD	0.76	0.22	0.05	RSD	0.54	RSD	0.42	0.19	0.15	RSD	0.36
C8	35.4	12.1	0.91	SD8	122	C17	0.90	18.6	0.94	EN6	349
RSD	0.17	0.20	0.03	RSD	0.97	RSD	0.07	0.11	0.06	RSD	0.45
C9	32.6	42.7	0.89	SD9	108					EN7	330
RSD	0.12	0.39	0.01	RSD	0.83					RSD	1.18

RSD – % relative standard deviation; Caf – caffeine; ChlAc – chlorogenic acid; CafAc – caffeic acid

tions of caffeine, caffeic and chlorogenic acid has been carried out. Data presented in Figure 3 have been transformed in order to show the content of these compounds in mg/l present in a can of soft or energy drink and in a cup of instant coffee or tea. In the case of instant coffee and tea it has been considered that 2.5 g of the product are solved or extracted, respectively, with 200 ml of water (a yield of 75% has been used for tea extraction). By drawing the obtained results a clear separation between regular and decaffeinated coffee is noticed. Soft, energy drinks and teas are together in a big group characterised by the absence of chlorogenic and caffeic acids; this group contains a small one that includes all the energy drinks (except EN1), characterised by higher amounts of caffeine. In the figure loadings for each considered variable have been localised (Caf, CafAc, and ChlAc).

Distribution patterns of unfermented tea

In the case of tea, the total amounts of each class of compounds are shown in Figure 4 for unfermented and fermented tea, respectively.

Unfermented tea includes green (GT) and white (WT) tea. Concentrations of individual phenolic acids, flavan-3-ols, flavonols, flavones, and alkaloids found in the analysed samples are presented in Table 4 and total amounts of each class are shown in Figure 4A. The chromatogram of a green tea sample is shown in Figure 2.

As seen in Figure 4A, in most of the unfermented teas, flavan-3-ols were the compounds found at higher concentrations, followed by alkaloids. Phenolic acids were the compounds present at a lower concentration in all unfermented tea samples. Despite the fact that flavan-3-ols were compounds found at a higher concentration in green tea, a large variability between samples was observed, with levels ranging from 10.7 (GT3) to 53.4 mg/l (GT11). A similar composition pattern was identified for white tea, with flavan-3-ols being major compounds [from 11.7 (WT3) to 31.2 mg/g (WT2)]. It can also be noted that higher concentrations of flavan-3-ols were found in green tea without any additive or blend, which can be due to the lower concentration of this compound class in the additives used.

Additional information can be retrieved from the individual concentration of each compound present in the samples (Table 4). In this regard, EGC was the main flavan-3-ol found in most samples; the highest concentration of individual flavan-3-ols was found in sample WT2, which is a blend of tea (white, green, and red tea). The second major compound found in the samples was EGCG in most cases, although it was not possible to determine a clear trend for flavan-3-ols due

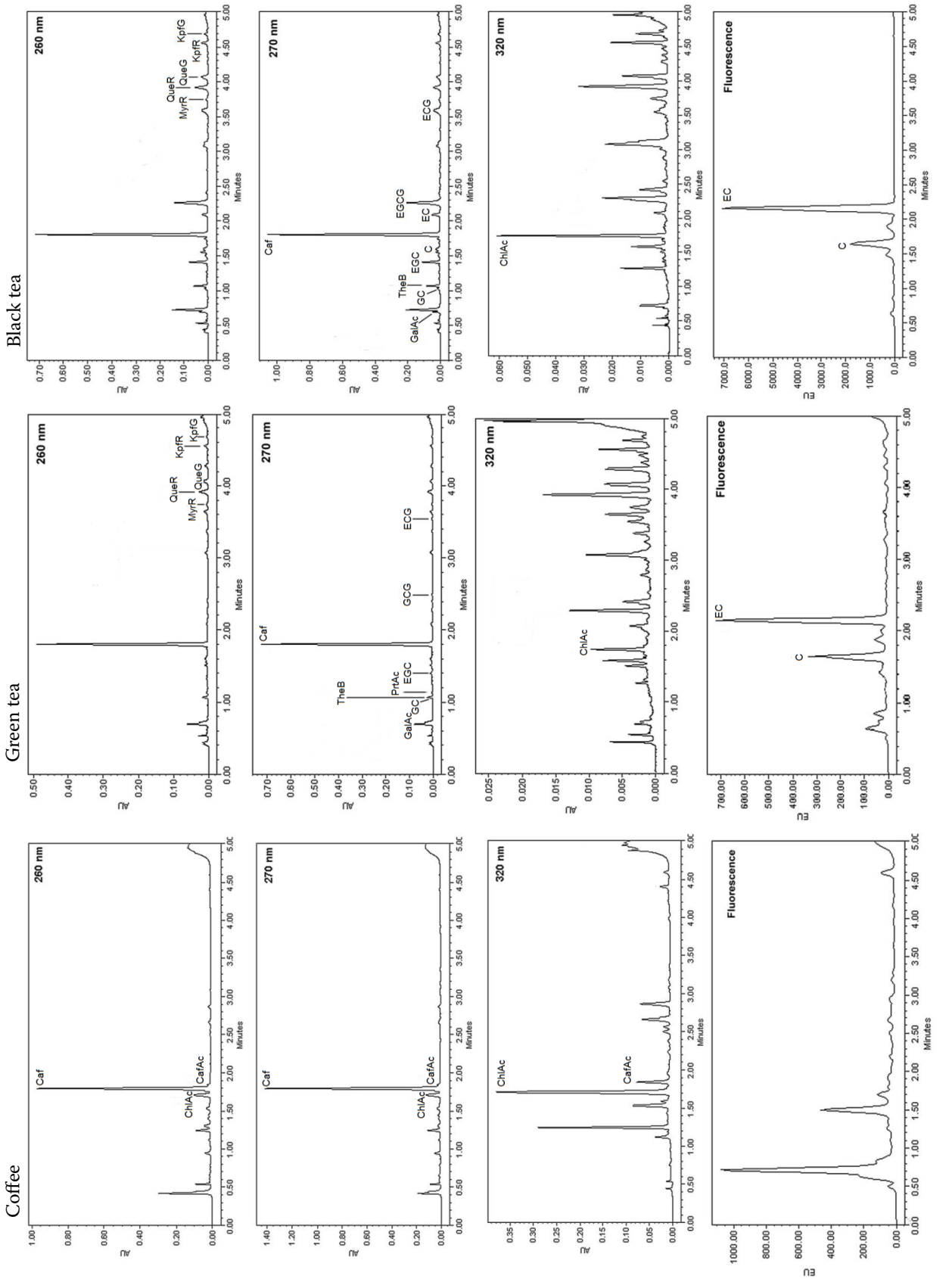

Figure 2. Chromatograms obtained in the analysis of a coffee, a green tea, and a black tea samples

