Talanta 94 (2012) 232–239

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Simultaneous determination of caffeic acid phenethyl ester and its metabolite caffeic acid in dog plasma using liquid chromatography tandem mass spectrometry

Caiming Tang [a], O. Samuel Sojinu [b,*]

[a] State Key Laboratory of Organic Geochemistry, Guangzhou Geochemistry Institute of Chinese Academy of Sciences, Guangzhou 510640, China
[b] Department of Chemical Sciences, Redeemer's University, Klm 46/48 Lagos-Ibadan Express Way, Nigeria

ARTICLE INFO

Article history:
Received 25 February 2011
Received in revised form 5 July 2011
Accepted 9 July 2011
Available online 21 March 2012

Keywords:
Caffeic acid phenethyl ester
Caffeic acid
Dog plasma
Stability
Liquid chromatography tandem mass spectrometry

ABSTRACT

A simple, reliable and sensitive method for the simultaneous determination of caffeic acid phenethyl ester (CAPE) and its metabolite caffeic acid (CA) in dog plasma was developed using liquid chromatography tandem mass spectrometry (LC–MS/MS). The sample pretreatment generally involved protein precipitation treatment (PPT) and direct dilution. CAPE and CA were separated with a C18 reversed-phase column. Electrospray ionization (ESI) interface operated in negative mode was chosen for ionization. Multiple reaction monitoring (MRM) mode was selected for data acquisition. The quantification range was 10.0–10,000.0 ng mL^{-1}. The intra- and inter-batch accuracies were within 92.5–107.0% with relative standard deviation (RSD, %) no more than 10.5%. CAPE and CA were proved to be stable in stabilizer-treated dog blood and PPT-treated plasma during the sampling and pretreatment period. The applicability has been evaluated with real samples from treated dogs.

© 2012 Published by Elsevier B.V.

1. Introduction

Caffeic acid phenethyl ester (CAPE), a polyphenolic ester firstly reported in 1987 [1], is an important bioactive compound present in propolis. In recent years, CAPE has attracted increasing scientific interest due to its various pharmacological activities, *e.g.*, anticancer/tumor [2–6], antiviral [7,8], anti-inflammatory [9–13], antioxidant [14–17], cytoprotection, [18–22] and vasorelaxant effects [23,24]. The pharmacological effects and mechanisms of CAPE have been comprehensively reviewed recently [25]. Rodent experiment has proved that CAPE can be easily hydrolyzed both *in vivo* and *in vitro* [26]. The main hydrolyzed metabolite is caffeic acid (CA), a natural product also, possessing several biological and physiological activities [27]. These activities include antioxidant [27,15], bactericidal [28,29], anti-tumor [30,31] and anti-obesity effects [32]. In light of the activities of CA, the major metabolite of CAPE *in vivo*, it is necessary to analyze the bioactivities of CA when studying CAPE. Accordingly, the pharmacokinetic parameters of CAPE and CA in animal or human body should be determined when CAPE is studied as a therapeutic agent.

To obtain better pharmacokinetic and metabolic information of CAPE and CA in animal and human body, the analytical methodologies should be established to quantitatively analyze CAPE and CA in biological matrices. So far, several methods have been developed for the determination of CAPE using high performance liquid chromatography connected to ultraviolet detector (HPLC–UV) [26,33,34], liquid chromatography mass spectrometry (LC–MS) [35] and LC–MS/MS [36,37]. Moreover, a variety of methods has been established to identity or quantify CA in various matrices including plant tissues [38–41], rat plasma [42,43], human plasma [44,45] and human serum [46]. Those methods involved high performance liquid chromatography coupled with electrochemical detector [47–49], HPLC–UV [50], gas chromatography coupled with mass spectrometry [51], LC–MS [39,40] and LC–MS/MS [41,43–45]. Nevertheless, no study has reported a method for the simultaneous determination of CAPE and CA in biological matrices. In addition, the stability of the two compounds, particularly for CAPE, in plasma and blood challenges the analytical chemists during the method development and validation. CAPE is liable to be enzyme-hydrolyzed to CA in plasma both *in vivo* and *in vitro*. Moreover, both CAPE and CA can be easily oxidized at ambient temperature. Therefore, the stability of CAPE and CA in plasma and blood is a critical concern for developing a reliable method to analyze the two compounds.

In this study, we aimed to develop and validate a stable and reliable method for the simultaneous determination of CAPE and

* Corresponding author. Tel.: +234 7066489038.
E-mail addresses: tcmcasgig@gmail.com (C. Tang), sojinuok2000@yahoo.com (O.S. Sojinu).

0039-9140/$ – see front matter © 2012 Published by Elsevier B.V.
doi:10.1016/j.talanta.2012.03.029

CA in dog plasma. The sampling procedures and sample pretreatment were optimized to enhance and ascertain the stability of the analytes during the experimental processes.

2. Materials and methods

2.1. Chemicals and reagents

CAPE (≥99.3%), CA (≥99.0%) and the isotope-labeled internal standard caffeic acid-$^{13}C_9$ (CA-$^{13}C_9$, ≥99.1%) were purchased from Sigma–Aldrich Corp. (St. Louis, MO, USA). Benzyl caffeate (BC, ≥99.0%), used as the internal standard for CAPE, was bought from Resealife (Burgdorf, Switzerland). HPLC-grade reagents including acetonitrile (ACN) and methanol (MeOH) were obtained from Merck (Darmstadt, Germany). Ethyl acetate, formic acid (FA), thenoyltrifluoroacetone (TTFA), citric acid (CTA) and ammonium acetate (NH_4AC) were of HPLC grade and purchased from Sigma–Aldrich Corp. (St. Louis, MO, USA). HPLC-grade trifluoroacetic acid (TFA) was purchased from Fluka (Milwaukee, WI, USA). All the water used in this study was ultra-pure and prepared with an ELGA water purifying system (ELGA LabWater Corporation, Marlow, UK).

