High throughput qualitative analysis of polyphenols in tea samples by ultra-high pressure liquid chromatography coupled to UV and mass spectrometry detectors

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ARTICLE INFO

ABSTRACT

The analysis of polyphenols in tea extracts is important due to their potential health benefits. Therefore, efficient and high throughput analytical methods have been developed for the separation of seven predominant polyphenols, also known as catechin derivatives, present in tea extracts. Columns packed with sub-2-μm particles operating at elevated pressure (UHPLC strategy) were selected to improve chromatographic performance. The potential of UHPLC–UV was demonstrated with baseline resolution of all standard catechins in only 30 s using a 50-mm column packed with 1.7-μm particles. When dealing with real samples such as tea extracts, however, longer columns of up to 150 mm in length were employed to enhance the separation of catechin derivatives and other constituents within the tea samples while maintaining an acceptable analysis time. Two strategies based on 2-D experiments were proposed to clearly identify catechins. Firstly, a liquid–liquid extraction procedure was added prior to the UHPLC–UV analysis to decrease the complexity of the sample. Secondly, UHPLC was coupled to ESI-MS/MS to attain sufficient sensitivity and selectivity between catechin derivatives and other constituents of tea extract. These two strategies were found extremely promising as a clear discrimination of catechins from the matrix could be attained.

1. Introduction

Green and black teas are the most widely consumed beverages worldwide. Their potential health benefits, such as inhibitory effects against cancer and prevention of cardiovascular disease, have been extensively documented on a scientific basis [1]. It is generally believed that catechin flavonoids, whose content is around 60 mg/g of the dry leaf weight [2], possess antioxidant properties and biological activity responsible for the claimed therapeutic activity of tea [3,4]. For this reason, it is important to develop efficient analytical methods able to assess the nature and amount of catechins in various tea extract samples.

Reverse-phase LC carried out on C8- or C18-bonded silica columns and coupled with UV, UV–DAD or electrochemical detection remains the most widespread method for the determination of polyphenols contained in plant and biological samples [5–8]. Recently published studies have reported analytical methods that require 20–60 min of analysis time per sample [5,7–10] or between 20 and 40 min when only 5–10 compounds of interest have to be simultaneously measured [7,8,11–13]. Furthermore, when more detailed structural information is required to identify and confirm molecular structures of unknown compounds, or when quantitative analysis has to be performed, RPLC methods can be coupled to electrospray mass spectrometry (ESI-MS) to take advantage of its high sensitivity and selectivity [7,8,13,14].

While providing satisfactory resolution, the above-reported RPLC methods for catechin analysis appear to have not taken full advantage of recent advances in liquid chromatography [15,16,17]. Indeed, it could be beneficial to further improve chromatographic performance in terms of throughput and/or resolution particularly when numerous complex tea extracts have to be analyzed. In this context, a recent study shows the possibility of carrying out a separation of six epicatechin derivatives in 5–10 min using a conventional 100 mm, 3.5 μm RPLC material at an elevated flow rate (1.2 mL/min) [18]. Even if the authors claimed that the proposed method is repeatable, sensitive and can be used on a conventional HPLC instrument, the selectivity between the investigated compounds and other analytes contained within a real matrix (tea extract) is obviously too limited, and this makes an unambiguous determination and quantification of epicatechins difficult.

As an alternative, it would be interesting to evaluate the use of columns packed with sub-2 μm particles in conjunction with dedicated instrumentation able to withstand pressures of 1000 bar (ultra-high pressure liquid chromatography, UHPLC) [19,20]. This technology has become available from several providers [20,21] and has shown some clear benefits in terms of analysis time, resolv-

ing power, solvent consumption and, to a lesser extent, sensitivity [23–25]. On the other hand, the main drawback for the use of columns packed with small particles is the generated backpressure, which is inversely proportional to the particle size, dp3 when operating at the optimum mobile phase velocity according to Darcy's law [26]. This makes mandatory the use of dedicated instrumentation. Up to now, only a few applications using UHPLC have been reported for the analysis of catechins and with MS as detector [27–29]. In the first study [27], the authors evaluated the amount of only two catechins, namely catechin and epicatechin, in various commercial chocolate samples in 3 min. In the second study, Solich et al. demonstrated the possibility of successfully separating various standard phenolic compounds including catechins. The unambiguous discrimination of catechins in real samples, however, remains critical [28]. Finally, the most recent study [29] developed a method for the qualitative and quantitative determination of catechins with UHPLC–PDA.

The present paper reports the development of efficient and high throughput UHPLC–UV methods for the separation of the seven predominant catechins present in tea extracts and gallic acid. UHPLC was also coupled with ESI-MS operating in the tandem mode to attain sufficient sensitivity and selectivity together with unequivocal identification between catechin derivatives and other constituents of tea extracts.

2. Experimental

2.1. Chemicals and reagents

The standard catechin flavonoids (+)-catechin (99%, C), (−)-epicatechin (96%, EC), (−)-catechin gallate (100%, CG), (−)-epicatechin gallate (99.1%, ECG), (−)-gallocatechin gallate (99.9%, GCG), (−)-epigallocatechin gallate (95.1%, EGCG), (−)-epigallocatechin (98.2%, EGC) and also gallic acid (99%, AC) were all obtained from Sigma–Aldrich (Milan, Italy) and stored at −20 °C in darkness. A stock solution of these eight compounds at a concentration of 0.5 mg/mL was initially prepared in pure methanol, and working standard solutions were obtained by appropriate dilution with pure water. Chemical structures of the eight investigated catechins are reported in Fig. 1. Additional chemicals such as ether (99%), ethyl acetate (99.5%) and butanol (98%) used for the liquid–liquid extraction procedures were purchased from Sigma–Fluka (Buchs, Switzerland).

For the UHPLC–UV experiments, acetonitrile was of HPLC gradient grade from Panreac Quimica (Barcelona, Spain), and formic acid was obtained from SDS (Peypin, France). For the UHPLC–MS/MS experiments, formic acid and acetonitrile (ACN) were of ULC/MS grade and purchased from Biosolve (Valkenswaard, Netherlands). Finally, water was obtained from a Milli-Q Water Purification System from Millipore (Bedford, MA, USA).

2.2. Preparation of tea extract

Lipton brand black "tea time finest Ceylan" tea packaged in tea bags (2 g of tea per bag) was purchased at a local grocery store.