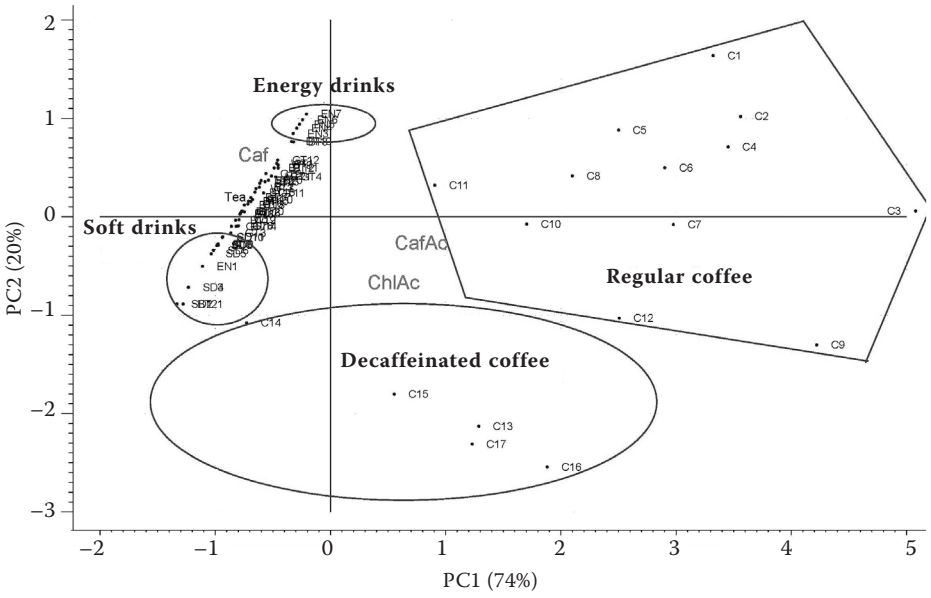

Figure 3. Principal component analysis results of concentration of chlorogenic acid (ChlAc), caffeic acid (CafAc), and caffeine (Caf) versus all analysed samples

to the great variability between tea types. Values for these compounds are in accordance with the concentrations reported in other studies; Khokhar and Magnusdottir (2002) analysed flavan-3-ols in 7 unfermented teas consumed in the UK, using water as extraction solvent and observed that EGC was the main compound in all teas, with levels ranging between 16.2 mg/g and 32.0 mg/g. Similar concentrations were found by Sultana *et al.* (2008) in green tea where high concentrations of EGCG and EGC were reported. EGCG levels ranged between 20.3 and 42.6 mg/g and EGC levels between 19.0 and 34.6 mg/g. Fernández *et al.* (2002) found lower concentrations of flavan-3-ols using aqueous acetonitrile (60%) as solvent; in all teas, EGCG was the main compound with concentrations ranging between 4.9 and 10.2 mg/g. In another study, the main flavan-3-ol occurring in 14 different tea samples was also EGC, where its concentration ranged from 8.0 mg/g to 83.3 mg/g, depending of the type of tea and cultivation site (Lin *et al.* 2003).

Altogether, differences in the reported levels can be attributed to variety, cultivation site, processing conditions and to the analytical methodology used for the determination of present phytochemicals; the concentration in the final extract was reported to be different depending on extraction conditions (Sultana *et al.* 2008). Since compounds have different polarity and because they are subjected to different interactions in the sample matrix, one solvent may be effective for the extraction of one class of compounds and need not be effective to others. This situation may be the case of highly polar phenolic acids and relatively apolar flavonoids. In this report, we used three sequential extractions with solvents of decreasing polarity in order to ensure quantitative recoveries of all target analytes.

Alkaloids, whose total concentration ranged between 11.8 (GT5) and 29.6 mg/g (GT12), were the second main type of compounds present in unfermented tea. The lower concentration found in sample GT5 was expected due to the lower content of green tea (< 61%) in this product. In all samples, caffeine was the predominant alkaloid, with concentrations ranging between 11.6 (GT5) and 28.4 mg/g (GT12), the values that are within the range (11.5–38.6 mg/g) reported by other researchers (Fernández *et al.* 2002; Khokhar & Magnusdottir 2002; Lin *et al.* 2003).

Another alkaloid detected in lower concentrations was theobromine; in green tea, its concentration ranged between 0.16 (GT5) and 2.37 mg/g (GT4), while lower levels were found in white tea [0.30 (WT3) to 0.76 mg/g (WT4)]. There are only a few reports available in the literature where theobromine was analysed (Fernández *et al.* 2002; Mizukami *et al.* 2007; Hu *et al.* 2009); reported levels were between 0.002 and 0.84 mg/g but, in general, we found higher concentrations. A plausible explanation for these differences is different sample preparation mentioned above: using three sequential extractions with different solvents can lead to a higher extraction efficiency and improve overall yields, when compared to single extraction procedures (Rostagno *et al.* 2009).

Flavonols and flavones were also detected at relatively high concentrations [ranging from 2.28 (WT1) to 6.51 mg/g (GT1)] in unfermented tea

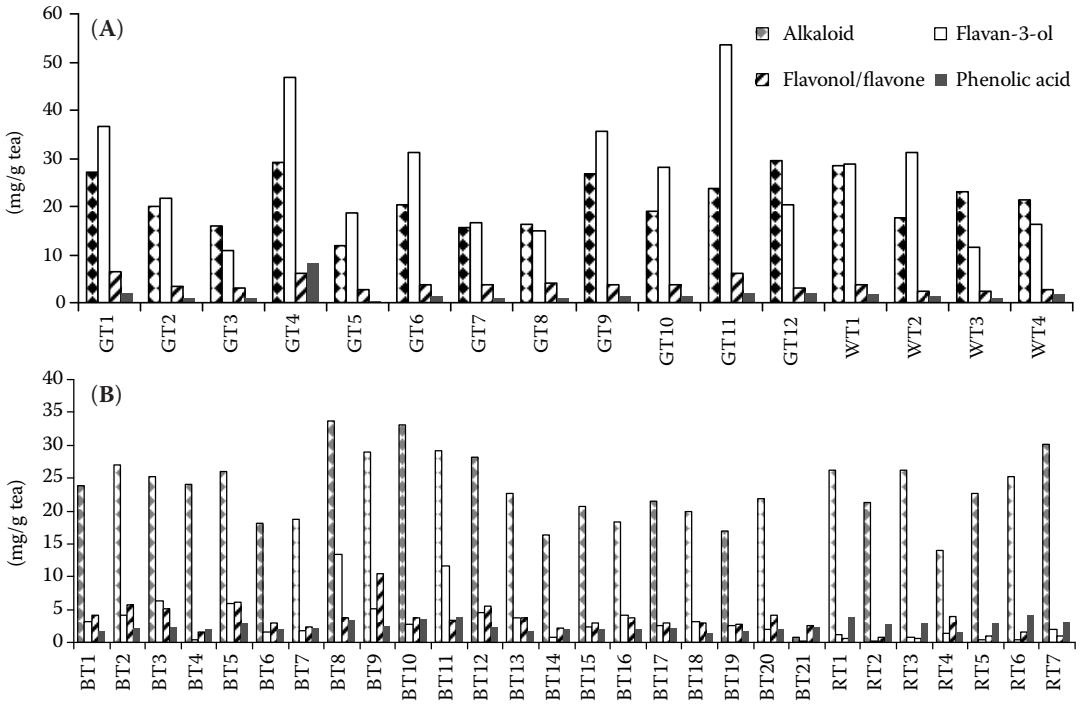

Figure 4. Distribution of compound classes in unfermented (**A**) and fermented (**B**) tea samples

samples. In general, they were higher in pure green tea than in tea blends. The major flavonol present in all samples was QueR, with concentrations between 1.15 (GT3) and 3.58 mg/g (GT1). Furthermore, quercetin derivatives were detected in higher concentrations than kaempferol and myricetin derivatives. Although there are a few reports about the concentration of flavonols in tea (Hertog *et al*. 1993; Perva-Uzunalic *et al*. 2006), reported levels of quercetin ranged between 1.40 and 2.30 mg/g, kaempferol between 0.91 and 1.50 mg/g, and myricetin from 0.52 to 1.20 mg/g, which supports our findings.