2.2. Equipments

The LC–MS/MS system consisted of a Shimadzu 20 series HPLC instrument (Shimadzu, Kyoto, Japan), a CTC auto-sampler (CTC Analytics, Zwingen, Switzerland) and an API 4000 (Applied Systems, Forster City, CA, USA) triple quadruple mass spectrometry equipped with an electrospray ionization source. Chromatographic separation was performed with a CAPCELL PAK C18 column (2.0 mm × 100 mm, 5 μm, Shiseido, Tokyo, Japan).

2.3. Working solutions

Around 2 mg of each analyte and internal standard was weighted and dissolved with corresponding volume of MeOH to get a stock solution at the concentration of 1.0 mg mL^{-1}. The working solutions of the analytes as well as the internal standards were prepared *via* serial dilution from the corresponding stock solutions with 50% MeOH (H_2O/MeOH, 1:1, v/v). The concentrations of the cocktailed calibration working solutions of CAPE and CA were 200.0–200,000.0 ng mL^{-1}, and those of the quality control working solutions were from 200.0 to 160,000 ng mL^{-1}. For the internal standards, the concentration of the cocktailed working solution was 1000.0 ng mL^{-1}. Amber glass bottles were used for preparing these solutions. The stock solutions and the working solutions were stored in a freezer at −20 °C and protected from light prior to use.

2.4. Calibration and quality control samples

The calibration samples and quality control samples (QCs) were prepared by spiking the relevant working solutions into the blank dog plasma with the dilution factor of 20. These samples were vortex-mixed sufficiently and MeOH was added at three-fold volume of the spiked plasma. The QCs were stored in dark at −80 °C condition prior to treatment and analysis.

2.5. LC–MS/MS conditions and parameters

The mobile phase A (MPA) was 0.5% FA in water containing 1 mM NH_4AC and the mobile phase B (MPB) was 0.5% FA in MeOH/ACN (1:1, v/v) containing 1 mM NH_4AC. The gradient elution program was set as follows: initial–1.00 min, linear from 10% to 20% MPB; 1.00–2.50 min, linear from 20% to 40% MPB; 2.50–3.00 min, 80% MPB; 3.00–3.10 min, linear from 80% to 90% MPB; 3.10–4.00 min, 90% MPB; 4.00–4.01 min, linear from 90% to 100% MPB; 4.01–4.5 min, 100% MPB; 4.50–4.51 min, linear from 100% to 10% MPB; and 4.51–6.3, 10% MPB. The flow rate was set at 0.30 mL min^{-1} and the injection volume was 10 μL. The column thermostatic oven and the autosampler were maintained at 20 °C and 4 °C, respectively.

The mass spectrometric parameters were optimized as follows: ESI was operated in negative mode; resolution of quadrupole-1 and quadrupole-3 was unit (0.7 ± 0.1 amu); the curtain gas, collision-activated dissociation gas, gas 1, and gas 2 were set at 25, 8, 50 and 55 psi, respectively; source temperature was 550 °C; ionization voltage was −4200 V; both entrance potential, and collision cell exit potential were set at −10 V; dwell time was 80 ms. All the gases used were high-pure nitrogen. MS acquisition was done with MRM mode. The MRM conditions and parameters including ion transitions, declustering potential and collision energy are reported in Table 1. The MRM transitions were chosen for the quantification and the identification of the investigated analytes. In addition, the retention time consistency of transitions in real samples and in standard samples was used to identify the analytes. The data acquisition and processing were performed with the software Analyst 1.4.2 (Applied Systems).

2.6. Sampling procedures

Sampling tube were prepared as follows: aliquot 100 μL of TTFA solution (TTFA in MeOH/ACN = 1:1, v/v, 5 mg mL^{-1}) and 100 μL of CTA solution (CTA in MeOH, 50 mg mL^{-1}) were placed in a polypropylene tube and evaporated to dryness under a gentle nitrogen stream at 40 °C. Those stabilizer-treated tubes were stored at −20 °C condition before use.

The experimental dogs (Beagle, Marshall BioResource, Beijing, China) were orally dosed with CAPE (dissolved in pure water) at a dose of 125 mg per one dog. Blood samples were sampled with lower limb venous sampling and collected at 0, 1, 2, 4, 8 and 24 h. Appropriate volume of dog blood (400 μL) was placed into a stabilizer-treated tube and vortex-mixed for 1 min. After mixing, the mixture was centrifuged with a rotating speed of 12,000 rpm at 1 °C for 5 min. Thereafter, the upper-layer plasma (100 μL) was transferred to a new tube followed by the addition of MeOH at three-fold volume of the plasma. The mixture was vortex-mixed for 3 min and stored at −80 °C condition prior to use. The sampling tubes and methanol were cooled with dry ice prior to use. The blank dog plasma was harvested using the same procedures for the real samples.

2.7. Sample pretreatment

The protein precipitation treated samples were centrifuged for 3 min with a rotating speed of 12,000 rpm at 0 °C. After

Table 1
Optimized mass spectrometric parameters for caffeic acid phenethyl ester (CAPE), caffeic acid (CA) and their internal standards.