2.2.1. Preparation of a conventional tea infusion

A brew of black tea was prepared following the instructions provided on the package by pouring 200 mL of boiling water into a glass flask and dipping a 2 g tea bag for 5 min. After cooling to ambient temperature, the solution was filtered through a nylon filter (0.45 μm × 47 mm from Millipore). The tea extract was kept frozen at 4 °C until the analysis. Then, this sample was directly injected into the UHPLC–UV system and subjected to a 10-fold dilution with pure water before the UHPLC–MS analysis.
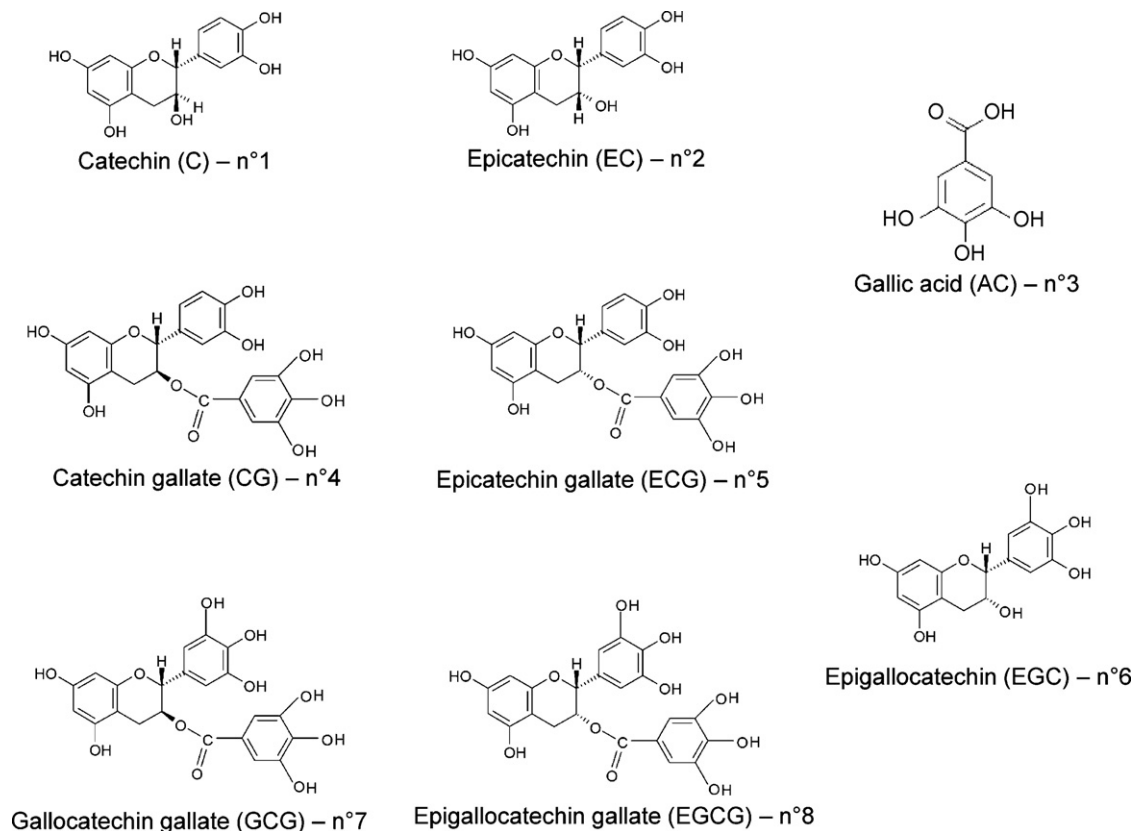

Fig. 1. Chemical structures of the eight investigated polyphenols.

2.2.2. Liquid–liquid extraction procedure

Before the purification, all tea samples were filtered through a nylon filter (0.45 μm × 47 mm). Then, 10 mL of the filtered tea sample was extracted 3-fold with 10 mL of organic solvent (ether, butanol or ethyl acetate). After vigorous stirring, the two phases were allowed to settle for couple of minutes. The aqueous layer (lower part) was recovered in a glass flask and further analyzed. The organic layer (upper part) was recovered and evaporated to dryness using a nitrogen stream to avoid the peak broadening that is always observed when a pure organic solvent is used for dilution purposes. The dry residue was subsequently dissolved in pure water before injection into the UHPLC system.

2.3. Instrumentation

2.3.1. UHPLC–UV experiments

UHPLC–UV experiments were performed on a Waters Acquity UPLC system (Milford, MA, USA). This instrument included a binary pumping system with a maximum flow rate of 2 mL/min, an auto-sampler with an injection loop volume of 2 or 5 μL used under full loop conditions, a UV–vis programmable detector and a column manager that included a column oven set at 30 °C. Data acquisition, data handling and instrument control were performed using the Empower Software v2.0. The UV detector time constant and data sampling rate were adjusted and could vary between 25–100 ms and 20–80 Hz, respectively, to obtain signals of highest quality even with ultra-fast separation. The detector wavelength was set at 265 nm as the best compromise to reach maximum absorbance for the compounds of interest.

2.3.2. UHPLC–MS/MS experiments

UHPLC–MS/MS experiments were performed on the same Waters ACQUITY UPLC system but hyphenated with a triple quadrupole (TQD) mass spectrometer from Waters. The TQD operated at a single mass resolution of m/z 0.7 FWHM and possessed an upper mass limit of m/z 2000. The ESCi® ionization source was used in the ESI negative mode, and ionization parameters, cone voltages and collision energies were optimized by infusing each compound (1 μg/mL) in 50:50 ACN:water plus 0.1% formic acid at a flow rate of 600 μL/min. Optimal cone voltage and collision energies values are summarized in Table 1. The capillary voltage and the source extractor voltage were set at 3000 and 3 V, respectively. The source temperature was maintained at 140 °C, the desolvatation gas temperature and flow at 400 °C and 800 L/h, respectively, and the cone gas flow at 50 L/h. MS/MS detection was carried out in the SRM mode, and the transitions are also indicated in Table 1. The collision gas flow was set at 0.2 mL/min of argon, and the entrance and exit potentials were adjusted to 1 and 0.5 V, respectively. Data acquisition, data handling and instrument control were performed using the Masslynx v4.1 software.

2.3.3. Stationary phases

Separations were carried out on various analytical columns: a Hypersil Gold C18 (50 mm × 2.1 mm ID, 1.9 μm) column provided by Thermo Fisher Scientific (Runcorn, UK), an Acquity BEH C18 (50 mm × 2.1 mm ID, 1.7 μm) column, an Acquity BEH Shield RP18 (50, 100 and 150 mm × 2.1 mm ID, 1.7 μm) column and an Acquity BEH phenyl (50 mm × 2.1 mm ID, 1.7 μm) column. All of these columns were provided by Waters. The solvent system A = 0.1 vol.% formic acid – water; B = 0.1 vol.% formic acid – acetonitrile was used in the entire study.