Among the analysed compounds, the lowest concentration [from 0.25 (GT5) to 8.19 mg/g (GT4)] was found for phenolic acids in green tea samples. The sample with the lowest amount of phenolic acids (GT5) also had a low content of green tea (75%). In contrast, the highest amounts of phenolic acids were observed in a sample with 98.2% of green tea and 0.4% of orange blossom. It is feasible to assume that besides a naturally higher concentration of the green tea used, the added component may have increased the relative concentration of phenolic acids since it is known that they are present in several citric species.

Among the analysed phenolic acids, GalAc was the major compound detected in samples [0.14 (GT5) to 5.65 mg/g (GT4)] followed by ChlAc [0.00 (GT9) to 2.54 mg/g (GT4)], while the rest of phenolic acids were in concentrations below 0.3 mg/g. In part, due to their high concentration among phenolic acids, GalAc, and ChlAc were the only compounds analysed in other studies (Fernández *et al*. 2002; Zuo *et al*. 2002; Nishitani & Sagesaka 2004). Reported concentrations of GalAc ranged between 0.02 mg/g and 3.62 mg/g while ChlAc concentrations ranged between 0.01 mg/g and 0.03 mg/g. The concentration of most samples was within reported ranges but relatively higher amounts of both acids were detected, which may be due to the sample preparation procedure used.

Distribution patterns of fermented tea

Phytochemical distribution patterns of two types of fermented tea, black tea (BT) and red tea (RT), were determined in this study. Total concentration of the different compound classes evaluated is presented in Figure 4B and individual concentration of each compound is given in Table 5; the chromatogram of a black tea sample is shown in Figure 2.

Alkaloids were the compounds found in the highest concentration in all fermented teas. It was not possible to identify a clear distribution pattern of flavonols/flavones and flavan-3-ols since a large variability was observed between

Table 4. Concentration of analysed compounds in unfermented tea

Samples	Caf	TheB	GC	EGC	CAT	EC	EGCG	GCG	ECG	MyrR	QueR	QueG	LutG	KpfR	KpfG	GalAc	PrtAc	ChlAc	CafAc	CouAc
GT1	25.9	1.27	5.86	13.1	6.28	2.50	6.11	nd	2.85	0.30	3.58	1.16	nd	0.88	0.59	0.82	nd	1.36	nd	nd
RSD	1.23	1.17	1.14	1.43	1.28	1.06	2.05	nd	2.23	3.57	1.09	2.09	nd	3.10	2.44	1.05	nd	1.02	nd	nd
GT2	18.4	1.58	2.16	7.92	0.54	4.62	3.77	nd	2.63	0.11	1.92	0.62	0.02	0.56	0.29	0.62	nd	0.51	nd	nd
RSD	2.11	1.33	2.40	2.36	1.04	1.22	2.41	nd	2.24	3.30	1.13	3.61	3.08	3.53	2.48	1.20	nd	1.54	nd	nd
GT3	14.3	1.61	1.89	5.43	0.40	2.82	0.13	0.02	nd	0.10	1.15	0.77	0.59	0.36	0.13	0.49	0.04	0.30	0.02	nd
RSD	2.09	2.22	1.17	1.37	2.05	2.02	3.18	3.73	nd	3.14	1.90	3.46	3.35	3.47	3.41	2.92	2.67	2.96	3.15	nd
GT4	26.8	2.37	5.71	15.70	1.78	7.04	11.16	nd	5.53	0.27	3.20	1.09	nd	0.76	0.76	5.65	nd	2.54	nd	nd
RSD	1.06	1.08	1.14	1.12	2.03	1.06	2.14	nd	1.12	3.87	1.61	2.16	nd	3.50	3.51	1.15	nd	2.08	nd	nd
GT5	11.6	0.16	1.98	10.08	0.36	4.49	1.13	nd	0.71	0.13	2.00	0.24	0.05	0.13	0.08	0.14	nd	0.14	nd	nd
RSD	2.04	1.35	2.37	1.22	2.18	1.23	1.17	nd	3.55	3.29	1.22	2.57	3.93	3.21	3.09	1.17	nd	2.14	nd	nd
GT6	19.8	0.57	0.25	6.91	nd	nd	4.40	nd	19.6	0.11	2.07	0.75	nd	0.52	0.26	1.00	nd	0.32	nd	nd
RSD	2.05	2.40	2.27	2.16	nd	nd	2.37	nd	2.38	3.13	1.25	2.18	nd	3.26	3.27	2.20	nd	1.44	nd	nd
GT7	15.4	0.40	1.25	5.42	0.29	2.98	3.58	0.07	3.12	nd	2.03	0.75	nd	0.51	0.36	0.97	0.02	0.18	nd	nd
RSD	2.14	1.48	2.41	2.05	1.10	1.08	1.11	3.63	1.41	nd	1.14	3.41	nd	3.53	3.80	2.16	2.68	2.57	nd	nd
GT8	15.8	0.36	1.38	8.11	0.29	3.16	nd	nd	2.10	nd	2.03	1.24	0.47	0.14	0.05	0.50	0.18	0.15	0.05	0.21
RSD	1.21	2.76	2.33	1.18	1.12	1.02	nd	nd	1.09	nd	1.08	2.28	3.55	3.16	3.25	2.08	2.28	1.33	3.21	0.04
GT9	25.6	1.12	3.70	10.4	0.77	5.27	8.69	0.49	6.33	nd	2.14	0.75	nd	0.60	0.32	1.21	nd	nd	nd	nd
RSD	2.10	1.07	1.11	2.08	1.14	1.21	1.16	3.74	1.48	nd	1.22	2.68	nd	3.58	3.52	1.14	nd	nd	nd	nd
GT10	18.4	0.47	2.21	7.67	0.44	4.50	7.32	0.34	5.74	nd	2.09	0.76	nd	0.51	0.28	1.08	nd	0.29	nd	nd
RSD	1.19	1.41	1.14	1.28	1.13	1.69	1.09	3.85	1.11	nd	1.32	3.32	nd	3.61	3.85	2.25	nd	1.36	nd	nd
GT11	22.7	1.16	5.06	12.6	1.59	6.80	14.8	0.34	12.2	0.28	3.28	1.20	nd	0.65	0.58	0.67	0.09	1.23	nd	nd
RSD	1.13	2.20	1.21	1.12	2.86	1.11	0.18	0.54	1.52	0.83	1.88	2.24	nd	3.54	3.14	3.45	4.17	2.11	nd	nd
GT12	28.4	1.18	1.22	4.80	0.38	2.36	6.48	nd	4.94	0.19	1.86	0.45	nd	0.22	0.24	1.78	0.04	0.30	nd	Tr
RSD	1.09	2.17	2.66	1.30	3.04	2.03	0.36	nd	1.08	0.05	2.38	3.77	nd	3.66	3.09	2.14	4.64	3.77	nd	nd
WT1	27.9	0.70	2.22	8.81	5.67	1.99	5.81	nd	4.28	0.16	2.54	0.54	nd	0.30	0.26	1.08	nd	0.45	nd	nd
RSD	1.46	3.05	1.47	1.10	1.06	2.07	1.04	nd	1.09	4.24	2.21	3.52	nd	4.04	4.46	2.29	nd	3.49	nd	nd
WT2	16.9	0.61	2.12	24.9	0.59	3.30	0.20	0.02	nd	0.14	1.24	0.44	0.01	0.29	0.16	0.92	nd	0.52	nd	nd
RSD	1.09	3.34	1.27	1.76	2.30	2.15	3.13	4.18	nd	3.95	2.22	3.54	4.27	4.11	4.11	2.30	nd	3.38	nd	nd
WT3	22.7	0.30	0.76	5.78	0.15	2.36	1.33	nd	1.27	0.14	1.61	0.43	nd	0.09	0.08	0.99	0.02	0.04	nd	nd
RSD	1.08	3.36	3.09	1.26	3.09	1.02	2.18	nd	2.35	3.19	2.25	3.10	nd	4.50	4.30	0.39	4.91	3.28	nd	nd
WT4	20.4	0.76	2.33	6.75	2.59	0.89	2.09	nd	1.70	0.15	1.44	0.51	nd	0.31	0.19	1.11	0.02	0.45	Tr	nd
RSD	1.10	3.18	1.41	1.29	1.91	3.03	0.13	nd	2.23	0.19	2.35	3.25	nd	3.17	4.25	2.23	4.76	3.72	nd	nd

