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Analysis of polyphenols and methylxantines in tea samples by means of nano-liquid chromatography utilizing capillary columns packed with core–shell particles

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ABSTRACT

In this study, a rapid separation of eleven polyphenols and three methylxanthines was obtained by means of nano-liquid chromatography (nano-LC), employing a 100 μm I.D. capillary column packed with C18 core–shell particles (2.7 μm) for 10 cm. All compounds were baseline resolved with a step gradient elution in less than 15 min. The developed analytical method was validated and the resulting RSD% for intra-day and inter-day repeatability, related to retention time, retention factor and peak area, were below 5.1 and 5.7%. LOD and LOQ values corresponded to 0.300 and 0.625 μg/mL, while linearity range assessed gave R^2 no lower than 0.990. Then, the method was used to determine studied compounds in tea extracts. Further, the nano-LC system was coupled with a mass spectrometer to confirm the components present in real samples. Finally the results were compared with those obtained using a capillary column (100 μm I.D. × 10 cm) packed with C18 sub-2 μm particles, applying the same nano-LC experimental conditions.

1. Introduction

Tea (*Camellia sinensis*) is a widespread beverage, largely consumed in Asia and Europe. It is classified into three types based on the manufacturing process used: black, green and oolong tea. Differences are due to the fermentation process of tea leaves after harvesting: green tea (non-fermented), oolong tea (semi-fermented) and black tea (fermented). The highest worldwide production, about 78%, includes the black tea type, while green tea is produced in minor amount (about 20%) especially in China and Japan. A small percentage of overall production, about 2%, is represented by Oolong tea [1]. Several studies have shown beneficial effects of tea consumption on human health, including the prevention of cancer [2,3], cardiovascular disease [4] and obesity [5] together with antioxidant [6], antibacterial, antiviral, antitoxin and antifungal activities [7].

Compounds considered responsible for beneficial health effects of tea are polyphenols, particularly flavanols and flavonols which account for 30% of leaf dry weight [8]. In green tea the main flavonoids are catechins. They are members of the class of flavan-3-ols which structure is characterized by C6–C3–C6 with two aromatic rings di- or tri-hydroxyl groups. They can be divided into free and esterified catechins. The four major tea catechins are: (−)-epigallocatechin gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), and (−)-epicatechin (EC); EGCG is usually present at the highest concentration [1]. Catechins are oxidised during the fermentation process and enzymatically catalysed to form theaflavins and thearubigins. For black tea the oxidation is advanced and catechins are completely oxidised but for oolong tea, it is usually stopped before completion [9]. Additionally green and black tea are known to contain lower amount of phenolic acids, being gallic acid the most abundant [10]. Additionally purine alkaloid in tea including caffeine, theobromine and theophylline are present [11].

Considering the importance of tea polyphenolic compounds on human health it is essential to develop rapid, sensitive and specific methods for their simultaneous qualitative and quantitative analysis. A wide variety of analytical methods have been reported for their analysis in tea samples. The classical approach combines high-performance liquid chromatography (HPLC) with UV–vis (DAD) or electrochemical detection [11–13].

Mass spectrometry was also used as detector for HPLC separation based method to obtain more sensitive and selective analysis of tea constituents [14–19]. A great number of these papers describes HPLC based methods for the simultaneous analysis of several tea polyphenols with the aim to have short analysis time, efficient

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conditions of analytes extraction, detection and characterization. In this view an important aspect of any chromatographic separation is the column characteristics. The great majority of methods use conventional 3 or 5 μm, C18 microparticulate columns of 250 mm.

However recently Guillarme et al. developed an efficient and high throughput analytical method for the separation of seven polyphenols in tea extracts by using an ultra performance liquid chromatography (UHPLC) strategy. Very short time of analysis and improved chromatographic performance were obtained by using column packed with 1.7-μm particles [20]. Disadvantages of this technique are due to the significant increase in column back pressure with the necessity to use instrumentation and consumables of higher costs.

An alternative to improve separation efficiencies and speed without reducing particle size is the use of superficially porous particles, also termed core–shell or fused-core particles. These particles are composed of a 1.7-μm solid core encompassed by a 0.5-μm porous silica layer (d_p = 2.7 μm) to reduce mass transfer and increase peak efficiency. Advantages of this technology are given to the ability to reach high peak efficiency without using very-high-pressure LC instrumentation necessary for sub-2-μm column particles [21].

To the best of our knowledge no paper on the analysis of tea constituents by using core–shell particle technology utilizing a miniaturized system like a nano-LC apparatus has been reported. The nano-LC technique has also the advantage to offer good efficiency, short analysis time, low sample dilution, low consumption of reagents and mobile phase, by using capillary columns of I.D. <100 μm for the analytes separation [22–25]. The potentiality of this environmentally friendly and cost-effective analytical technique, is not yet largely exploited in food analysis.

Therefore the aim of this work was to develop and validate a nano-LC based method for the simultaneous analysis of 14 highly represented polyphenols in tea samples employing UV–vis detection. In addition, mass spectrometry (MS) with electrospray (ESI) interface was used for accurate mass detection of the sample analytes.