2.4. HPLC modeling software

The optimal conditions for each sub-2 μm stationary phase were found thanks to HPLC modeling software (Osiris 4.1.1.2, Datalys,

Table 1
m/z, MS/MS transitions, optimal cone voltages and collision energies for investigated catechin derivatives.

No	Compound	m/z	MS/MS transitions	Cone voltage (V)	Collision energy (V)
1	C	290.3	289.3 > 109.3	40	25
2	EC	290.3	289.3 > 109.3	40	25
3	AC	170.1	169.1 > 125.2	30	20
4	CG	442.4	441.4 > 169.3	40	25
5	ECG	442.4	441.4 > 169.3	40	25
6	EGC	306.3	305.3 > 125.3	40	25
7	GCG	458.4	457.4 > 169.3	40	25
8	EGCG	458.4	457.4 > 169.3	40	25

Grenoble, France). For this purpose, the eight analytes were individually injected using two gradient runs that differ in slope, namely 2–40% B linear gradient in 14 and 4.8 min, respectively. Optimal conditions for separation with all stationary phases were determined for k_{min} equal to 0.2 (no peak eluted before 0.3 min) and k_{max} equal to 30 (no peak after 3.8 min).

3. Results and discussion

3.1. Determination of catechins by UHPLC–UV

There is a wide variety of polyphenols present in green or black tea extract. This study was dedicated to the determination of catechin flavonoids in particular, however, because it has been demonstrated that these compounds possess rather potent antioxidant properties and a significant degree of bioavailability, which results in beneficial health effects [1,3,4].

3.1.1. Comparison of various columns packed with sub-2 μm particles

Despite some successful attempts in the analysis of catechins using capillary zone electrophoresis [30], micellar electrokinetic chromatography [31], gas chromatography after derivatization [32] or thin-layer chromatography [33], the method of choice, which encompasses approximately 80% of the literature, remains RPLC with UV absorbance detection. An RPLC–UV baseline separation of the eight most abundant catechins was first published by Goto et al. [34] in which a C18 stationary phase and a complex gradient system made of water, acetonitrile and phosphoric acid were used. This interesting study demonstrated that four catechins (EGC, EGCG, EC and ECG) were predominant in tea extract while the other catechins were only present in minor amounts. In 1998, Dalluge et al. [35] showed that acidic conditions were mandatory to both attain a complete resolution of the catechins and eliminate peak tailing. The same study [35] also demonstrated that the complete separation of catechins was column-dependent and concluded that endcapped, deactivated, monomeric C18 columns were preferable.

Therefore, four different endcapped, deactivated columns packed with sub-2-μm particles from two different providers were tested in acidic conditions to attain the baseline separation of seven important catechins and gallic acid. Among these supports, a conventional C18 material (Hypersil Gold C18), a hybrid BEH C18 support (Acquity BEH C18), a hybrid BEH RP18 support with a polar (carbamate) embedded group (Acquity BEH Shield RP18) and a hybrid BEH phenyl material (Acquity BEH phenyl) were initially selected. A systematic procedure was employed to determine the best conditions for the separation of the eight compounds of interest. This operation essentially consists of performing two gradient runs that differ in slope with the eight compounds and the four supports using a water–acetonitrile mobile phase with 0.1% formic acid. Then, the data were computed in optimization software to model the behavior of each compound in the whole composition
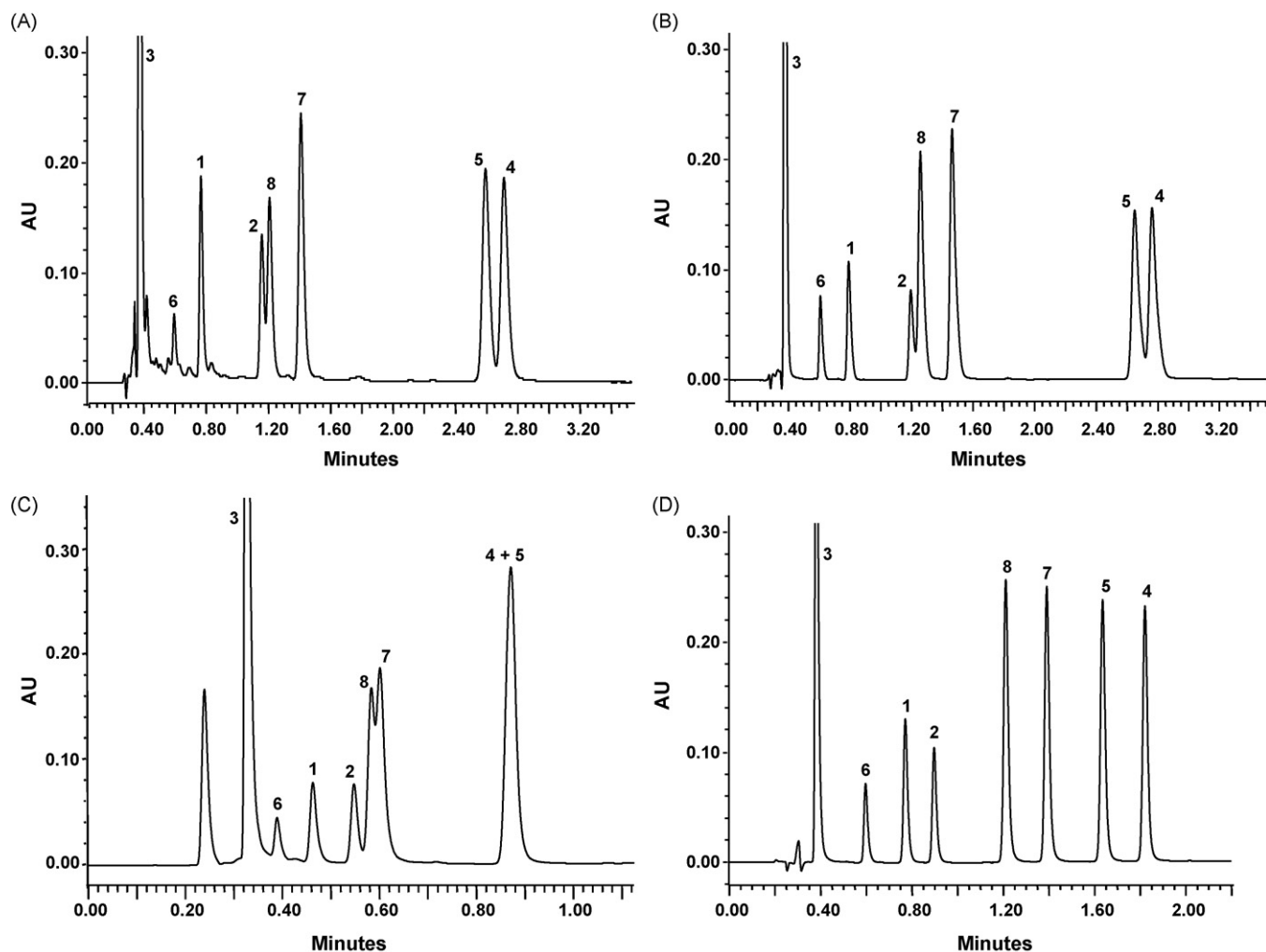
Fig. 2. Optimal UHPLC chromatograms of an eight standard polyphenols mixture at 20 μg/mL obtained with various RP columns packed with sub-2μm particles at 30 °C. Numbers correspond to Fig. 1. F = 500 μL/min and a gradient procedure was used with A as pure water and B as ACN. 0.1% formic acid was present in both solvents. (A) Acquity BEH C18 50 mm × 2.1 mm ID, 1.7 μm column, gradient 11–16.6%B in 3.52 min. (B) Hypersil GOLD C18 50 mm × 2.1 mm ID, 1.9 μm column, gradient 10–16.3%B in 3.52 min. (C) Acquity BEH phenyl 50 mm × 2.1 mm ID, 1.7 μm column, gradient 16.8–23.4%B in 0.6 min. (D) Acquity BEH Shield RP18 50 mm × 2.1 mm ID, 1.7 μm column, gradient 13.3–22.2%B in 3.46 min. 1: C, 2: EC, 3: AC, 4: CG, 5: ECG, 6: EGC, 7: GCG, 8: EGCG.