RSD – % relative standard deviation; nd – not detected (< limit of detection); Tr – traces (< limit of quantitation)

samples. The lowest total alkaloid concentration of black tea samples was 16.3 mg/g (BT14 – the use of additives could reduce the actual amount of tea in the product), while the highest reached a double value [33.7 mg/g (BT8)]. It can also be observed in Figure 4B that tea blends and flavoured tea presented the highest concentration of alkaloids, while the addition of fruits produces tea with lower concentrations of this compound class. The decaffeinated tea sample (BT21) had a very low concentration of alkaloids (0.71 mg/g) in accordance with the manufacturer's information. Total concentration of alkaloids in red tea samples was similar to values observed in black tea, ranging from 14.0 (RT4) to 30.0 mg/g (RT7).

Caffeine was the main alkaloid in all fermented teas. In black tea, its concentration ranged between 15.7 (BT14) and 32.3 mg/g (BT8) and slightly lower concentrations were observed for tea with added fruits and flavours; similar results were reported by Khokhar & Magnusdottir (2002). Astill *et al*. (2001) observed that the caffeine content of black tea was influenced by variety, growing environment and manufacturing conditions. Caffeine concentrations in red tea samples were comparable to those observed for black tea, which is in accordance with other reports (Lin *et al*. 2003). The other analysed alkaloid was theobromine [0.21 (BT21) to 1.45 mg/g (BT8)], which was found in much lower concentrations than caffeine in all fermented samples, in concentrations consistent with reported values.

Total flavan-3-ols in black tea were found in concentrations ranging from 0.43 mg/g (BT4) to 13.5 mg/g (BT8). In the case of the decaffeinated tea sample (BT21), total flavan-3-ols (0.18 mg/g) were much lower than in the rest of black teas. Possible explanations for this observation are losses and/or degradation of flavan-3-ols caused by the decaffeination process. It was also observed that in red tea [from 0.28 (RT2) to 1.36 mg/g (RT4)] flavan-3-ol values were lower than most values found in black tea samples. In this case, not only processing conditions may be responsible for the lower concentration of this compound class, but also it may be mainly caused by the different raw material used. It is noteworthy however that flavan-3-ols are very sensitive to degradation provoked by oxidation reactions. Among flavan-3-ols, EC was the major compound of the class in most fermented tea. In black tea, its levels ranged from 0.11 (BT21) to 2.79 mg/g (BT11), while in red tea they ranged from 0.11 (RT2) to 0.80 mg/g (RT4). Other major flavan-3-ols in some black teas were EGC (< 1.69 mg/g) and EGCG (< 3.74 mg/g). The minor flavan-3-ol present was GCG, which was not detected in most samples. Although there was a great variability in flavan-3-ol levels of fermented tea, this fact was reported previously (Fernández *et al*. 2002; Wang *et al*. 2010); the lower concentration of flavan-3-ols in samples with fruits added was also revealed when compared to pure samples (Khokhar & Magnusdottir 2002).

Other compound classes analysed are flavonols and flavones. Levels in black tea ranged from 1.64 (BT4) to 10.5 mg/g (BT9). In red tea, concentration values were lower: from 0.50 (RT1) to 1.49 mg/g (RT6). Several factors may influence the concentration of these compound classes in red tea, but it is most likely that it is determined by the raw material used. Among the detected flavonols and flavones, QueR was the main compound in all black tea samples (1.04–3.18 mg/g). Again, quercetin derivatives were found in higher levels than kaempferol and myricetin derivatives. The total phenolic acid concentration of black tea was not significantly different from red tea; concentration values ranged from 1.43 (BT18) to 3.96 mg/g (BT11) for black tea, while for red tea they ranged between 1.58 (RT4) and 4.13 mg/g (RT6); the main phenolic acid in fermented tea was GalAc and relatively high amounts of ChlAc were found. CafAc was not detected in all samples and CouAc was not detected at all or the concentration in the sample was below quantitation limits (but above detection limits); in the case of PrtAc, small amounts were observed in most samples. There are only a few studies where phenolic acids were analysed in black tea samples and reported levels of gallic and chlorogenic acids are consistent with our results (Fernández *et al*. 2002; Cabrera *et al*. 2003).

Differentiation of unfermented and fermented tea

Distribution patterns can be used to differentiate teas, not only by raw materials and processing conditions, but also by their phytochemical profile. Taking this into account, pattern recognition procedures were applied to different groups of data sets with the objective of classifying samples according to the tea type. PCA was used to process data on the phytochemical distribution of all types of tea; firstly PCA was formed by the

matrix of all tea samples (except decaffeinated ones) and analysed compounds: 43 samples and 20 variables. The graph of PC1 vs. PC2 is shown in Figure 5A, where it is possible to identify three different groups for red, black and unfermented tea. This PCA was capable of explaining about 50% of the observed variance. Considering the great differences in sample types (green, white, black, and red tea) and tea content (a wide variety of additives have been used), these results are exceptionally good. Furthermore, these results also imply that it is possible to differentiate most of the unfermented teas (negative PC2 scores) from fermented teas (positive PC2 scores). Unfermented tea samples that were wrongly classified as fermented ones (GT4 and GT12) represented less than 5% of the total number of samples. Unfermented and fermented teas have similar amounts of alkaloids, although some unfermented teas showed a slightly lower concentration of caffeine than fermented tea. In both types of samples, flavonol and flavone levels were similar; QueR was the major compound, and the concentration of quercetin derivatives was higher than that of kaempferol and myricetin derivatives.