2. Experimental

2.1. Chemicals

All chemicals were of analytical reagent grade and were used without further purification. Formic acid, acetonitrile (ACN) and methanol (MeOH) were purchased from Carlo Erba (Milan, Italy). Gallic acid (GA), theobromine (TB), protocatechuic acid (PA), theophylline, anhydrous, (TP), (−)-epigallocatechin (EGC), (+)-catechine hydrate (C), caffeic acid (CA), (−)-epigallocatechin gallate (EGCG), (−)-epicatechin (EC), p-coumaric acid (p-CA), (−)-epicatechin gallate (ECG), (−)-catechin gallate (CG), and o-coumaric acid (o-CA) were purchased from Sigma (St. Louis, MO, USA), while caffeine (CAF) was from Merck (Darmstadt, Germany). Bidistilled water was obtained with a Milli-Q system (Millipore, Bedford, MA, USA). Stock standard solutions of studied compounds (1 mg/mL) were prepared dissolving each analyte in ACN/H_2O mixtures and stored at −18 °C. Exactly, ECG, EC, EGCG, PA, CG, EGC, GA, TB, TF, CA and CAF were solubilized in 20/80 (v/v), ACN/H_2O, while o-CA, p-CA and C, in 40/60 (v/v), ACN/H_2O. For nano-LC analyses, sample solutions and mobile phases were daily prepared diluting stock standard solutions with water at the desired concentration and mixing the appropriate volumes of organic modifiers and formic acid, respectively. Each mobile phase was sonicated before use.

Different brands of green and black tea were purchased in a local supermarket and pharmacy.

2.2. Instrumentation

A Spectra System P2000 conventional gradient (HPLC) pump, with a Spectra System SCM1000 vacuum membrane degasser and a UV–vis on-column Spectra System UV 1000 detector (each one purchased from Thermo Separation Products, San José, CA, USA) was used for nano-LC experiments.

To reduce the flow rate from μ- to nL/min^{-1}, a split-flow system was assembled in our laboratory. The pump was connected to a stainless steel tee (VICI Valco, Houston, TX, USA) with a polyetheretherketone (PEEK) tube (50 cm × 130 μm I.D.). The second exit of the tee was directed to the eluent reservoir of the pump through a fused silica capillary (50 cm × 50 μm I.D.). By doing so, part of the solvent delivered by the pump was recycled. Finally, the third exit of the tee was connected with a stainless steel tube (3 cm × 500 μm I.D.) to the injection valve. At the optimized conditions, the flow rate through the column was about 1200 nL/min. It was calculated measuring the time needed by the mobile phase, eluting from the capillary column, to fill an open micro-syringe (Hamilton, Reno, NV, USA), with a known volume, connected by a Teflon tube.

Detection wavelengths were selected at 200 and 280 nm, and data were collected by Spectra System Software PC 1000 (Fremont, CA, USA). Samples were introduced into the column by a Separserve GmbH modified six-port injector valve (Munster, Germany), injecting for 15 s and washing the loop immediately after with the mobile phase. The loop of about 50 μL was also used as mobile phase reservoir to perform analyses in a step gradient mode. For the gradient elution the procedure was as follows: the HPLC pump delivered methanol to the splitting tee and then to the modified injection valve opportunely filled with the selected mobile phase at the desired time. The valve was manually controlled.

Since the loop was of about 50 μL and the flow rate of the column of 1200 nL/min, methanol could not enter into the column and interfere the applied step gradient. In fact, it took about 40 min before methanol could reach the column, while mobile phase in the loop was changed at about each 5 min (see Section 2.3) and total analysis time was less than 16 min. Methanol had only the function to push the mobile phase in the loop.

A Perkin Elmer series 10 LC pump (Palo Alto, CA, USA) was used for packing the capillaries.

Accurate identification and characterization of analytes were done with an LCQ IT electrospray mass spectrometer (Thermo-Finnigan, St. Jose, CA, USA) controlled by Xcalibur™ 1.3 software (Thermo-Finnigan). The capillary column was connected to the MS through a commercial nano-spray interface (Thermo-Finnigan). The emitter tip was prepared in our laboratory shaping a fused silica capillary (length, 10.5 cm, 25 μm I.D., 375 μm O.D., from Composite Metal Services, Hallow, UK) with sand paper positioned on a rotating disk. The tip was placed at about 1–2 mm from the MS orifice using an x-y-z manipulator and checking the appropriate alignment by a camera.

Positive ion detection mode was chosen with capillary and spray voltages of 12 V and 1.8 kV, respectively.

2.3. Column preparation and chromatographic conditions

Nano-LC experiments were performed in fused silica capillaries (100 μm I.D., 375 μm O.D., purchased from Composite Metal Services), packed in our laboratory with Kinetex® C18 stationary phase, 2.6 μm particle size, 100 Å. This stationary phase was obtained emptying a commercial Kinetex® C18 column (150 mm × 4.6 mm, Phenomenex, Torrance, CA, USA). The packing procedure was the same described in previous works [26,27].

Mobile phases for the step gradient mode consisted of different mixtures of ACN/MeOH/water containing 0.5% formic acid.

Exactly, gradient started with a mobile phase composed by ACN/MeOH/water/formic acid 7/3/89.5/0.5 (v/v/v/v). After 5.5 min ACN was increased at 12% despite of water, and at 9.5 min it was still increased up to 17%. After each run, the column was equilibrated with the starting mobile phase for 15 min.

2.4. Sample preparation

About 10 mg of each tea bag, differing for brand and type of tea, were accurately weighed and put in a glass flask. After this, 5 mL of boiling water was added as in brewing a cup of tea. After 20 min, the cooled down tea infusion was filtered through 0.20 μm nylon syringe filter and injected into the chromatographic system. The tea extracts were stored in the dark at −18 °C. Polyphenols and methylxanthines were identified comparing their retention time and UV absorption with those of standards.

3. Results and discussion

Obtain faster analysis time and improved resolution power are common goals of modern HPLC practitioners. Reducing the particle size has been the strategy of many column's manufacturers, anyway the drawback of such approach is increased backpressure over the system, which often requires the purchase of expensive ultra-high pressure instrumentation or the employment of short columns, useful to speed up analysis as well [28].