range. Fig. 2 presents the optimal chromatograms obtained with the four stationary phases. Firstly, it is worth mentioning that the elution order of non-epi and epi forms was identical for all investigated columns and was in close agreement with previous studies [36,37]. Indeed, epi forms with gallate (EGCG and ECG) were eluted prior to non-epi forms with gallate (GCG and CG), and a non-epi form without gallate (C) was eluted prior to non-epi forms without gallate (EGC and EC).

Fig. 2A and B, which corresponds to the hybrid BEH C18 and conventional C18 materials, depict a very similar chromatographic profile obtained with close mobile phase conditions. The separation, however, remained critical for both EC and EGCG (peaks number 2 and 8) and also for CG and ECG (peaks number 4 and 5). These results confirm that selectivity was too limited on these two stationary phases and that the only way to attain a baseline separation would be to increase efficiency by lengthening column. The separation obtained with the phenyl BEH stationary phase (Fig. 2C) was also not satisfactory as it was difficult to separate EC, GCG, EGCG (peaks number 2, 7 and 8). There was also no resolution for CG and ECG (peaks number 4 and 5). Finally, Fig. 2D presents the separation achieved on the BEH RP18 support that contains a polar embedded group. In this case, using a 13.3–22.2% ACN gradient in 3.46 min gave a baseline separation with high selectivity and

a minimal resolution of 3.6 between of C and EC (peaks number 1 and 2). Additionally, the analysis time was quite short at less than 2 min. The different separations provided confirmation that endcapped, monomeric C18 columns are required for the separation of the naturally occurring tea catechins as stated by Dalluge et al. [35]. Furthermore, we also demonstrated that columns containing a polar embedded group were beneficial to improve selectivity of the structural isomer pairs of catechins. It is worth mentioning that columns packed with larger particles but with the same chemical properties (Waters Xterra RP18 or Xbridge shield RP18) can address the issue of separation on conventional HPLC instrumentation and generate strictly equivalent selectivity and resolving power with a longer analysis time (data not shown).

3.1.2. Ultra-fast and highly efficient separations of catechins

One of the main advantages of UHPLC technology is related to the possibility of attaining either ultra-fast or high resolving power separations. Indeed, with the Van Deemter curves obtained for columns packed with small particles [26] and the elevated backpressure attainable (up to 1000 bar in UHPLC instrumentation [22]), it is possible to tune column geometry, the mobile phase flow rate and other chromatographic conditions to attain fast or highly efficient separations.
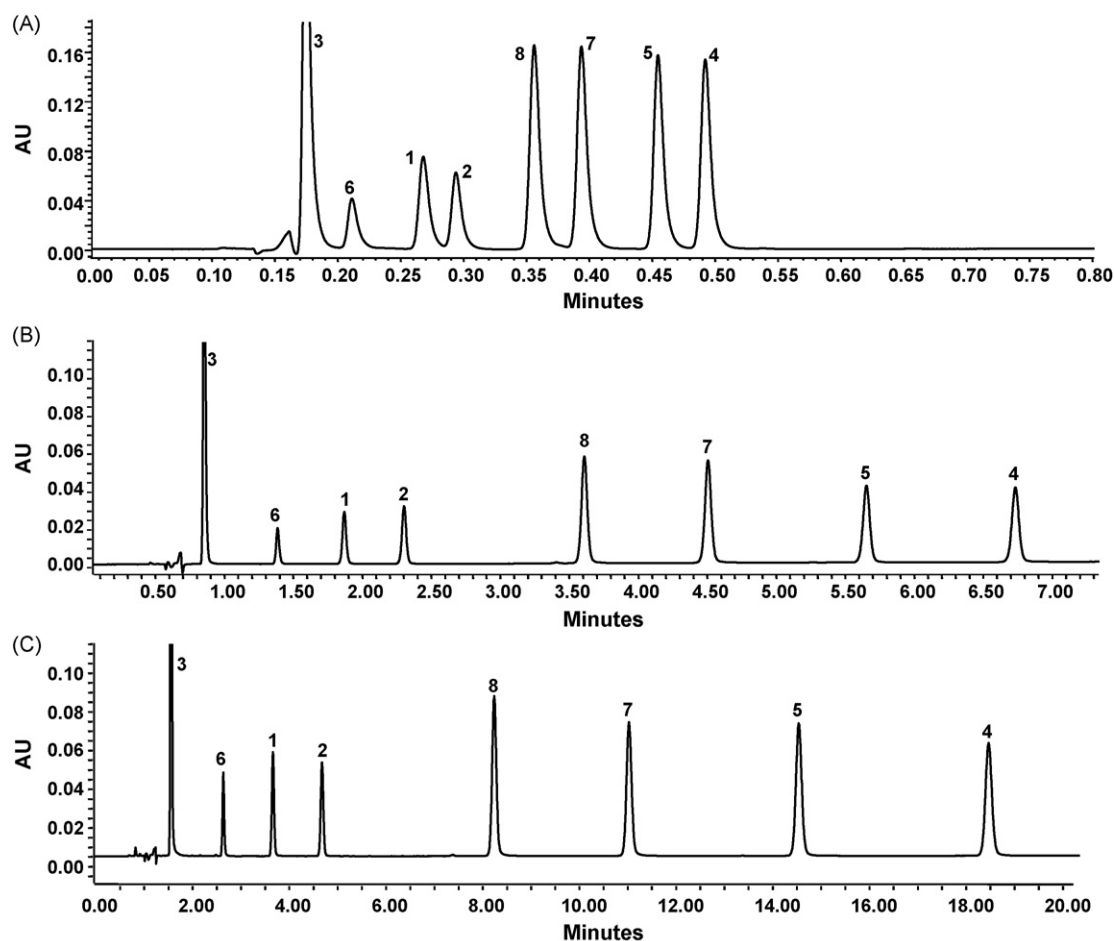
Fig. 3. Ultra-fast and high-resolution UHPLC separations of the mixture of eight polyphenols at 10 µg/mL, with T = 30 °C, using an Acquity BEH Shield RP18 2.1 mm ID, 1.7 µm column. (A) 50 mm column length, F = 1 mL/min, gradient 18–30%B in 0.3 min. (B) 100 mm column length, F = 500 µL/min, gradient 13.3–22.2%B in 7.05 min. (C) 150 mm column length, F = 500 µL/min, gradient 13.3–22.2%B in 30 min. 1: C, 2: EC, 3: AC, 4: CG, 5: ECG, 6: EGC, 7: GCG, 8: EGCG.