The comparison of distribution patterns indicates that the main difference between unfermented and fermented tea is the content of flavan-3-ols and phenolic acids. Unfermented tea has high concentrations of flavan-3-ols, while these are present only in low amounts in fermented tea. Illustratively, in unfermented tea, total flavan-3-ol content ranged from 10.7 mg/g to 53.4 mg/g, while for fermented tea it ranged from 0.28 mg/g to 13.5 mg/g. Besides differences in concentrations, the phytochemical fingerprint of both types of tea is quite different. While in unfermented tea the main flavan-3-ol was EGC (5.42–13.1 mg/g), in fermented tea the main flavan-3-ol was EC (0.11–1.63 mg/g) and EGC was found in concentrations lower than 1.69 mg/g. Another relevant flavan-3-ol present in both kinds of samples was EGCG, which showed a much lower concentration in fermented tea. These large concentration differences were expected since flavan-3-ols are oxidised or condensed to other large polyphenolic molecules such as theaflavins and thearubins during the fermentation process (Gardner *et al*. 2007).

An inverse trend was observed in the phenolic acid pattern distribution: these compounds were found in higher concentrations in unfermented tea (0.25–8.19 mg/g) than in fermented tea (1.43–3.96 mg/g), although there were some exceptions. The higher level of GalAc (unfermented: 0.14–1.78 mg/g; fermented: 1.15–3.01 mg/g) is the main difference observed between both types of samples, and may be linked to its release from flavan-3-ol gallates during the fermentation process, increasing the concentration of this compound in the final product. This implies that flavan-3-ols and GalAc are potential markers for differentiation of unfermented and fermented tea. In fact, such patterns are the key to identify the phytochemical fingerprint of samples allowing a more accurate differentiation among tea types.

However, it is important to consider that some samples were blends of fermented and unfermented teas. With relatively high amounts of unfermented tea, these samples had sufficient levels of key components to produce a characteristic fingerprint of unfermented tea, and were correctly identified as unfermented (negative PC2 scores). Also, the great variability in PCA scores can be partially attributed to different types of samples and additives used, because some samples were also rich in polyphenol fruits and, depending on the amount added, they can influence the phytochemical fingerprint.

In order to provide a better insight into the distribution patterns of green, white, black and red tea, the variability caused by such samples needs to be eliminated. To this end, a second PCA (Figure 5B) was performed using only pure samples (according to the manufacturer) and all 20 analysed compounds, which was capable of explaining 57% of the observed variance. As can be seen, unfermented teas (green and white) form a group of samples with negative PC1 and PC2 scores, while black teas form another group. It is also possible to group red tea, which has positive PC1 scores and negative PC2 scores. However, differentiation between green and white tea was not possible.

Most samples were correctly identified using this PCA, with the exception of samples GT1, BT4 and BT7. In the case of differentiation of unfermented and fermented samples, the only sample wrongly classified was GT1. Clearly, differentiation between tea types can be improved by using more homogeneous groups of samples.

As mentioned in the previous sections, there was also a large variability in the concentration of several minor compounds and this variability affects the differentiation ability between tea types. Seeking to improve separation by eliminating this variability, only the main compounds of each class (Caf, EC, EGC, EGCG, QueR, and GalAc)

Table 5. Concentration of analysed compounds in fermented tea

Samples	Caf	TheB	GC	EGC	CAT	EC	EGCG	GCG	ECG	MyrR	QueR	QueG	LutG	KpfR	KpfG	GalAc	PrtAc	ChlAc	CafAc	CouAc
BT1	23.1	0.82	0.29	0.59	0.50	0.39	0.45	nd	0.89	0.95	1.77	0.68	nd	0.50	0.32	1.42	0.06	0.31	nd	Tr
RSD	1.12	2.22	3.04	3.34	3.58	2.12	3.14	nd	3.33	3.21	1.27	3.21	nd	3.23	3.16	1.17	3.20	3.47	nd	nd
BT2	25.7	1.38	0.34	0.60	0.37	1.02	0.47	nd	1.28	0.18	2.55	1.43	nd	0.75	0.87	1.34	nd	0.80	nd	nd
RSD	1.11	1.07	3.35	3.32	3.05	2.05	3.61	nd	2.29	3.20	1.15	2.28	nd	3.02	3.64	1.23	nd	3.21	nd	nd
BT3	24.4	0.91	0.42	0.84	0.35	1.20	1.48	nd	2.00	0.23	3.13	0.56	nd	0.63	0.48	1.82	0.04	0.52	nd	Tr
RSD	1.25	2.13	3.07	3.22	3.12	1.25	1.86	nd	1.23	3.14	1.64	3.05	nd	2.36	3.21	1.26	3.18	3.07	nd	nd
BT4	23.7	0.35	0.04	0.16	0.04	0.15	0.04	nd	nd	0.03	1.04	0.08	0.08	0.28	0.14	1.90	0.07	0.05	nd	Tr
RSD	1.12	3.60	3.02	3.72	3.12	3.03	2.81	nd	nd	3.54	1.07	3.45	3.19	3.78	4.25	1.07	3.17	3.25	nd	nd
BT5	24.8	1.24	0.46	1.50	0.65	1.63	0.58	nd	1.05	0.26	3.18	1.21	nd	0.72	0.65	2.12	nd	0.81	nd	nd
RSD	1.09	2.09	3.13	1.97	2.06	2.03	2.46	nd	2.38	3.75	1.36	2.25	nd	4.30	4.56	1.15	nd	2.42	nd	nd
BT6	17.7	0.43	0.13	0.22	0.25	0.90	nd	0.03	nd	0.13	1.77	0.44	nd	0.33	0.21	1.60	0.08	0.21	nd	Tr
RSD	1.04	3.19	3.17	3.13	3.14	2.08	nd	4.34	nd	3.09	1.32	3.57	nd	3.59	3.13	1.16	4.13	3.12	nd	nd
BT7	18.3	0.37	0.13	0.53	0.76	0.39	nd	nd	nd	0.11	1.39	0.20	nd	0.22	0.54	1.81	0.10	0.16	nd	Tr
RSD	1.53	3.54	3.52	3.78	3.84	2.31	nd	nd	nd	3.18	1.14	3.40	nd	3.43	3.25	1.10	4.80	3.60	nd	nd
BT8	32.3	1.45	1.33	2.05	0.71	2.68	3.74	nd	2.98	0.12	2.12	0.54	nd	0.52	0.43	2.74	nd	0.69	nd	nd
RSD	1.21	2.05	2.40	2.86	3.32	3.19	1.11	nd	1.17	3.14	1.21	2.40	nd	3.28	3.76	1.15	nd	2.13	nd	nd
BT9	27.7	1.28	0.28	nd	0.85	1.37	0.58	nd	1.96	0.25	2.61	6.31	nd	0.65	0.64	1.87	nd	0.74	nd	Tr
RSD	1.25	2.09	3.31	nd	2.19	2.08	3.22	nd	1.19	3.46	1.17	1.16	nd	3.66	4.78	2.13	nd	2.29	nd	nd
BT10	32.2	0.86	0.15	0.63	0.46	1.40	0.15	nd	nd	0.32	1.70	0.75	nd	0.66	0.39	2.98	0.17	0.43	nd	Tr
RSD	1.37	3.22	3.31	3.19	3.32	3.56	3.54	nd	nd	2.95	2.83	3.08	nd	3.37	3.11	1.06	4.68	3.66	nd	nd
BT11	27.4	1.80	0.85	1.22	0.97	2.79	2.91	nd	2.88	0.16	1.92	0.52	nd	0.43	0.25	3.01	nd	0.95	nd	Tr
RSD	1.05	2.35	3.11	2.56	2.03	2.23	2.95	nd	1.22	2.21	2.09	3.59	nd	3.02	4.22	1.10	nd	2.18	nd	nd
BT12	27.0	1.15	0.36	0.43	0.46	0.69	0.70	nd	1.87	0.23	2.68	1.22	nd	0.70	0.62	1.88	0.02	0.49	nd	nd
RSD	1.49	3.16	3.22	3.47	3.54	3.08	3.04	nd	2.12	3.82	1.13	2.17	nd	3.14	4.38	2.15	4.72	3.14	nd	nd
BT13	22.1	0.68	0.19	0.71	0.19	0.58	0.70	nd	1.39	0.21	2.10	0.68	nd	0.48	0.32	1.59	0.05	0.20	nd	nd
RSD	1.10	3.28	3.24	3.19	3.13	4.04	3.36	nd	2.11	3.70	1.19	3.15	nd	3.54	3.98	2.19	4.57	4.20	nd	nd
BT14	15.7	0.61	0.04	0.20	0.23	0.41	nd	nd	nd	0.16	1.26	0.30	0.02	0.34	0.13	1.15	0.06	0.70	nd	Tr
RSD	1.02	3.08	3.17	4.12	3.18	3.09	nd	nd	nd	0.24	2.35	3.03	0.31	3.53	4.36	2.28	4.17	3.08	nd	nd
BT15	20.3	0.34	0.10	nd	0.33	1.06	0.22	nd	0.71	0.16	1.88	0.45	nd	0.26	0.20	1.75	0.08	0.18	nd	Tr
RSD	1.44	3.38	3.58	nd	3.13	2.05	0.60	nd	3.71	0.22	2.28	3.13	nd	3.61	3.29	2.18	4.13	4.51	nd	nd