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	Declustering potential (V)	Collision energy (eV)	Dwell time (ms)
CAPE	283.0	135.0	−90	−60	80
Benzyl caffeate	268.8	138.8	−90	−45	50
CA	178.8	134.8	−60	−33	80
CA-$^{13}C_9$	188.0	142.8	−95	−30	50

centrifugation, an aliquot 50 μL of the supernatant was transferred to a well of a 96-well-plate followed by adding 100 μL of MeOH, 20 μL of internal standard working solution and 150 μL of water. Then the plate was sealed and vortex-mixed for 5 min. Thereafter, 10 μL of the mixture was injected onto the LC–MS/MS system.

2.8. Method validation

2.8.1. Calibration curves

Quantification was performed with internal standard method, using benzyl caffeate and CA-$^{13}C_9$ as the internal standards for CAPE and CA, respectively. In consideration of the impact of matrix effect, the calibration samples were prepared using the stabilizer-treated blank dog plasma. The concentrations of the spiked calibration samples were 10.0, 20.0, 50.0, 100.0, 500.0, 2000.0, 5000.0, and 10,000.0 ng mL^{-1}. Two groups of calibration samples were set for each analytical batch. The first one was injected at the beginning of the batch and the other at the end. The two-group calibration samples were combined to achieve an 8-point calibration curve with the weight factor of $1/x^2$. The linear range (10.0–10,000.0 ng mL^{-1}) was ascertained to encompass the expected concentration in the real samples as much as possible.

2.8.2. Accuracy and precision

Six replicates of QCs were prepared at four concentration levels including lower limit of quantification QC (LLQC, 10.0 ng mL^{-1}), low QC (LQC, 30.0 ng mL^{-1}), medium QC (MQC, 400.0 ng mL^{-1}) and high QC (HQC, 8000.0 ng mL^{-1}). These QCs, interspersed among individual batch, were analyzed for the determination of inter-batch accuracy and precision. The inter-batch accuracy and precision were determined using three independent analytical batches of the QCs. The accuracy was expressed as the percentage of the determined concentration to the nominal concentration of the QCs and calibration samples. RSD was calculated to evaluate the precision of the method. The acceptance criteria were in accordance with the USFDA guidance for industry [52]. For the acceptable calibration samples and QCs, the relevant accuracies should be within 85–115% with the precision $\leq$ 15%, except for LLQC samples, for which the accuracies should be within 80–120% with the precision $\leq$ 20%.

2.8.3. Limit of quantification

The limit of quantification was defined as the concentration of LLQC sample that should be capable of producing the signal at least five times above the noise level produced by a blank sample.

2.8.4. Matrix effect, recovery and carryover effect

The matrix effect was expressed as the percentage of the peak area from the post-extraction spiked samples to that from the neat solutions at the same nominal concentrations. The recovery was evaluated in percentage comparing the peak area from the normal QCs to that from the post-extraction spiked samples. The carryover was assessed with the comparison of the peak area at the retention time of the analyte in the blank sample which was injected following the injection of the upper limit of quantification sample to that of the upper limit of quantification sample.

2.8.5. Stability

The stability was a main challenge of this study. The stability was assessed with the percentage of the concentration from the stability samples to that from the samples quantified just after preparation at the same nominal concentrations. The stability of CAPE, as well as CA, in dog blood and plasma was studied. The stability of CAPE and CA in stabilizer-treated dog blood at room temperature (RT) condition and on ice bed was studied. The stability of CAPE and CA in protein precipitation treated plasma at RT, on ice bed and at −80 °C was tested. The freeze-thaw stability was tested after the protein precipitation treated samples have undergone three freeze-thaw cycles. The auto-sampler stability was assessed by reanalyzing the analytical batch after it was stored in auto-sampler at 4 °C for 48 h.

3. Results and discussion

3.1. Optimization for sampling procedures

As described in the previously published literatures [26,34,36], CAPE is liable to be enzyme-hydrolyzed in rat plasma under ambient temperature condition. In those studies, stabilizer additives sodium fluoride (NaF) was added into the samples to enhance the stability of CAPE in rat plasma during sample pretreatment. Additionally, low temperature and low pH environment has been proved to be helpful to improve the stability of CAPE in those studies.

In this study, we chose TTFA and CTA as the stabilizer additives. TTFA, an inhibitor of carboxylesterase activity [53], would more specially inhibit the enzymatic degradation of CAPE compared with NaF. Therefore, TTFA was preferentially tested as the stabilizer in this study. Instead of acetate buffer, CTA was selected to prepare the sample tubes. Because CTA is a nonvolatile acid and in possession of relatively low pK_a value, it is feasible to stay in tube in solid state and can offer enough acidity for the sample. During the experiment of the blood stability of CAPE and CA, NaF was also evaluated. NaF was chosen as an additional additive after the addition of TTFA and CTA. Yet, the experimental result showed the stability could not become better when NaF was additionally added. In addition, due to its low solubility in organic solvents, NaF should be dissolved in water before preparing the sampling tubes. This could lead to difficulty and long time consumption to evaporate the water during the preparation of sampling tubes. Eventually, NaF was not used in this study.

No study has reported the stability of CAPE and CA in blood samples. However, the time range from venous blood collection to plasma harvesting should not be neglected, particularly for the susceptive and unstable compounds such as CAPE and CA. Therefore, we prepared stabilizer-treated sampling tubes before blood collection. Once infused into sampling tubes, the blood samples were mixed with stabilizer additives by vortex-mixing.