Because the separation presented in Fig. 2D presents an elevated selectivity, it is possible to further increase the mobile phase flow rate, the value of %Binitial and the gradient slope while maintaining acceptable separation. In this perspective, an 18–30% ACN gradient in 0.3 min was carried out at a flow rate of 1 mL/min to speed up the original separation as much as possible. The final chromatogram presented in Fig. 3A shows a baseline separation of the eight phenolic compounds in only 30 s with a minimal resolution of 1.72 for C and EC (peaks number 1 and 2). For this separation, the generated backpressure was around 850 bar, and this remained acceptable even for routine use of the method.

On the other hand, the determination of some minor compounds contained within a complex matrix such as tea extract, which possesses hundreds of constituents, requires a high resolving power. Thus, the conditions used for Fig. 3A cannot be applied to real tea samples. Additionally, even if the separation in Fig. 2D presents a high selectivity for catechins, the global resolving power is still too limited to deal with real samples. The performance in gradient mode can be estimated with the peak capacity (P), which is the number of peaks that can be separated in a given time window. The latter is based on the gradient time (t_{grad}) and peak width at the baseline in time units (W_t) according to the following equation:

$$P = \frac{t_{grad}}{W_t} + 1. \tag{1}$$

For the separation reported in Fig. 2D, the peak capacity was equal to 35. To increase peak capacity, longer columns packed with small particles have been tested with extended gradient time. How-

ever, to avoid changes in selectivity, the basic rules for method transfer in gradient mode, which have been presented elsewhere [38], have been strictly applied. In Fig. 3B, a 100 mm × 2.1 mm, 1.7 µm column was used at a flow rate of 0.5 mL/min. As the column length varied, the gradient time was scaled accordingly (3.46 vs. 7.05 min) while the initial and final composition remained identical. In Fig. 3B, the quality of the separation was significantly improved and peak capacity was 2-fold higher (70). Finally, the column length was further increased to 150 mm and the gradient time extended to the maximal acceptable value (30 min). The obtained chromatogram is presented in Fig. 3C. Under these conditions, the value of P was further increased to 85 in 20 min. Therefore, the gain in peak capacity between Fig. 3C and B, which was equal to 20%, was quite limited in proportion to the increase in analysis time (4-fold) and to the generated backpressure. This observation is in good agreement with recent UHPLC studies [39–41] that show the longest column does not necessarily provide an important increase in gradient performance as the latter depends on both isocratic efficiency and the column dead time, which is elevated with longer columns operating at low flow rate.

Eqs. (2) and (3) show the interdependency of peak capacity with isocratic efficiency (N) and column dead time (t_0) [42,43]:

$$P = 1 + \frac{\sqrt{N}}{4} \times \frac{1}{b+1} \ln\left(\frac{b+1}{b}e^{S \times \Delta\Phi} - \frac{1}{b}\right), \tag{2}$$