Table 5 to be continued

Samples	Caf	TheB	GC	EGC	CAT	EC	EGCG	GCG	ECG	MyrR	QueR	QueG	LutG	KpfR	KpfG	GalAc	PrtAc	ChlAc	CafAc	CouAc
BT16	17.9	0.42	0.15	1.69	0.32	1.06	0.26	nd	0.70	0.16	1.94	1.02	nd	0.32	0.28	1.70	0.08	0.18	nd	Tr
RSD	1.11	3.28	3.03	2.32	3.44	2.13	0.45	nd	3.62	0.20	2.20	2.41	nd	3.11	3.90	2.16	4.10	4.61	nd	nd
BT17	21.0	0.46	0.14	0.53	0.27	1.06	0.13	nd	0.35	0.18	1.80	0.44	nd	0.27	0.18	1.92	0.09	0.14	nd	Tr
RSD	1.06	3.37	3.45	3.19	3.06	2.16	0.40	nd	3.51	0.14	2.44	3.54	nd	3.94	3.07	2.15	4.22	4.15	nd	nd
BT18	19.4	0.44	0.14	0.95	0.27	0.91	0.26	nd	0.69	0.15	1.72	0.54	nd	0.28	0.20	1.36	0.07	nd	nd	Tr
RSD	1.24	3.30	3.09	2.21	3.07	3.43	0.49	nd	3.79	0.18	3.11	3.60	nd	3.17	3.20	2.19	4.32	nd	nd	nd
BT19	16.5	0.36	0.12	0.28	0.31	1.09	0.20	nd	0.53	0.16	1.73	0.48	nd	0.26	0.20	1.52	0.07	0.17	nd	Tr
RSD	1.13	3.35	3.26	3.48	3.16	2.25	0.35	nd	3.42	0.06	2.17	2.54	nd	3.12	3.15	2.07	4.29	4.35	nd	nd
BT20	20.8	0.98	0.20	0.53	0.37	0.55	0.27	nd	nd	0.21	2.09	0.85	nd	0.56	0.48	1.51	0.04	0.51	nd	Tr
RSD	1.20	2.26	3.14	3.21	3.14	3.11	0.52	nd	nd	1.69	1.34	2.29	nd	3.59	3.51	2.20	4.52	3.31	nd	nd
BT21	0.50	0.21	nd	nd	0.07	0.11	nd	nd	nd	0.16	1.73	0.35	nd	0.25	0.03	1.55	0.03	0.69	Tr	nd
RSD	3.52	3.11	nd	nd	4.17	4.02	nd	nd	nd	4.20	2.32	3.07	nd	3.11	4.26	2.24	3.77	3.26	nd	nd
RT1	25.0	1.25	0.17	0.16	0.20	0.61	nd	nd	nd	0.08	0.27	0.08	nd	0.04	0.03	3.59	0.11	0.16	nd	Tr
RSD	1.09	2.08	2.23	3.12	3.21	3.18	nd	nd	nd	3.37	3.24	3.42	nd	2.88	3.79	1.49	3.24	3.15	nd	nd
RT2	20.3	0.92	nd	nd	0.17	0.11	nd	nd	nd	0.11	0.34	0.12	nd	0.10	0.05	2.62	0.11	0.10	nd	Tr
RSD	1.06	3.16	nd	nd	3.01	3.04	nd	nd	nd	3.18	2.79	4.39	nd	2.26	3.28	1.13	3.19	3.26	nd	nd
RT3	25.1	1.13	0.12	0.13	0.12	0.48	nd	nd	nd	0.08	0.32	0.09	nd	0.05	nd	2.87	0.10	0.04	nd	Tr
RSD	1.10	2.22	3.00	3.32	3.23	3.17	nd	nd	nd	4.42	2.38	4.48	nd	3.21	nd	1.12	3.14	3.21	nd	nd
RT4	13.4	0.59	0.20	nd	0.36	0.80	nd	nd	nd	0.38	1.88	0.73	0.08	0.47	0.41	1.01	0.02	0.55	nd	Tr
RSD	1.15	3.27	3.33	nd	3.03	3.12	nd	nd	nd	3.15	2.04	3.18	3.30	2.72	2.55	2.27	3.72	3.15	nd	nd
RT5	21.9	0.72	0.07	nd	0.05	0.20	nd	nd	nd	0.13	0.68	0.07	nd	0.08	0.05	2.85	0.11	0.02	nd	Tr
RSD	1.01	3.16	3.98	nd	3.04	3.02	nd	nd	nd	3.20	2.66	3.41	nd	3.39	2.88	1.10	3.19	3.55	nd	nd
RT6	24.0	1.13	nd	nd	0.09	0.27	nd	nd	nd	0.27	0.98	0.11	nd	0.09	0.04	3.41	0.09	0.63	nd	Tr
RSD	1.09	2.21	nd	nd	3.60	3.14	nd	nd	nd	3.14	2.17	3.05	nd	2.36	2.90	1.99	3.11	3.45	nd	nd
RT7	28.8	1.22	0.10	nd	0.10	0.26	0.01	nd	0.69	nd	0.70	0.14	nd	0.08	0.07	3.02	0.11	0.02	nd	Tr
RSD	1.07	2.17	3.57	nd	3.10	3.13	3.99	nd	3.65	nd	3.82	3.16	nd	3.30	3.52	1.93	3.12	3.93	nd	nd