Core–shell particles technology has recently gained renewed interest in the chromatographic field since it was possible to obtain particles with an overall diameter of about 2.7 μm, a fused core of 1.7 μm and a porous shell of 0.5 μm [29]. The use of core–shell particles allows to obtain comparable efficiency to that of sub-2 μm particles, at half the backpressure. For this reason, columns packed with these stationary phases can be used with conventional pumps. Moreover, the reduced mass transfer of the analytes into the stationary phases reduces band broadening phenomena, which most contributes to the so-called "C term" in Van Deemter equation. As a practical consequence, it is possible to use higher flow rates and, therefore, speed-up analysis time, without impairing the resolution [29].

Studies on comparison between core–shell and sub 2-μm particles demonstrated that retention factors and sample loading capacity on the fused-core particle column were slightly lower than those observed for the sub-2 μm particle column. However, similar efficiency separations to the sub-2 μm particles could be achieved [30].

Miniaturized HPLC systems are becoming increasingly popular and represent a major trend in such fields, like proteomics, and whenever dealing with limited amount of samples. The low flow rates typically employed in fact lead to less sample dilution, resulting in a sensitivity gain. Also, the considerable saving in terms of solvent consumption is not to be overlooked, both for economical reasons and, more important, for the impact on the environment.

When capillary columns packed with sub 2-μm particles are employed in a nano-LC system obtained by means of a splitting device, rarely high backpressure are generated and consequently conventional chromatographic instrumentation can be used [31,32].

However, the convenience of innovative stationary phases with fast kinetic and able to reduce analyses time, i.e. the core–shell one, are attractive for this miniaturized technique, too.

In this work we selected the Kinetex C18 stationary phase with core–shell particle technology for packing a 10 cm long capillary column (100 μm I.D.) and evaluating its performance in nano-LC for the separation of a mixture of polyphenols and methylxanthine.

Table 1
Repeatability intra-day ($n = 4$; RSD%).

Compound	t_R	k	A (200 nm)	A (280 nm)
Gallic acid (GA)	2.45	5.09	3.89	
Teobromine (TB)	3.26	4.33	4.95	
Protocatecuic acid (PA)	4.63	3.02	4.15	
Theophylline (TP)	2.77	3.04	4.92	
Epigallocatechin (EGC)	3.43	3.56	4.90	
Catechin (C)	2.56	2.05	4.99	
Caffeine (CAF)	2.15	1.49	4.86	
Caffeic acid (CA)	2.42	1.83	-	3.73
Epicatechin (EC)	2.66	2.12	4.61	
Epigallocatechin gallate (EGCG)	2.65	2.11	4.65	
p-Coumaric acid (p-CA)	2.13	1.38	–	4.61
Epicatechin gallate (ECG)	2.48	1.32	4.74	
Catechin gallate (CG)	2.39	1.22	4.81	
o-Coumaric acid (o-CA)	2.29	1.38	–	3.84

Optimized separation conditions were applied to the analysis of tea extracts and, for an accurate identification of tea components, the nano-LC system was coupled with the MS.

Further, the same standard mixture was analyzed in the same separative condition with an analogous capillary column (length, 10 cm; 100 μm I.D.) packed with C18 sub-2 μm particle stationary phase to make a comparison.

3.1. Nano-LC separation

The selected compounds, namely GA, TB, PA, TP, EGC, C, CAF, CA, EC, EGCG, p-CA, ECG, CG, and o-CA, present in some cases very similar chemical structures (see Fig. 1), differing from each other by the presence and/or the position of either hydroxyl or methyl groups in the skeleton (i.e. C and EC, ECG and CG). For this reason, their baseline resolution is a quite challenging task in the analytical field.

In this work, with the aim to obtain baseline resolution of all analytes in a reasonable time, columns of different packed length (25, 15 and 10 cm, 100 μm I.D.), containing Kinetex® C18 2.6 μm particles, were employed with a number of mobile phases, working in isocratic or gradient elution mode (data not shown). Although the use of longer capillary columns offered a good resolution for the studied compounds, long analysis time were obtained. Best results were achieved with the capillary column packed for 10 cm, applying the step gradient mode described in the experimental section and operating with a flow rate of 1200 nL/min. The presence of small concentration of MeOH and formic acid was necessary for improving the selectivity of the system. The presence of the acid in the mobile phase was also useful for improving the ionization efficiency for MS characterization [33].

The absorbance of CA, p-CA and o-CA was higher at a wavelength of 280 nm, and consequently this wavelength was used for their quantification.

Due to the fact that the nano-LC apparatus does not possess a thermostating system, analyses were carried out at room temperature, conditioning the room at 26 °C. At temperature below this value, the separation between C and CAFF was lost.

A representative chromatogram of a standard mixture containing 10 μg/mL of each compound is reported in Fig. 2.

3.2. Method validation

In order to validate the nano-LC method, intra-day and inter-day precision, expressed as RSD% of retention time (t_R), retention factor (k), and peak area (A), limit of detection (LOD), limit of quantitation (LOQ), linearity range, and recovery, were evaluated.