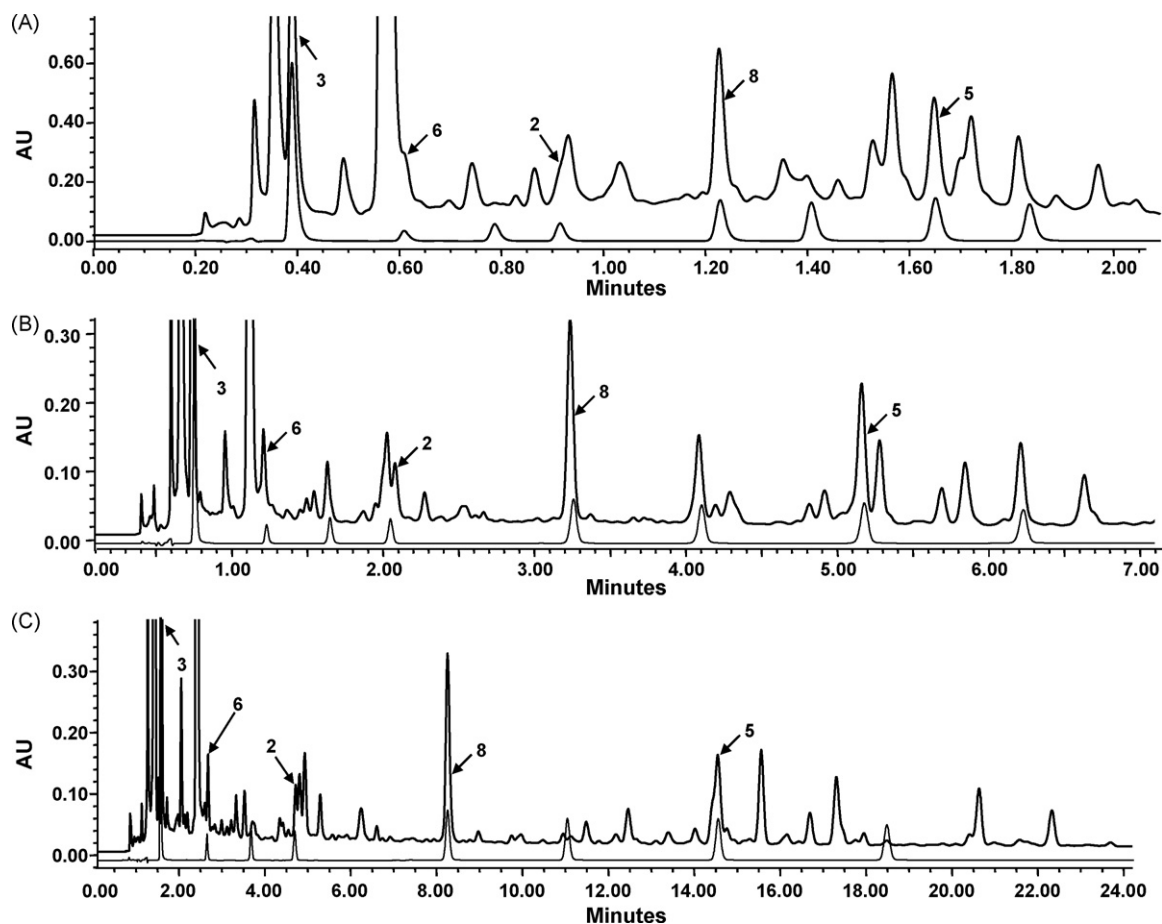$$b = \frac{t_0 \Delta\Phi S}{t_{grad}}, \tag{3}$$

Fig. 4. Conditions identical to that of Fig. 3. Bold chromatogram corresponds to a commercial tea extract infused for 5 min while the light chromatogram represents the mixture of the eight standards. 2: EC, 3: AC, 5: ECG, 6: EGC, 8: EGCG.

where t_{grad} is the gradient time, $\Delta\Phi$ is the change in solvent composition during the gradient, ranging from 0 to 1, and S is a parameter (slope of the logarithmic plot: $d(\log k)/d\Phi$) related to the solute nature, molecular weight and organic modifier nature.

In conclusion, the separation presented in Fig. 3B represents the best compromise between throughput and resolving power for the separation of the seven-catechin derivatives and gallic acid.

3.1.3. Application to commercial tea extract

When dealing with standard polyphenols, the UHPLC–UV method provides excellent selectivity and resolution as demonstrated above. This proposed strategy was thus applied to the determination of catechin derivatives in real tea samples.

To illustrate the complexity of the matrix, a real tea sample infused for 5 min was injected into the UHPLC–UV system using the methods described in Figs. 2D, 3B and C, which correspond to analysis times of 2, 7 and 24 min, respectively. The corresponding profiles are presented in Fig. 4 (bold chromatograms) with a simultaneous overlay of the separations of the eight standard catechins (light chromatograms). It appears from these figures that tea extracts are very complex matrices, and, as expected, the different proposed methods do not present enough resolving power to easily discriminate catechins contained in tea samples.

Despite these critical separations, it is possible to draw some qualitative conclusions regarding the content of catechins in our commercial tea sample. Firstly, it is possible to assess that there was an important amount of EGCG (peak 8 at 1.23 min in Fig. 4A) and ECG (peak 5 at 1.66 min in Fig. 4A) in the tea extract as the corresponding peaks in the three chromatograms were quite sig-

nificant. It is a bit more difficult to make the same conclusion on the presence of EC (peak 2 at 0.92 min in Fig. 4A) and EGC (peak 6 at 0.62 min in Fig. 4A) because quantities were lower. The small peaks were observed identically on the three chromatograms and confirmed by standard injection. These results are in good agreement with a previous study [2], which demonstrated that EGC, EGCG, EC and ECG were the predominant catechins in tea samples. Regarding AC (peak 3 at 0.39 min in Fig. 4A), its presence in large quantity was confirmed by the three chromatograms of Fig. 4, and this is logical as it is a degradation product of catechin derivatives. Finally, regarding C (peak at 0.78 min in Fig. 4A), GCG (peak at 1.42 min in Fig. 4A) and CG (peak at 1.89 min in Fig. 4A), there is no consensus between results provided by the three profiles presented in Fig. 4. Indeed, there is no evidence for these three compounds in Fig. 4A and C while there were some important peaks for these three catechins in the chromatogram of Fig. 4B. This clearly demonstrates the limitations of the UHPLC–UV strategy for the qualitative evaluation of catechins and the difficulty of carrying out the quantitative evaluation of complex tea extracts.

To avoid these problems, two strategies were investigated: (i) addition of a selective sample preparation, namely LLE prior to the UHPLC–UV separation (discussed in Section 3.2) and (ii) switching from the universal UV to the selective MS detector (discussed in Section 3.3).

3.2. Determination of catechins by LLE–UHPLC–UV

A sample preparation step was added to the analytical procedure to decrease the complexity of the tea extract samples and to
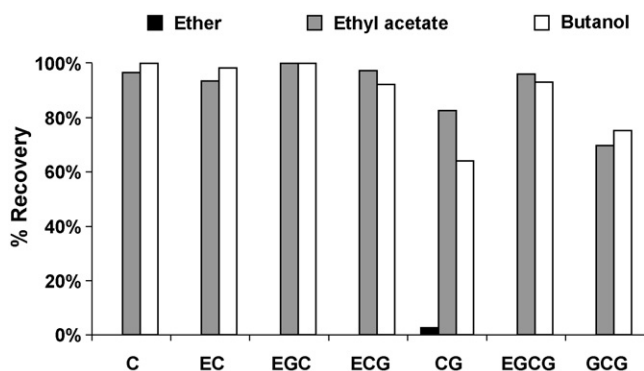
Fig. 5. Recoveries for the seven-catechin derivatives using three different extraction procedures. Recoveries were determined with the UHPLC–MS/MS method presented in Fig. 8A.

obtain a better evidence about the presence or absence of catechin derivatives. Various generic, simple liquid–liquid extraction (LLE) procedures were evaluated prior to the UHPLC–UV analysis. After maceration of tea in pure water, LLE with butanol, ether, ethyl acetate…, were performed according to the literature [5,44,45].