Caf – caffeine; TheB – theobromine; GC – (–)-gallocatechin; EGC – (–)-epigallocatechin; CAT – (–)-catechin; EC – (–)-epicatechin; EGCG – (–)-epigallocatechin gallate; GCG – (–)-gallocatechin gallate; ECG – (–)-epicatechingallate; MyrR – myricetin-3-*O*-rhamnoside; QueR – quercetin-3-*O*-rutinoside; QueG – quercetin-3-*O*-glucopyranoside; LutG – luteolin-7-*O*-glucoside; KpfR – kaempferol-3-*O*-rutinoside; KpfG – kaempferol-3-*O*-glucoside; GalAc – gallic acid; PrtAc – protocatechuic acid; ChlAc – chlorogenic acid; CafAc – caffeic acid; CouAc – *p*-coumaric acid; RSD – % relative standard deviation; nd – not detected (< limit of detection); Tr – traces (< limit of quantitation)

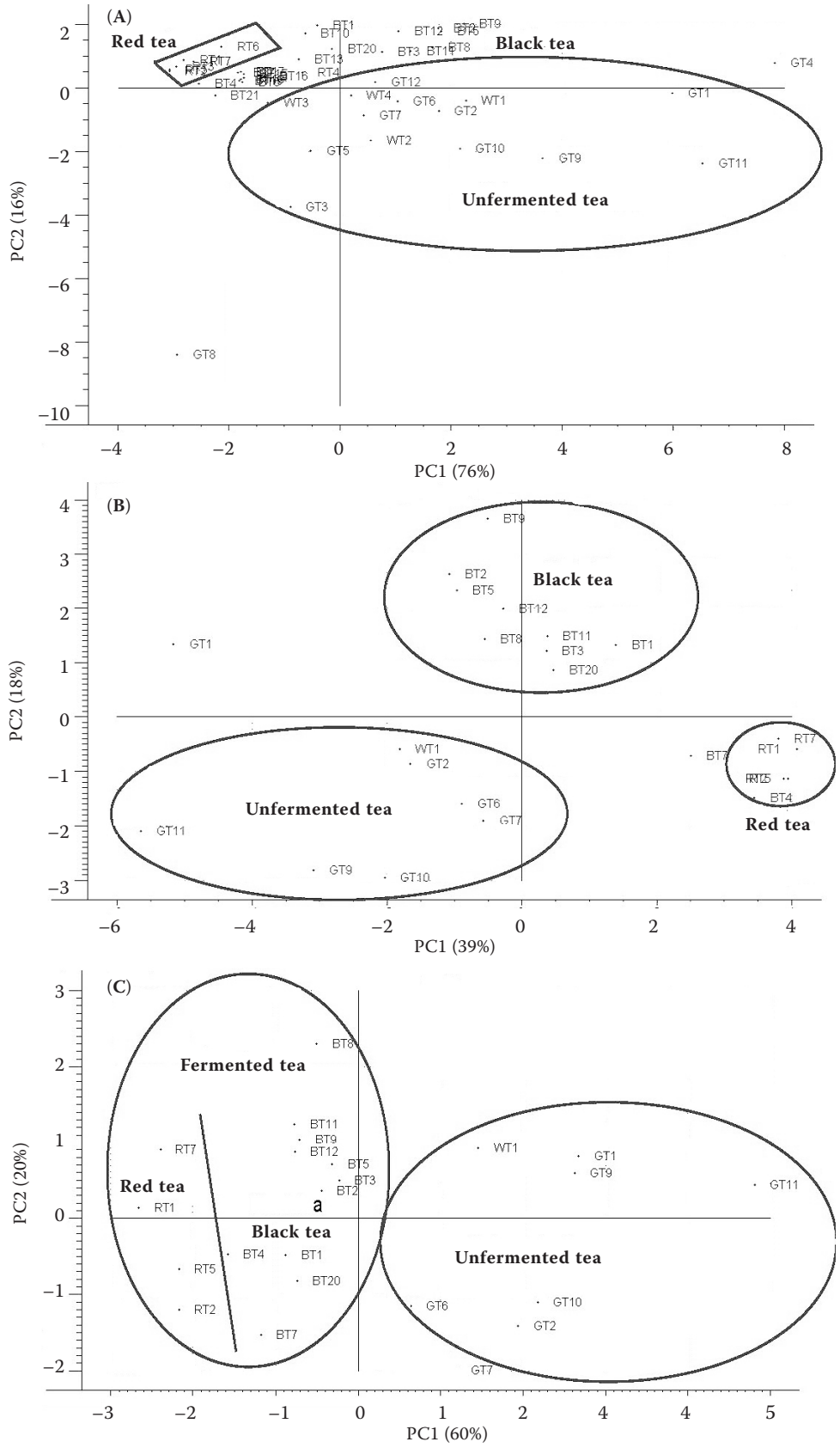

Figure 5. Principal component analysis results of (**A**) concentration of the 20 analysed compounds versus all samples (except decaffeinated tea – BT21), (**B**) concentration of the 20 analysed compounds versus pure samples, and (**C**) concentration of main compounds (Caf, EC, EGC, EGCG, QueR, and GalAc) versus pure samples

were included in the next PCA (Figure 5C) with pure samples. In this case, the explained variance increased to 80%, indicating that it is possible to use phytochemical distribution patterns to identify tea types. All unfermented teas had positive PC1 scores, while fermented teas had negative scores. It is also possible to discern between black and red tea: although both tea types have negative PC1 scores, red tea had lower PC1 scores (< –2) than black tea.

CONCLUSIONS

Concentrations of 20 compounds of major phytochemical classes (phenolic acids, flavan-3-ols, flavones, flavonols, and alkaloids) present in beverages were used to identify distribution patterns. For all studied samples it has been possible to establish a classification according to the content in caffeine, chlorogenic and caffeic acid, and well differentiated groups have been established for regular and decaffeinated coffee, teas, and soft and energy drinks.

Unfermented and fermented teas had similar levels of alkaloids, flavones, and flavonols but differences were observed in the levels of flavan-3-ols and phenolic acids. In general, a high variability in the distribution of analysed compounds was observed between and within groups of samples. However, using pattern recognition procedures, the differentiation between unfermented and fermented teas based on their phytochemical distribution patterns was possible. Additionally, separation of different types of fermented teas (black and red) was also achieved using the same distribution patterns. Differentiation of unfermented teas (green and white) was not possible due to their similar phytochemical profiles. However, using selected compounds as markers and homogeneous groups of samples increases the accuracy of sample differentiation up to 80%. Therefore, the reported results indicate that phytochemical distribution patterns can be used for the classification of most tea types, although further researches are still needed to improve separation between unfermented tea types.

Acknowledgments. Noellia Manchón had a predoctoral scholarship FPI-INIA. The authors also would like to thank the invaluable work of Rosario Vicente in the experimental part of this work.

References

Astill C., Birch M.R., Dacombe C., Humphrey P.G., Martin P.T. (2001): Factors affecting the caffeine and polyphenol contents of black and green tea infusions. Journal of Agricultural and Food Chemistry, **49**: 5340–5347.

Barone J.J., Roberts H.R. (1996): Caffeine consumption. Food and Chemical Toxicology, **34**: 119–129.

Cabrera C., Gimenez R., Lopez M.C. (2003): Determination of tea components with antioxidant activity. Journal of Agricultural and Food Chemistry, **51**: 4427–4435.

Cabrera C., Artacho R., Gimenez R. (2006): Beneficial effects of green tea – a review. Journal of the American College of Nutrition, **25**: 79–99.