Tables 1 and 2 show results concerning intra-day and inter-day precision of the mixture analyzed four times in the same day and

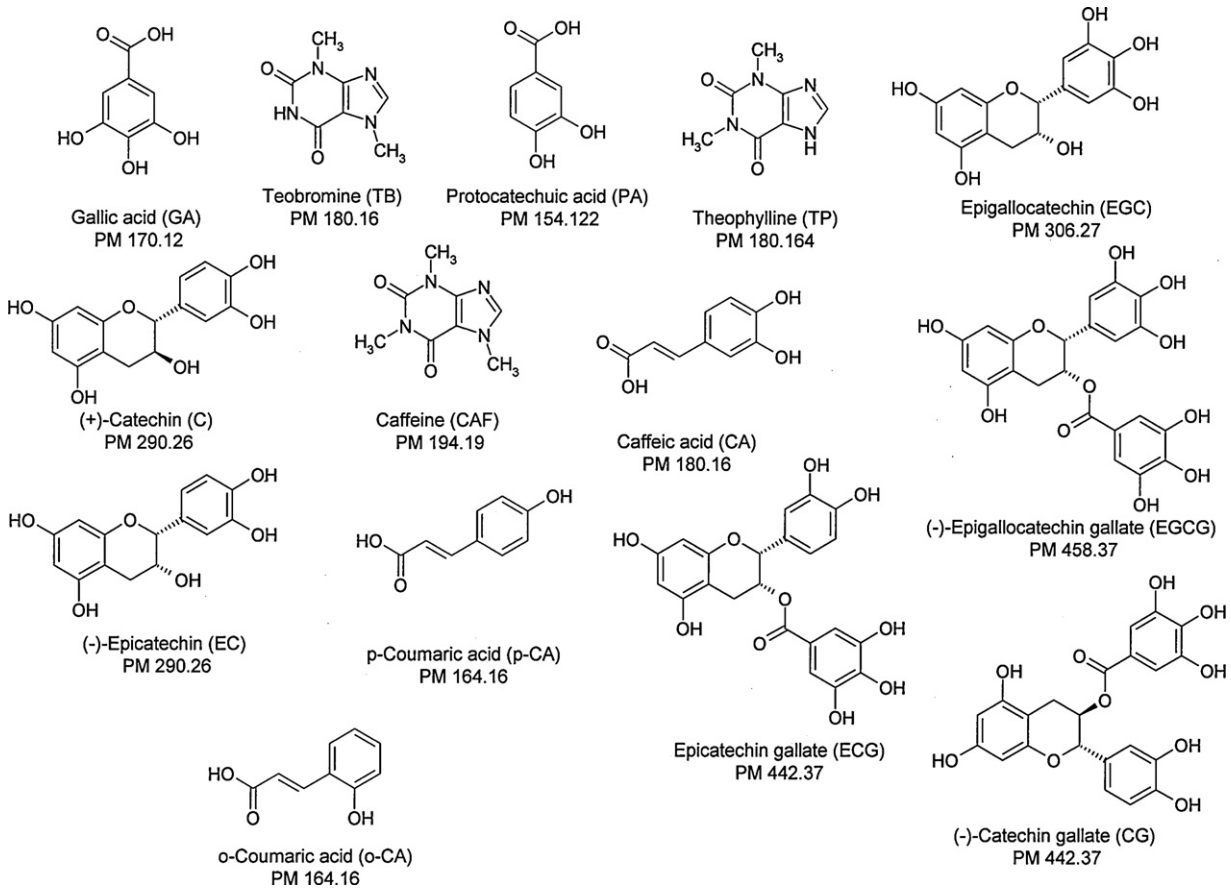

Fig. 1. Chemical structures of studied compounds.

over three days ($n = 9$). RSD% values for t_R, k, and A were in the 2.13–4.63, 1.22–5.09 and 3.73–4.99 range, respectively, for intra-day precision, while for inter-day precision were between 2.21 and 4.39, 3.57 and 5.68, and 2.51 and 5.46, correspondingly.

The sensitivity of the method was characterized by a LOD value of 0.300 μg/mL (signal to noise ratio of 3) and a LOQ of 0.625 μg/mL (signal to noise ratio of 10), for all compounds. LOD and LOQ values were calculated at 280 nm for CA, p-CA and o-CA, while for other compounds at 200 nm.

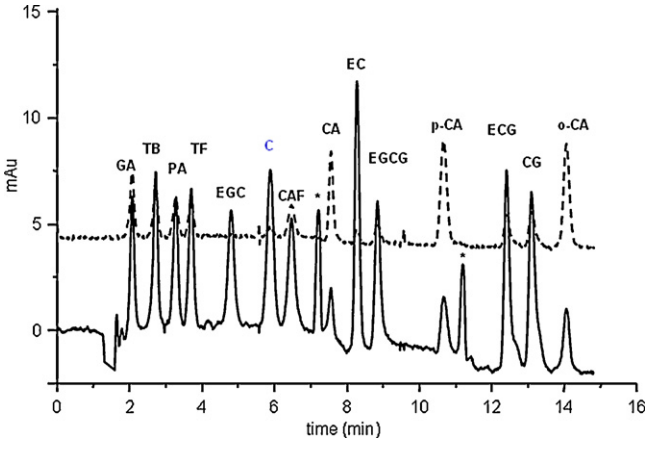

Fig. 2. Chromatogram of a standard mixture of polyphenols and methylxantines (10 μg/mL each). Capllary column (100 μm I.D. × 10 cm) packed with C18 Kinetex® 2.6 particles; gradient elution, as reported in the experimental section, flow rate, 1200 nL/min; peak identification: GA, gallic acid; TB, theobromine; PA, protocatechuic acid; TF, theophylline; EGC, epigallocatechin; C, catechin; CAF, caffeine; CA, caffeic acid; EC, epicatechin; EGCG, epigallocatechin gallate; p-CA, p-coumaric acid; ECG, epicatechin gallate; CG, catechin gallate; o-CA, o-coumaric acid. Detection wavelength: solid and dashed lines were 200 and 280 nm, respectively.

Table 2
Repeatability inter-day ($n = 9$, days = 3; RSD%).