These procedures were compared in term of recoveries, calculated as the ratio of catechin content in organic solvent and total catechin content in both water and organic solvent, for the seven investigated catechins. The recovery values were obtained with the selective and sensitive UHPLC–MS/MS method described in Section 3.3. Fig. 5 shows the results obtained for all catechins using the three different extracting solvents. Firstly, ether is too nonpolar for extracting catechins, and it was rejected because all catechins were recovered in the aqueous phase. The results for ethyl acetate or butanol were very close with recoveries between 93.7% and 100% for ethyl acetate and 92% and 100% for butanol. Only the recoveries of CG and GCG were below these values with both solvents. The lower values observed for these compounds were attributed to their rather low concentration in real tea samples, which are below the limit of quantitation (LOQ). This can make the determination of these compounds in both phases inaccurate and imprecise.

Considering these observations, ethyl acetate was selected as the extraction solvent, because it was easier to use than butanol, which can lead to mixing of the two phases. The two chromatograms presented in Fig. 6 correspond to the direct injection of a commercial tea extract infused for 5 min (light chromatogram) and the organic fraction of the same extract after a liquid–liquid extraction with ethyl acetate (bold chromatogram). This separation
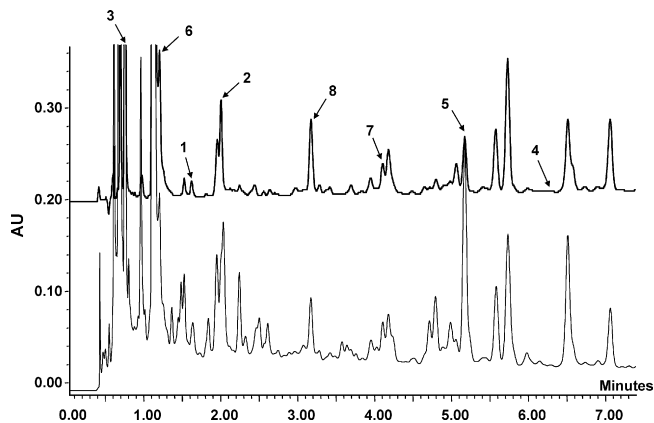

Fig. 6. Conditions similar to that of Fig. 3B. The bold chromatogram corresponds to a commercial tea extract infused for 5 min while the light chromatogram represents the organic fraction of the same extract after a liquid–liquid extraction with ethyl acetate. 1: C, 2: EC, 3: AC, 4: CG, 5: ECG, 6: EGC, 7: GCG, 8: EGCG.

clearly demonstrates the importance of adding a sample preparation step such as LLE with ethyl acetate prior to the analysis. From this separation, it is now possible to confidently identify catechins present in tea samples. In the tested tea extract, the abundance of catechin derivatives can be classified as follows: EGC > EC > EGCG > ECG > GCG > C > CG.

3.3. Determination of catechins by UHPLC–MS

Another strategy was alternatively proposed to further enhance the UHPLC–UV separation of catechins in real tea extracts. Even if UV remains the gold standard detection mode for routine catechin determination, a more selective detector could be useful in some instance [46]. The addition of LLE prior to UHPLC–UV was found to be promising (Section 3.2) but, mass spectrometry (MS) was also successfully reported for catechin analysis to minimize problems associated with inadequate separation quality [2,47,48].

3.3.1. Selectivity of UHPLC–MS and UHPLC–MS/MS towards catechins

In this study, the possibility of coupling UHPLC with MS detection, without any sample preparation, was evaluated for the determination of polyphenols. For the UHPLC–MS experiments, the seven-minute method (Fig. 3B) was selected as it presents the best compromise between analysis time and resolving power. The corresponding chromatograms are presented in Fig. 7. In these separations, real tea samples were diluted 10-fold with water before analysis to avoid contamination of the ESI source and eventual clogging of the heated capillary. The ESI source parameters (i.e., cone voltage, capillary voltage, source extractor voltage, source temperature, desolvatation gas temperature and flow and cone gas flow) were tuned by infusion experiments in the single ion monitoring (SIM) mode to attain the highest possible intensity of ions. In all cases, the negative mode provides a better sensitivity than the positive mode, and the [M−H]- adduct always presented the highest signal-to-noise ratio. Using the optimal ESI settings reported in Section 2.3, product ion scans were acquired with collision energies ranging between 10 and 60 eV, and the most intense transition was selected for each monitored precursor m/z. The optimal transitions and the corresponding collision energies are reported in Table 1.

The selected transitions were identical for both non-epi and epi form of an isomeric pair, and the major fragments were 109, 125 and 169 m/z. The catechin structures reported in Fig. 1 enable a discussion of the fragmentation pathways. First, the product ion 109 m/z was mainly observed for C and EC and should arise from the cleavage of a C–C bond to yield the 2-hydroxyphenol ion (109 m/z). The ion 125 m/z (109 m/z + OH) was the major ion for AC and EGC, but it was also observed on the product ion scans of CG, ECG, GCG and EGCG at high collision energy. This ion should correspond to the 2,3-dihydroxyphenol group common to all these molecules. Finally, the ion 169 m/z, which corresponds to a loss of gallate, was obviously the most intense fragment for the four catechins possessing a gallate moiety (CG, ECG, GCG and EGCG).