Daghbouche Y., Garrigues S., Vidal M.T., Delaguardia M. (1997): Flow injection Fourier transform infrared determination of caffeine in soft drinks. Analytical Chemistry, **69**: 1086–1091.

Dorea J.G., Da Costa T.H.M. (2005): Is coffee a functional food? British Journal of Nutrition, **93**: 773–782.

Drewnowski A., Gomez-Carneros C. (2000): Bitter taste, phytonutrients, and the consumer: a review. American Journal of Clinical Nutrition, **72**: 1424–1435.

Duarte G.S., Pereira A.A., Farah A. (2010): Chlorogenic acids and other relevant compounds in Brazilian coffees processed by semi-dry and wet post-harvesting methods. Food Chemistry, **118**: 851–855.

Fernández P.L., Pablos F., Martín M.J., González A.G. (2002): Study of catechin and xanthine tea profiles as geographical tracers. Journal of Agricultural and Food Chemistry, **50**: 1833–1839.

Frary C.D., Johnson R.K., Wang M.Q. (2005): Food sources and intakes of caffeine in the diets of persons in the United States. Journal of the American Dietetic Association, **105**: 110–113.

Fujioka K., Shibamoto T. (2008): Chlorogenic acid and caffeine contents in various commercial brewed coffees. Food Chemistry, **106**: 217–221.

Garcia-Lafuente A., Guillamon E., Villares A., Rostagno M.A., Martinez J.A. (2009): Flavonoids as anti-inflammatory agents: implications in cancer and cardiovascular disease. Inflammation Research, **58**: 537–552.

Gardner E.J., Ruxton C.H.S., Leeds A.R. (2007): Black tea – helpful or harmful? A review of the evidence. European Journal of Clinical Nutrition, **61**: 3–18.

Hertog M.G.L., Hollman P.C.H., Vandeputte B. (1993): Content of potentially anticarcinogenic flavonoids of tea infusions, wines, and fruit juices. Journal of Agricultural and Food Chemistry, **41**: 1242–1246.

Hu B., Wang L., Zhou B., Zhang X., Sun Y., Ye H., Zhao L. (2009): Efficient procedure for isolating methylated catechins from green tea and effective simultaneous

analysis of ten catechins, three purine alkaloids, and gallic acid in tea by high-performance liquid chromatography with diode array detection. In: Abstracts of Papers of the American Chemical Society: 242. – Paper with this title is publish in Journal of Chromatography A, **1216**: 3223–3231 (authors: Hu B., Wang L., Zhou B., Zhang X., Sun Y., Ye H., Zhao L., Hu Q., Wang G., Zeng X.).

Khokhar S., Magnusdottir S.G.M. (2002): Total phenol, catechin, and caffeine contents of teas commonly consumed in the United Kingdom. Journal of Agricultural and Food Chemistry, **50**: 565–570.

Lin Y.S., Tsai Y.J., Tsay J.S., Lin J.K. (2003): Factors affecting the levels of tea polyphenols and caffeine in tea leaves. Journal of Agricultural and Food Chemistry, **51**: 1864–1873.

Mattila P., Kumpulainen J. (2002): Determination of free and total phenolic acids in plant-derived foods by HPLC with diode-array detection. Journal of Agricultural and Food Chemistry, **50**: 3660–3667.

Meinhart A.D., Bizzotto C.S., Ballus C.A., Prado M.A., Bruns R.E., Teixeira-Filho J., Godoy H.T. (2010): Optimisation of a CE method for caffeine analysis in decaffeinated coffee. Food Chemistry, **120**: 1155–1161.

Mizukami Y., Sawai Y., Yamaguchi Y. (2007): Simultaneous analysis of catechins, gallic acid, strictinin, and purine alkaloids in green tea by using catechol as an internal standard. Journal of Agricultural and Food Chemistry, **55**: 4957–4964.

Nishitani E., Sagesaka Y.M. (2004): Simultaneous determination of catechins, caffeine and other phenolic compounds in tea using new HPLC method. Journal of Food Composition and Analysis, **17**: 675–685.

Obanda M., Owuor P.O., Mang'oka R. (2001): Changes in the chemical and sensory quality parameters of black tea due to variations of fermentation time and temperature. Food Chemistry, **75**: 395–404.

Owuor P.O., Obanda M. (2007): The use of green tea (*Camellia sinensis*) leaf flavan-3-ol composition in predicting plain black tea quality potential. Food Chemistry, **100**: 873–884.

Perva-Uzunalic A., Skerget M., Knez Z., Weinreich B., Otto F., Gruner S. (2006): Extraction of active ingredients from green tea (*Camellia sinensis*): Extraction efficiency of major catechins and caffeine. Food Chemistry, **96**: 597–605.

Peterson J., Dwyer J., Jacques P., Rand W., Prior R., Chui K. (2004): Tea variety and brewing techniques influence flavonoid content of black tea. Journal of Food Composition and Analysis, **17**: 397–405.

Rostagno M.A., Palma M., Barroso C.G. (2003): Ultrasound-assisted extraction of soy isoflavones. Journal of Chromatography A, **1012**: 119–128.

Rostagno M.A., Villares A., Guillamón E., Garcia-Lafuente A., Martínez J.A. (2009): Sample preparation for the analysis of isoflavones from soybeans and soy foods. Journal of Chromatography A, **1216**: 2–29.

Rostagno M.A., Manchon N., D'Arrigo M., Guillamón E., Villares A., Garcia-Lafuente A., Ramos A., Martínez J.A. (2011): Fast and simultaneous determination of phenolic compounds and caffeine in teas, mate, instant coffee, soft drink and energetic drink by high-performance liquid chromatography using a fused-core column. Analytica Chimica Acta, **685**: 204–211.

Santini A., Ferracane R., Mikusova P., Eged S., Srobarova A., Meca G., Manes J., Ritieni A. (2011): Influence of different coffee drink preparations on ochratoxin A content and evaluation of the antioxidant activity and caffeine variations. Food Control, **22**: 1240–1245.

Sharangi A.B. (2009): Medicinal and therapeutic potentialities of tea (*Camellia sinensis* L.) – A review. Food Research International, **42**: 529–535.

Sultana T., Stecher G., Mayer R., Trojer L., Qureshi M.N., Abel G., Popp M., Bonn G.K. (2008): Quality assessment and quantitative analysis of flavonoids from tea samples of different origins by HPLC-DAD-ESI-MS. Journal of Agricultural and Food Chemistry, **56**: 3444–3453.

Wang Y.Y., Yang X.R., Li K.K., Li C.R., Li L.L., Li J.X., Huang H.L., He Y.M., Ye C.X., Song X.H. (2010): Simultaneous determination of theanine, gallic acid, purine alkaloids, catechins, and theaflavins in black tea using HPLC. International Journal of Food Science and Technology, **45**: 1263–1269.

Zuo Y.G., Chen H., Deng Y.W. (2002): Simultaneous determination of catechins, caffeine and gallic acids in green, Oolong, black and pu-erh teas using HPLC with a photodiode array detector. Talanta, **57**: 307–316.

Received for publication November 8, 2012
Accepted after corrections January 30, 2013

Corresponding author

Dr. Mauricio Ariel Rostagno, Centro para la Calidad de los Alimentos, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), C/José Tudela s/n, 42004, Soria, Spain; E-mail: mauricio.rostagno@gmail.com