The stability of CAPE and CA in stabilizer-treated dog plasma was tentatively studied without the addition of organic solvent at first. However, the stability was less than 2 h on ice bed. Accordingly, MeOH was added into the tube just after the plasma harvesting, which could enhance the stability and ascertain CAPE was stable in dog plasma (TTFA and CTA inside) on ice bed at least for 2 h and CA at least for 4 h. In addition, the other objective of MeOH addition was to suppress the mutual conversion between the analytes and their glucuronide metabolites in real samples. It has been reported the glucuronide metabolites can be back-converted to their parent drugs, which could lead to the inaccurate results in bioanalytical assay [54]. Celli et al. reported that the concentration of CAPE glucuronide metabolites was much higher than that of CAPE [36]. Accordingly, it was necessary to inactivate the glucuronidase with MeOH to suppress the conversion and back-conversion between the analytes and their glucuronide metabolites. This could help to achieve reliable and repeatable experimental results.

3.2. Sample pretreatment optimization

Liquid–liquid extraction was preliminarily applied to the pretreatment in study, because it was used in most published studies [34,36,37,45]. Although liquid–liquid extraction is a feasible protocol to process the samples, it is more tedious and time-consuming compared with direct dilution treatment. Moreover, the

evaporation procedure in liquid–liquid extraction protocol may lead to oxidization of the analytes. In this study, with direct dilution pretreatment, no significant matrix effect and no interferent were observed for the analytes, which demonstrated the pretreatment was feasible. The final reagent composition of the injection solution was MeOH/H_2O (1:1, v/v), which was optimized to enhance the autosampler stability and reduce the carryover effect.

3.3. Optimization of LC–MS/MS conditions

The polyphenols, such as the CAPE and CA, can be detected with negative ESI mass spectrometry. Therefore, ESI operated in negative mode was preferentially selected as the ionization source for CAPE and CA. For optimizing the parameters and working conditions of mass spectrometry, a neat solution containing individual analyte or internal standard compound (1.0 μg mL^{-1} in 50% MeOH) was directly infused into the mass spectrometer with a Harvard syringe pump at a flow rate of 10 μL min^{-1}. The precursor ion was firstly found using MS-1 scan mode. Then, product ion scan mode was applied to find the product ions. The mass spectrometric fragmentation patterns of the analytes and the internal standards are provided in Fig. 1.

The most abundant product ion observed was *m*/*z* 135 for CAPE and CA both, which was in accordance with the previous studies [26,37,41]. The ion *m*/*z* 135 is the decarboxylated product from the caffeate ion (*m*/*z* 179) which is the precursor ion of CA and the ester-cleaved product ion of CAPE. The other major product ion *m*/*z* 161 may be due to the dehydration from the caffeate ion (*m*/*z* 179). For the internal standard benzyl caffeate, the most abundant product ion was *m*/*z* 134, which was in accordance with the study of Wang et al. [37]. In their study, methyl caffeate was used as the internal standard and its most abundant product ion was *m*/*z* 134 also.

After the precursor ions and the most abundant product ions were found, the MRM transitions were ascertained to monitor the analytes and the internal standards. The mass spectrometric parameters and working conditions were optimized sequentially.

Yet the optimum declustering potential and the collision energy were not used in the final method. We observed CAPE could produce higher signal response compared with CA at the sample concentration level with the optimum MRM parameters. Unsuitably high signal response may lead to the signal saturation in mass spectrometry. Therefore, the declustering potential and the collision energy were enhanced to decrease the signal response of CAPE and CA. The baseline in the MRM transition channel of CA was very high. For this troubleshooting, collision energy was enhanced, which evidently decreased the baseline of CA.

Reversed-phase liquid chromatography column was commonly used for the determination of CAPE or CA in most previous studies. Several types of reversed-phase columns including Zorbax-C18 (2.0 mm × 50.0 mm, 5 μm, Agilent), Luna-C18 (2.0 mm × 50.0 mm, 5 μm, Phenomenex), CAPCELL PAK C18 (2.0 mm × 50 mm, 5 μm, Shiseido) and CAPCELL PAK C18 (2.0 mm × 100 mm, 5 μm, Shiseido) were tested in this study. Sound peak shape of the analytes could be achieved using all of those columns. However, only when the 100-mm CAPCELL PAK C18 column was used, could the sufficient chromatographic resolution for the analytes and their metabolites in the real samples be obtained.

The mixture of ACN and MeOH (1:1, v/v) was chosen as the organic mobile phase in order to achieve appropriate retention time and satisfying chromatographic resolution for the analytes. In light of its polar properties, CA can be easily ionized and deprotonated in aqueous solution. This might result in poor retention of CA on reversed-phase column. Therefore, FA was added into both MPA and MPB at a relatively high concentration of 0.5% (v/v) to suppress