Compound	t_R	k	A (200 nm)	A (280 nm)
Gallic acid (GA)	2.21	4.71	4.85	
Teobromine (TB)	3.22	4.61	4.02	
Protocatecuic acid (PA)	3.05	4.00	2.73	
Theophylline (TP)	2.99	4.25	2.51	
Epigallocatechin (EGC)	3.07	3.72	3.87	
Catechin (C)	2.33	4.25	3.36	
Caffeine (CAF)	2.61	4.30	3.55	
Caffeic acid (CA)	2.79	3.57	–	4.14
Epicatechin (EC)	3.27	4.68	4.74	
Epigallocatechin gallate (EGCG)	2.97	4.48	5.46	
p-Coumaric acid (p-CA)	2.69	4.16	–	4.37
Epicatechin gallate (ECG)	2.49	3.60	3.70	
Catechin gallate (CG)	4.39	5.68	3.62	
o-Coumaric acid (o-CA)	3.02	4.20	–	4.05

Linearity of the method was assessed from LOQ value to a concentration of 70 μg/mL, selecting 9 points for the calibration curves. For caffeine, the linearity range was tested up to 100 μg/mL. For

Table 3
Mean recovery data and RSD of selected catechins and methylxantines in tea.

Compound	Recovery (%) ($n = 3$)	
	5 μg/mL (RSD, %)	20 μg/mL (RSD, %)
Teobromine (TB)	99.15 (4.26)	102.01 (6.49)
Epigallocatechin (EGC)	85.30 (3.32)	88.17 (3.45)
Caffeine (CAF)	92.83 (2.02)	85.03 (2.55)
Epicatechin gallate (ECG)	100.76 (4.67)	78.22 (5.13)

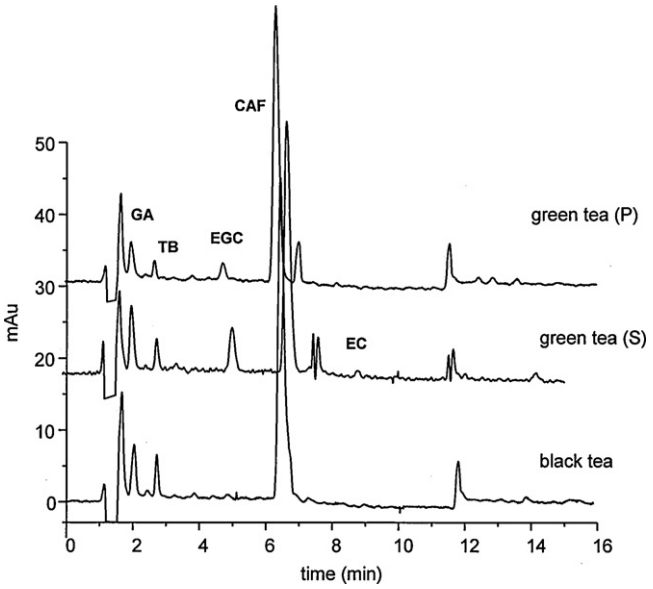

Fig. 3. Chromatograms of tea extracts. For chromatographic conditions and peak identification see Fig. 2 and text.

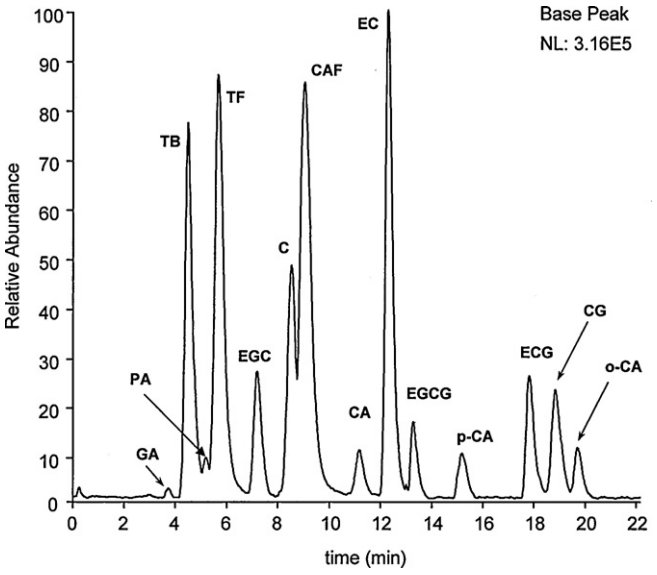

Fig. 4. Chromatogram of a standard mixture in positive ion detection mode. For chromatographic conditions see Fig. 2. MS conditions: capillary voltage 12 V; spray voltage 1.8 kV; capillary temperature, 200° C.

each point, analyses were repeated in triplicate. Peak areas were plotted as a function of concentration expressed as μg/mL, obtaining acceptable values of correlation coefficients, R^2, between 0.990 and 0.998, without the use of an internal standard.

In order to verify column-to-column reproducibility, three columns were prepared and the same standard mixture was analyzed using the optimized conditions. Satisfactory results were obtained with RSD% values lower than 8.15% for peak areas, 6.22% for retention time and 5.48% for retention factor.

3.3. Analysis of tea sample

The validated method was applied to the analysis of one black tea and three different brand tea (B, biological green tea; P, green tea from pharmacy; S, green tea from supermarket). In addition, to corroborate peak identification, analyses of the same samples were performed coupling the nano-LC system to a MS detector by means of a nano-spray interface.