Fig. 7A presents the analysis of the eight standard catechins using MS detection operating in the tandem mode. This separation demonstrates the high quality chromatograms that can be attained in UHPLC–MS/MS. It is indeed impossible to assess the different catechins on the basis of MS response alone as the different epimers possess a similar m/z ratio and fragmentation pathways. This leads to identical MS/MS transitions. As the chromatographic separation of epimers was complete, however, the differentiation of catechin derivatives can be safely assessed in UHPLC–MS/MS. In this separation, the sensitivity was far better than that of UV with signal-to-noise (calculated with an internal algorithm from the Masslynx software) comprised between 200 for GCC and 1000 for AC at a concentration of 1 μg/mL.
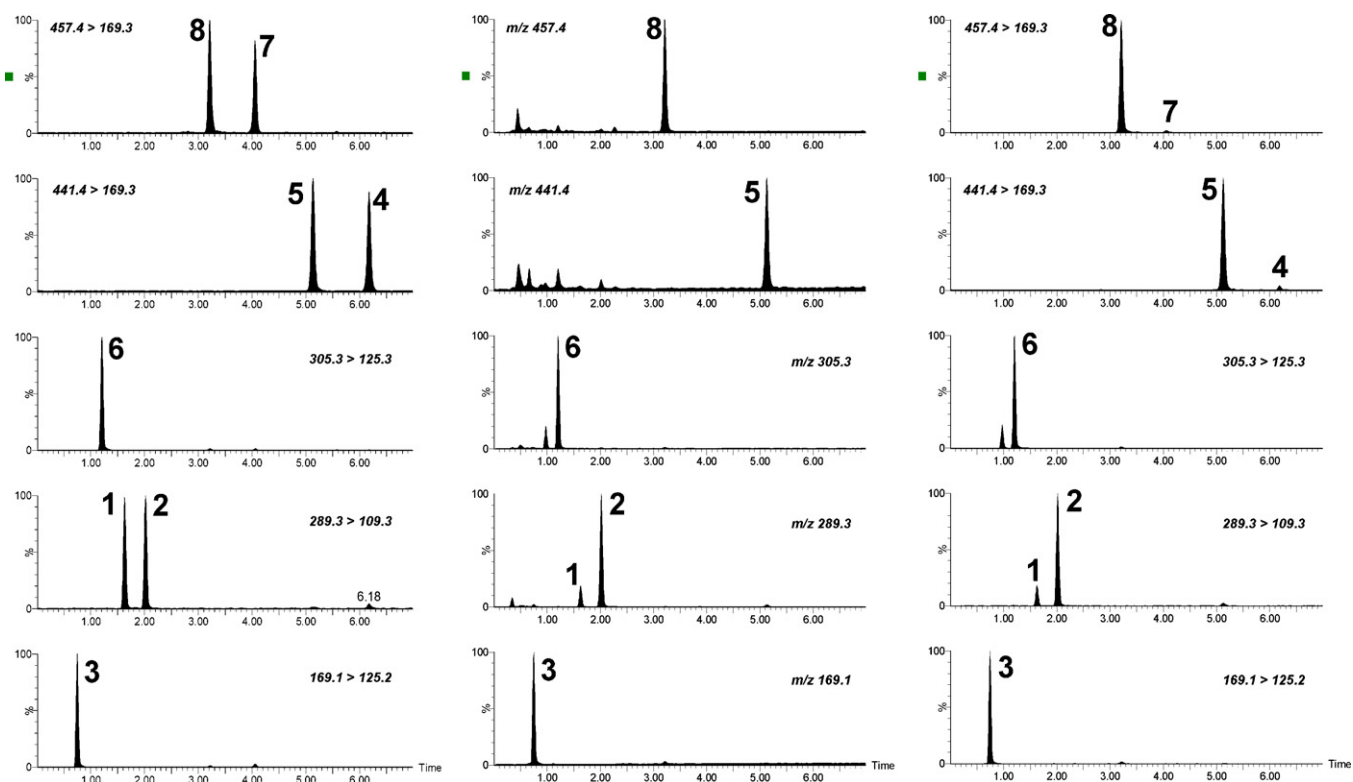
Fig. 7. UHPLC–MS/MS analysis of seven-catechin derivatives and gallic acid. Chromatographic conditions were similar to that of Fig. 3B. (A) Mixture of eight polyphenols standards at 1 μg/mL in the SRM mode. (B) 10-fold diluted commercial tea extract infused for 5 min with polyphenols detected in the SIR mode (UHPLC–MS). (C) 10-fold diluted commercial tea extract infused for 5 min with polyphenols detected in the SRM mode (UHPLC–MS/MS). 1: C, 2: EC, 3: AC, 4: CG, 5: ECG, 6: EGC, 7: GCG, 8: EGCG.

A real sample of diluted tea extract was injected into the system to highlight the elevated selectivity of the MS device. Initially, the detector was used as a single quadrupole instrument operating in the SIM mode, and the chromatogram is reported in Fig. 7B. Under these conditions, numerous contaminants were observed on all channels, particularly for the corresponding m/z ratio of EGCG and ECG, and this led to limited sensitivity and selectivity. The low sensitivity and selectivity can obviously be attributed to the low resolution of the single quadrupole instrument, which is around 0.7–1 FWMH. The same tea extract sample was also analyzed with an MS instrument operating in the tandem mode. As observed in Fig. 7C, the selectivity was significantly improved, and the qualitative discrimination of each catechin was very straightforward based on their retention time and MS/MS transitions. Under these conditions, the sensitivity was excellent with signal-to-noise higher than 500 for AC in the 10-fold diluted tea sample. Only a contaminant peak at 0.99 min was observed for the transition of EGC, but this peak possessed a different retention time from that of peak number 6. Similarly to the LLE–UHPLC–UV, it is also possible to establish a qualitative ranking between the different catechins present in our commercial tea sample using the UHPLC–MS/MS method. The abundance of catechins can now confidently be classified as follow: EGC > EC > EGCG > ECG > C > CG > GCG, by comparing the chromatogram of tea extract with that of standard catechins in pure water.

4. Concluding remarks

The aim of this study was to show the possible benefits of using columns packed with sub-2-μm particles in ultra-high pressure conditions (UHPLC) for the qualitative determination of various catechin derivatives in tea extracts. After a careful selection of the most appropriate column chemistry (Acquity BEH Shield RP18), it was demonstrated that the separation of eight standard polyphenols could be achieved in about 30 s while maintaining sufficient resolution. When dealing with complex matrices such as tea extracts, however, which could possess hundreds of constituents, the resolving power becomes more important than throughput. For this reason, longer columns have been tested, and a good compromise was obtained with a 100-mm column using a gradient from 13% to 22.2% ACN in 7.05 min. In real tea extracts, the unambiguous UV identification of catechins always remains tedious, and quantitation also remains critical.

For this reason, two procedures involving 2-D experiments were implemented. On the one hand, the complexity of tea extract samples was reduced with the help of a simple, rapid purification procedure. A liquid–liquid extraction with ethyl acetate as the organic solvent was employed prior to the UHPLC–UV analysis, and this demonstrated some evident benefits for catechin determination. On the other hand, UHPLC was coupled to MS/MS detection to attain a sufficient sensitivity and selectivity between catechin derivatives and other constituents of the tea extract sample. This strategy was found to be extremely well suited as selectivity remains excellent in the SRM mode even with complex tea extracts.

As shown in this paper, the two proposed strategies, namely LLE–UHPLC–UV (gold standard for routine analysis) and UHPLC–MS/MS, are very useful for an unambiguous qualitative determination of catechins in tea. A study about quantitation of catechins in tea by these two approaches is under way.

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