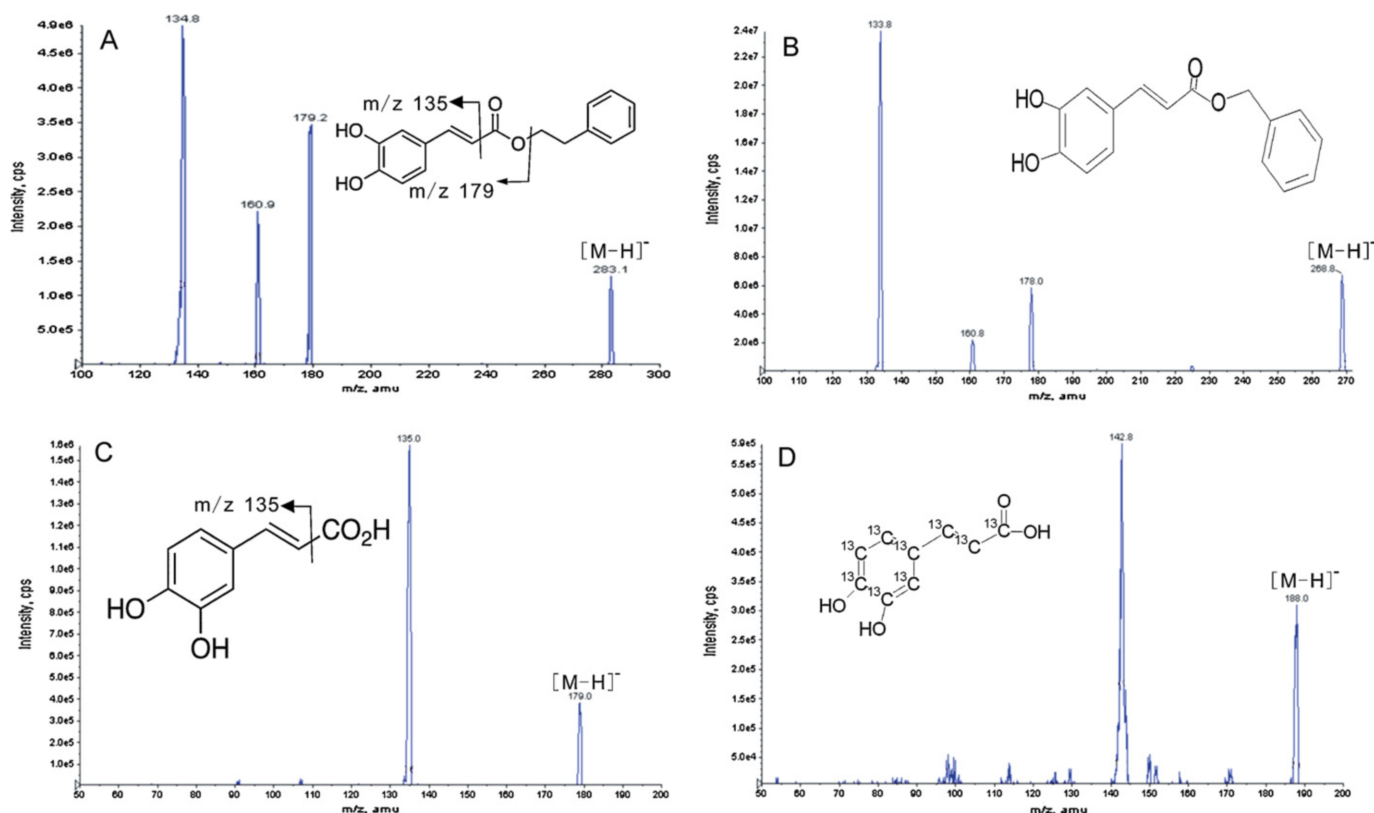

Fig. 1. Ion fragmentation patterns and chemical structures of caffeic acid phenethyl ester (A), benzyl caffeate (B), caffeic acid (C) and caffeic acid-$^{13}C_9$ (D). CAPE: caffeic acid phenethyl ester; CA: caffeic acid.