Even if catechins cannot be completely extracted with infusion with hot water, this procedure was selected because the way by which tea is daily taken, and the investigation of obtained data can give an immediate idea of what we are introducing in our body. Furthermore, during method optimization, it was noted how the sample solvent composition can affect peak symmetry, efficiency and retention of first eluting compounds. The solvent must be as similar as possible to the initial gradient mobile phase and in this case best results were achieved dissolving compounds in pure water [33]. This phenomenon, well-known in nano-LC, is on the basis of on-column focusing procedure. Extraction with even only 10% of ACN caused the elution of first eluting compounds with the dead time of the column.

Fig. 3 reports the chromatograms obtained from a black and two green tea samples. The peak identification was done comparing retention time, UV absorbance and spiking each supposed component with a standard solution.

The recovery of extraction procedure was estimated by spiking the three tea samples (S type) with TB, EGC, CAF and ECG standard solutions at two different concentration levels in the range of calibration curve (5 and 20 μg/mL, final concentration added) and calculating the average values of three repeated runs. Recoveries study were satisfactory as can be observed in Table 3.

Table 4, instead, reports the amounts of catechins and methylxanthines found in the different tea samples analyzed. The amount of polyphenols and methylxanthines in tea extracts can be different depending on several factors, such as the origin of tea, the fermentation process and the type of extraction performed. Data obtained in this study, where the amount of CAF found was notable compared to other components, are in accordance with other works [34–39]. Considering the research papers where infusion was selected as extraction procedure and similar compounds were studied, amounts of polyphenols found in the analyzed tea samples are in considerable agreement with data reported by Ref. [39].

3.4. Nano-LC/MS coupling

Nano-LC represents the ideal front-end separation technique, if MS detection is straightforward, since there is no need to split the flow prior to the MS interface and, therefore, no sample waste occurs [40,41].

With the aim of a certain identification of analyzed compounds, the nano-LC system was hyphenated with the ion-trap MS detector through a nano-ESI interface, operating at the same optimized conditions above discussed.

In literature catechins are detected by MS in both positive or negative polarity. Usually the ionization efficiency and the mass signal in negative polarity is worst compared to positive polarity [42]. However, both modes were tested, considering the presence of acidic compounds in the selected mixture as well. In any case, best results, in term of good signal and number of detected peaks, were achieved working with positive polarity. Although MS measurements were satisfactory, considering the overall compounds determination, UV detection gave higher sensitivity.

Fig. 4 shows the base peak chromatogram of the standard mixture. Even if with different intensity, it was possible to identify all the components of the mixture. In Fig. 5a and b, instead, are reported the base peak and the extracted ion chromatogram of two different green tea. A study of fragmentation was done on theobromine, obtaining the same product ions (precursor ion, $[M+H]^+ = 181.2$; product ions, 110.07 and 137.0) found in literature [43].

Table 4
Amounts of catechins and methylxantines in different tea samples.

Sample	GA[a] (%)	TB (%)	PA (%)	TP (%)	EGC (%)	C (%)	CAF (%)	CA (%)	EC (%)	EGCG (%)	p-CA (%)	ECG (%)	CG (%)	o-CA (%)
Black tea	4.19 ± 4.33	2.27 ± 2.56	–	–	–	–	40.52 ± 3.44	–	–	–	–	–	<LOD	–
Green tea B	6.12 ± 0.08	2.32 ± 3.43	–	–	2.65 ± 3.97	–	29.09 ± 3.96	–	0.66 ± 5.01	–	–	nq	nq	–
Green tea S	5.46 ± 2.38	2.17 ± 2.	–	–	1.59 ± 2.14	–	29.89 ± 3.98	–	0.87 ± 4.87	–	–	–	–	–
Green tea P	3.87 ± 3.60	0.97 ± 1.63	–	–	1.75 ± 4.34	–	42.98 ± 4.65	–	–	–	–	–	<LOD	–

nq, not quantifiable.
[a] Values expressed as μg/mg of tea ($n = 3$, value = X ± RSD%).

3.5. Comparison between the Kinetex® 2.6 μm particle and the bidentate sub 2 μm particle stationary phases

As already mentioned, last advances in chromatographic field concern predominantly with the use, on the one hand, of small particles size stationary phase (i.e. sub 2 μm particles), and on the other hand, with the use of core–shell particles. Both types of stationary phase offer very high efficiency and short analysis time. This last advantage, is due mostly to the high selectivity of sub 2 μm particle stationary phase, which allows to reduce the packed length of the column and consequently analysis time. In addition, a rapid kinetic is offered by the core–shell technology [44].

Quite often is possible to find in literature comparative studies between these two typologies of stationary phases that aim to better characterize their properties and to establish which of them can offer better performance [30,45–47].

The major drawback related with the use of sub-2 μm particles in combination with classical chromatographic instrumentation is the occurrence of a high backpressure in most of cases not compatible with the one supported by conventional pumps.

However, working in nano-LC, Fanali's group did not encounter this problem, due to the use of a splitting device system to obtain nano-flows and to the short length of the packed column [32].

For this reason, the performances of the column packed with the Kinetex phase was compared with that one of a column of the same length and I.D. packed with sub 2 μm particles. Analyses were performed at the same linear velocity and applying the same step gradient. In this conditions, a complete loss of resolution for most compound was observed, i.e. between TF and EGC and EC and EGCG (data not showed). Further, the system peaks related to change of mobile phase affected the detection of analyte peaks. For this reason, changes of mobile phase were done at different time but after the elution of the same peaks, e.g., after EGC elution (first step) and after EGCG peak (second step).

As a result, longer analysis time were recorded comparing with the column packed with Kinetex stationary phase but, even if retention factor were higher, it was not possible to separate TB from PA and C from CAF (see Fig. 6).