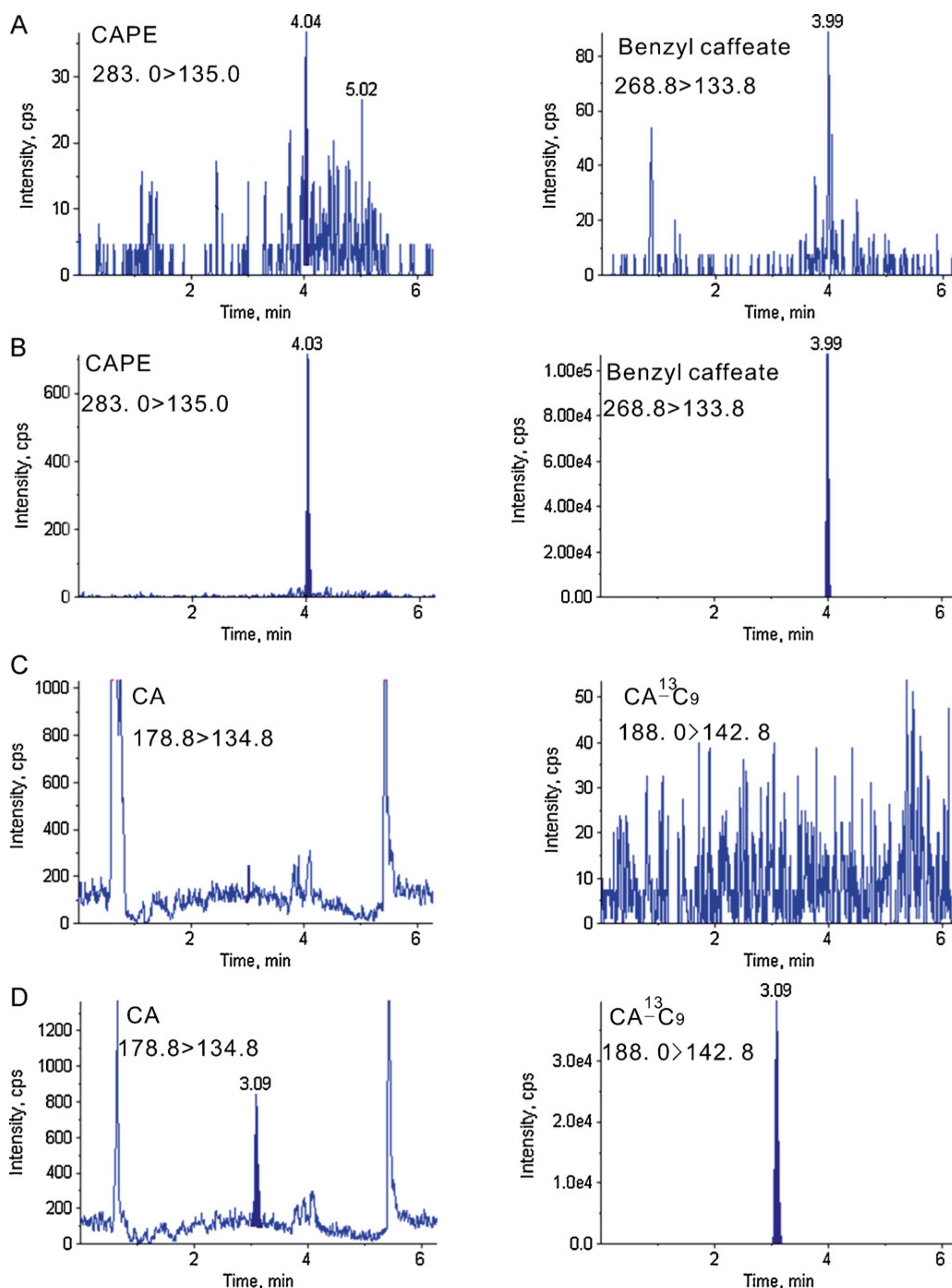

Fig. 2. Representative chromatograms of caffeic acid phenethyl ester (CAPE) and caffeic acid (CA) in dog plasma. (A): Chromatograms of CAPE (left) and benzyl caffeate (right) in blank dog plasma sample; (B): chromatograms of CAPE (left) and benzyl caffeate (right) in lower limit of quantification quality control (LLQC) sample (10.0 ng mL^{-1}); (C): chromatograms of CA (left) and caffeic acid-$^{13}C_9$ (CA-$^{13}C_9$, right) in blank dog plasma sample; (D): chromatograms of CA (left) and CA-$^{13}C_9$ (right) in LLQC sample (10.0 ng mL^{-1}). The peaks of the analytes and internal standards are color-filled.

the deprotonation process of CA during chromatographic separation. The retention and the peak shape were improved after the addition of FA in mobile phase. The addition of NH_4AC in mobile phase could decrease the signal response. However, it was found to be helpful for improving the linearity of the analytes. Accordingly, NH_4AC was added at a low concentration to improve comprehensive experiment results. With the optimized chromatographic condition, satisfactory peak shape and suitable retention time of the analytes and internal standards were obtained (Fig. 2).

Some metabolites, probably the glucuronide metabolites of CAPE and CA, were eluted from the column very close to their parent compounds. Therefore, a gentle gradient elution program was set to enhance the chromatographic resolution. As illustrated in Fig. 3, several peaks produced by the metabolites were observed in the real samples. Nevertheless, with the optimum chromatographic elution program, sufficient chromatographic resolution of those compounds was achieved.

Comparing the chromatograms from the spiked samples with those from the real samples (Figs. 2 and 3), we concluded that using real samples to conduct the method development was necessary. In this study, several phase II metabolites were found. These metabolites, generated in the *in vivo* metabolic processes including glucuronidation, sulfation and methylation, can be in-source-dissociated to the precursor ion of their parent compounds.

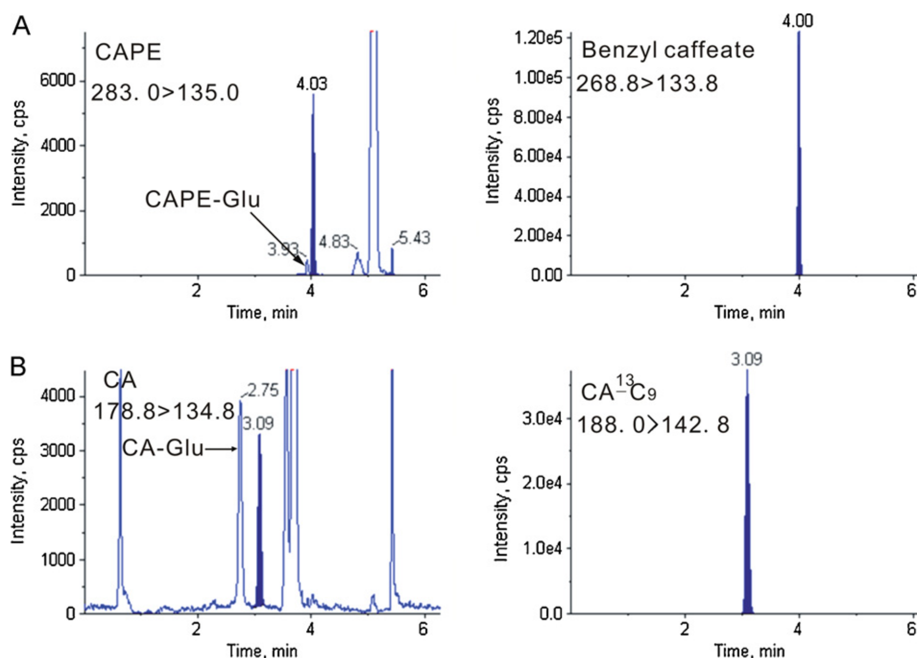

Fig. 3. Representative MRM chromatograms of caffeic acid phenethyl ester (CAPE) and caffeic acid (CA) in real sample (plasma from dosed dog). (A): Chromatograms in the MRM-transition channels of CAPE (left) and benzyl caffeate (right) in real sample; (B): chromatograms in the MRM-transition channels CA (left) and CA-$^{13}C_9$ (right) in real sample. Glu: glucuronide. The color-filled peaks belong to the analytes and internal standards.

If these metabolites cannot be separated form their parent compounds, the quantification of the target analytes will be influenced probably. Up to now, no study has reported the separation of CAPE, CA and their phase II metabolites in the quantitative studies of CAPE and CA.

The glucuronide metabolites of CAPE and CA (indicated in Fig. 3) were identified using precursor ion scan mode and neutral loss scan mode. The Supplementary data of this paper provides the details for identifying these glucuronide metabolites. The study of other metabolites, generating or not generating peaks in the current MRM transition channels, is ongoing in our further studies.

3.4. Quantification

3.4.1. Calibration curves and limit of quantification

The calibration curves for CAPE and CA were performed between 10 and 10,000 ng mL^{-1}. The intra-batch accuracies of the calibrators were within 98.3–101.2% and 97.2–103.0% for CAPE and CA, respectively. The inter-batch accuracies of the calibrators for CAPE were 95.2–104.0% with precision (RSD, %) no more than 4.8%. For CA, the inter-batch accuracies were 97.9–102.0% with RSD within 2.1–4.7%. The comprehensive accuracy and precision results of the calibration are summarized in Table 2. The representative quadratic calibration equations from the validation run were $Y = -5.2e-008X^2 + 0.00277X + 0.00274$ ($R = 0.9998$) and $Y = -7.7e-008X^2 + 0.00195X + 0.00072$ ($R = 0.9983$) for CAPE and CA, respectively. As shown in Fig. 2, the signal to noise ratio of the analyte peaks from the LLQC samples (10.0 ng mL^{-1}) are more than 5, which can fulfill the acceptance criterion.

3.4.2. Accuracy and precision

The accuracy and precision experiments were performed with four levels of QCs (LLQC, LQC, MQC and HQC). The intra-batch accuracies were 95.0–104.9% with RSD ≤ 7.6% and 92.5–104.3% with RSD ≤ 5.1% for CAPE and CA, respectively. The inter-batch accuracies were 101.3–107.0% with RSD ≤ 10.5% for CAPE and 98.8–102.8% with RSD ≤ 6.6% for CA, respectively. The comprehensive intra-/inter-batch accuracy and precision results are provided in Table 3.

3.5. Recovery and matrix effect

Satisfactory recoveries of the analytes were obtained. The recoveries of CAPE and CA at the low, middle and high concentration levels were within 97.9–110.2% with the RSD less than 5.8% (Table 4). Six lots of single dog plasma were used to prepare the post-extraction spiked samples at the concentrations of LQC, MQC and HQC. And the neat solutions were prepared with 50% MeOH (MeOH/H_2O, 1:1, v/v) at the same nominal concentrations. No significant matrix effect was observed for CAPE and CA. The matrix effect was within 102.2–112.5% with RSD ≤ 6.4% for CAPE and 89.9–94.1% with RSD ≤ 5.3% for CA, respectively (Table 4). The results unambiguously demonstrated that the co-eluted endogenous compounds did not influence the ionization of the analytes, thus did not affect the assay. Therefore, the pretreatment using protein precipitation and direct dilution was capable to ensure the ruggedness and sensitivity of the assay.

3.6. Selectivity and carryover effect

MRM scan mode can provide high selectivity in the biological analysis. As Fig. 2 shows, no obvious interference peak was observed at the retention times of the analytes and internal standards in blank samples. Furthermore, the selectivity in the real samples was investigated also. As illustrated in Fig. 3, the analytes were sufficiently separated from their metabolites. Precursor ion scan mode was applied to the investigation of selectivity. Both the product ion and the precursor ion of the individual analyte were fixed and regarded as the product ion to perform the precursor ion scan. If no same precursor ion was found at the retention time of the individual analyte with the two fixed product ions, we could confirm that no co-eluted metabolite interferent present in the real sample. Our experiment results proved there was no co-eluted metabolite interferent present in the real samples (data not shown).

Table 2
Precision and accuracy of the assay for caffeic acid phenethyl ester (CAPE) and caffeic acid (CA) in dog plasma with the calibration samples at eight concentration levels.

Sample name	Nominal concentration ($ng\,mL^{-1}$)	CAPE				CA			
		Intra-batch ($n=2$)		Inter-batch ($n=6$)		Intra-batch ($n=2$)		Inter-batch ($n=6$)	
		Back-calculated concentration (mean, $ng\,mL^{-1}$)	Mean accuracy (%)	Mean accuracy (%)	RSD (%)	Back-calculated concentration (mean, $ng\,mL^{-1}$)	Mean accuracy (%)	Mean accuracy (%)	RSD (%)
Level-8	10.0	10.1	100.5	99.2	4.8	10.1	100.9	100.2	3.3
Level-7	20.0	19.7	98.3	100.5	3.9	19.5	97.2	99.6	2.9
Level-6	50.0	49.7	99.3	101.3	3.6	51.5	103.0	100.5	3.1
Level-5	100.0	101.2	101.2	101.9	3.3	99.8	99.8	99.8	2.1
Level-4	500.0	502.5	100.4	100.9	1.5	494.0	98.8	102.0	4.3
Level-3	2000.0	2015.0	100.7	97.1	3.1	1990.0	99.4	99.1	2.2
Level-2	5000.0	4920.0	98.5	95.2	4.3	5085.0	102.0	97.9	4.3
Level-1	10,000.0	10,075.0	100.8	104.0	4.7	9920.0	99.2	101.4	4.7

Table 3
Precision and accuracy of the assay for caffeic acid phenethyl ester (CAPE) and caffeic acid (CA) in dog plasma with the quality control samples at four concentration levels.