From these data, the following consideration can be done. The limited diffusion path of molecules in pores of core–shell particles, responsible of fast mass transfer kinetic and high efficiencies, can be very useful for the separation of compounds with very similar structure or chemical behaviours (closely related hydrophobicity). In fact, in the study here presented, the capillary column packed with core–shell particles offered better performance, in terms of resolution capability and analysis time, than sub-2 μm particles column, even if this last column showed larger interactions with analytes.

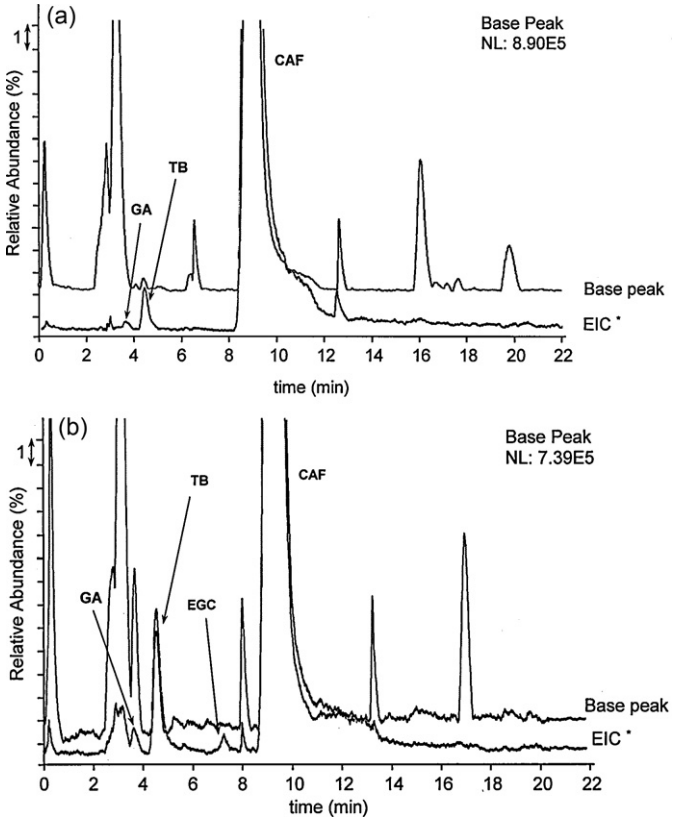

Fig. 5. Chromatograms of tea extracts in positive ion detection mode. (a) Green tea P and (b) green tea B. For other conditions, see Fig. 4 and text. *EIC – extracted ion chromatogram – $[M+H]^+$: 171.1, 181.2, 291.2, 307.0 (m/z).

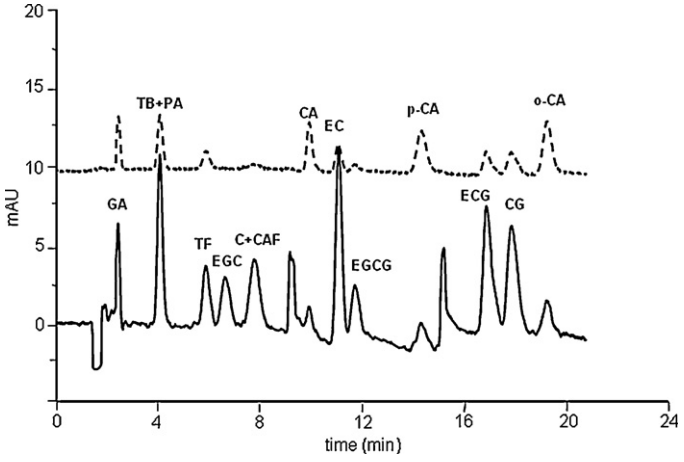

Fig. 6. Chromatogram of a standard mixture of polyphenols and methylxantines (10 μg/mL each). Capillary column (100 μm I.D. × 10 cm) packed with C18 bidentate sub 2-μm particles; gradient elution, as reported in the text; peak identification as in Fig. 2. Detection wavelength: solid and dashed lines were 200 and 280 nm, respectively.

4. Conclusions

A method for the simultaneous separation of several polyphenols and methylxanthines in a single run, employing a nano-LC system, was developed. Experiments were carried out in a capillary column (100 μm I.D. × 10 cm) packed with C18 Kinetex® 2.6 μm particles stationary phase. A good chromatographic performance, assessed in terms of repeatability, linearity, and sensitivity, permitted the determination of studied compounds in tea extract. The described chromatographic technique offers as main advantages the reduced consumption of reagents and it can be performed also with a "laboratory-made" system. As a consequence, it can be considered an analytical system environmentally friendly and cheap. As resulted from a comparison with a column packed with C18 sub-2 μm particles, stationary phases obtained from core–shell technology can be advantageously employed in nano-LC, as well.

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References

[1] S. Sang, J.D. Lambert, C.T. Ho, C.S. Yang, Pharmacol. Res. 64 (2011) 87.
[2] J.M. Yuan, C. Sun, L.M. Butler, Pharmacol. Res. 64 (2011) 123.
[3] E. Gonzalez de Mejia, M.V. Ramirez-Mares, S. Puangpraphant, Brain Behav. Immun. 23 (2009) 721.
[4] J.M. Hodgson, K.D. Croft, Mol. Aspects Med. 31 (2010) 495.
[5] K.A. Grove, J.D. Lambert, J. Nutr. 140 (2010) 446.
[6] B. Frei, J.V. Higdon, J. Nutr. 133 (2003) 3275S.
[7] M. Friedman, Mol. Nutr. Food Res. 51 (2007) 116.
[8] S.M. Chacko, P.T. Thambi, R. Kuttan, I. Nishigaki, Chin. Med. 5 (2010) 13.
[9] Q.V. Vuong, J.B. Golding, M. Nguyen, P.D. Roach, J. Sep. Sci. 33 (2010) 3415.
[10] Y. Wang, C.T. Ho, J. Agric. Food Chem. 57 (2009) 8109.
[11] B. Hua, L. Wang, B. Zhou, X. Zhang, Y. Sun, H. Ye, L. Zhao, Q. Hua, G. Wang, X. Zenga, J. Chromatogr. A 1216 (2009) 3223.
[12] A.P. Neilson, R.J. Green, K.V. Wood, M.G. Ferruzzi, J. Chromatogr. A 1132 (2006) 132.
[13] A. Kotani, K. Takahashi, H. Hakamata, S. Kojima, F. Kusu, Anal. Sci. 23 (2007) 157.
[14] M. Pelillo, B. Biguzzi, A. Bendini, T. Gallina Toschi, M. Vanzini, G. Lercker, Food Chem. 78 (2002) 369.
[15] D. Del Rio, A.J. Stewart, W. Mullen, J. Burns, M.E. Lean, F. Brighenti, A. Crozier, J. Agric. Food Chem. 52 (2004) 2807.
[16] D.J. Zeeb, B.C. Nelson, K. Albert, J.J. Dalluge, Anal. Chem. 72 (2000) 5020.
[17] A. Kiehne, U.-H. Engelhardt, Z. Lebensm. Unters. Forsch. 202 (1996) 299.
[18] W. Pongsuwan, T. Bamba, K. Harada, T. Yonetani, A. Kobayashi, E. Fukusaki, J. Agric. Food Chem. 56 (2008) 10705.
[19] L.-Z. Lin, P. Chen, J.M. Harnly, J. Agric. Food Chem. 56 (2008) 8130.
[20] D. Guillarme, C. Casetta, C. Bicchi, J.L. Veuthey, J. Chromatogr. A 1217 (2010) 6882.
[21] J.M. Cunliffe, T.D. Maloney, J. Sep. Sci. 30 (2007) 3104.
[22] J.P. Vissers, J. Chromatogr. A 856 (1999) 117.
[23] Y. Saito, K. Jinno, T. Greibrokk, J. Sep. Sci. 27 (2004) 1379.
[24] R. García-Villalba, A. Carrasco-Pancorbo, G. Zurek, M. Behrens, C. Bässmann, A. Segura-Carretero, A. Fernández-Gutiérrez, J. Sep. Sci. 33 (2010) 2069.
[25] J. Hernández-Borges, Z. Aturki, A. Rocco, S. Fanali, J. Sep. Sci. 30 (2007) 1589.
[26] S. Fanali, E. Camera, B. Chankvetadze, G. D'Orazio, M.G. Quaglia, J. Pharm. Biomed. Anal. 35 (2004) 331.
[27] S. Fanali, Z. Aturki, G. D'Orazio, A. Rocco, J. Chromatogr. A 1150 (2007) 252.
[28] E. Oláh, S. Fekete, J. Fekete, K. Ganzler, J. Chromatogr. A 1217 (2010) 3642.
[29] J.J. Kirkland, F. Gritti, G. Guiochon, J. Chromatogr. A 1166 (2007) 3046.
[30] A. Abrahim, M. Al-Sayah, P. Skrdla, Y. Bereznitski, Y. Chen, N. Wu, J. Pharm. Biomed. Anal. 51 (2010) 131.
[31] A. Rocco, S. Fanali, J. Chromatogr. A 1216 (2009) 7173.
[32] G. D'Orazio, A. Rocco, S. Fanali, J. Chromatogr. A 1228 (2012) 213–220.
[33] C.A. Hughey, B. Wilcox, C.S. Minardi, C.W. Takehara, M. Sundararaman, L.M. Were, J. Chromatogr. A 1192 (2008) 259.
[34] U.D. Uysal, Z. Aturki, M.A. Raggi, S. Fanali, J. Sep. Sci. 32 (2009) 1002.
[35] M.A. Rostagno, N. Manchón, M. D'Arrigo, E. Guillamón, A. Villares, A. García-Lafuente, A. Ramos, J.A. Martínez, Anal. Chim. Acta 685 (2011) 204.
[36] W.E. Bronner, G.R. Beecher, J. Chromatogr. A 805 (1998) 137.
[37] I. Novak, M. Seruga, S. Komorsky-Lovric, Food Chem. 122 (2010) 1283.
[38] Y. Zuo, H. Chen, Y. Deng, Talanta 57 (2002) 307.
[39] J.P. Aucamp, Y. Hara, Z. Apostolides, J. Chromatogr. A 876 (2000) 235.
[40] K. Lanckmans, A. Van Eeckhaut, S. Sarre, I. Smolders, Y. Michotte, J. Chromatogr. A 1131 (2006) 166.
[41] D. Pröfrock, Anal. Bioanal. Chem. 398 (2010) 2383.
[42] Z. Spácil, L. Nováková, P. Solich, Food Chem. 123 (2010) 535.
[43] M. Thevis, G. Opfermann, W. Schänzer, J. Mass Spectrom. 38 (2003) 1197.
[44] D. Guillarme, J. Ruta, S. Rudaz, J.-L. Veuthey, Anal. Bioanal. Chem. 397 (2010) 1069.
[45] M.C. Pietrogrande, F. Dondi, A. Ciogli, F. Gasparrini, A. Piccin, M. Serafini, J. Chromatogr. A 1217 (2010) 4355.
[46] S. Fekete, J. Fekete, K. Ganzler, J. Pharm. Biomed. Anal. 49 (2009) 64.
[47] J. Zheng, D. Patel, Q. Tang, R.J. Markovich, A.M. Rustum, J. Pharm. Biomed. Anal. 50 (2009) 815.