Analyte	Sample type	Nominal concentration ($ng\,mL^{-1}$)	Intra-batch ($n=6$)			Inter-batch ($n=18$)	
			Observed concentration (mean, $ng\,mL^{-1}$)	Mean accuracy (%)	RSD (%)	Mean accuracy (%)	RSD (%)
CAPE	LLQC	10.0	10.5	104.9	7.6	107.0	6.3
	LQC	30.0	30.5	101.7	5.7	105.5	6.4
	MQC	400.0	404.4	101.1	2.7	105.4	4.9
	HQC	8000.0	7595.0	95.0	1.8	101.3	10.5
CA	LLQC	10.0	9.3	92.5	5.1	98.8	6.6
	LQC	30.0	30.4	101.4	4.9	100.5	5.1
	MQC	400.0	416.5	104.3	4.8	102.8	4.1
	HQC	8000.0	8105.0	101.4	1.9	102.0	4.5

Table 4
Recovery and matrix effect for caffeic acid phenethyl ester (CAPE) and caffeic acid (CA) in dog plasma with the quality control samples.

Analyte	Sample type	Nominal concentration ($ng\,mL^{-1}$)	Mean recovery ($n=3$, %)	Precision (RSD, %)	Mean matrix effect ($n=6$, %)	Precision (RSD, %)
CAPE	LQC	30.0	110.2	5.8	102.2	2.1
	MQC	400.0	108.2	1.6	103.1	6.4
	HQC	8000.0	108.4	1.2	112.5	5.4
CA	LQC	30.0	100.2	3.1	90.1	5.3
	MQC	400.0	98.4	2.4	89.9	4.6
	HQC	8000.0	97.9	1.8	94.1	1.5

As mentioned in the literatures [36,38], carryover of CAPE was large when using LC–MS/MS for analysis. We used 0.5% TFA in ethyl acetate and 1% FA in ACN/MeOH/H_2O (1:1:1, v/v/v) as the strong washing solution and weak washing solution, respectively. This could sufficiently clean up the CAPE residues on syringe and valve. So, the carryover was mainly caused by the CAPE residue on the chromatographic column. The absolute carryover was about 0.2%. However, to reduce time consumption, we did not set any additional column-cleaning procedure in the elution program.

3.7. Stability

CAPE and CA were stable in stabilizer-treated dog blood at least for an hour at room temperature (Table 5), which indicated there was sufficient time for the blood sample collection and plasma harvesting. The stability of CAPE and CA in PPT-treated dog plasma (TTFA and CTA inside) was at least for 2 h and 4 h, respectively (Table 6). This could ascertain CAPE and CA were stable during the sample pretreatment (<2 h). Freeze-thaw stability experiment was performed with LQC and HQC samples stored at −80 °C and thawed at room temperature for three cycles within one week. The results indicated that CAPE and CA were stable after three freeze-thaw cycles and also proved the storage stability was at least for one week. The auto-sampler stability was investigated by re-injecting the batch after being stored in auto-sampler at 4 °C for 48 h. The accuracy and the precision of the re-injected batch were repeatable and acceptable.

Table 5
Stability of caffeic acid phenethyl ester (CAPE) and caffeic acid (CA) in stabilizer-treated dog blood in room temperature (RT).

Analyte	Sample type	Treatment	Mean percentage ($n=3$, %)
CAPE	LQC	RT 0 h	100.0
		RT 1 h	111.1
	HQC	RT 0 h	100.0
		RT 1 h	92.9
CA	LQC	RT 0 h	100.0
		RT 1 h	101.3
	HQC	RT 0 h	100.0
		RT 1 h	96.8

Table 6
Stability of caffeic acid phenethyl ester (CAPE) and caffeic acid (CA) in PPT-treated dog plasma (TTFA and CTA inside) on ice bed and after three freeze-thaw (FT) cycles.

Analyte	Sample type	Treatment	Mean percentage (n = 3, %)
CAPE	LQC	Ice bed 0 h	100.0
		Ice bed 2 h	96.7
		Ice bed 4 h	70.5
		3 FT-cycles	105.5
	HQC	Ice bed 0 h	100.0
		Ice bed 2 h	93.1
		Ice bed 4 h	80.1
		3 FT-cycles	104.4
CA	LQC	Ice bed 0 h	100.0
		Ice bed 2 h	100.6
		Ice bed 4 h	96.2
		3 FT-cycles	89.5
	HQC	Ice bed 0 h	100.0
		Ice bed 2 h	112.4
		Ice bed 4 h	98.2
		3 FT-cycles	100.6

4. Conclusions

In summary, we have developed and validated an LC–MS/MS method for the simultaneous determination of CAPE and CA in dog plasma. The sampling procedures, sample pretreatment, and LC–MS/MS working conditions were optimized to obtain reliable experimental results. Our results demonstrate that CAPE and CA are stable in the stabilizer-treated samples through the whole analysis. This method has been proved to be accurate, precise, selective and reliable. The application to real samples has proved the feasibility of the method.

Acknowledgments

The authors profoundly appreciate the assistance and some valuable suggestions from the colleague Mr. Bingtie Fu. The authors also wish to thank Dr. Qiuxin Huang and Dr. Chao Li (Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, Guangzhou, China) for their help in improving the quality of the English language of this paper. The authors specially appreciate the dedication from Dr. Zuojun Yu (Hawaii University, Honolulu, USA) for improving this paper. The valuable comments and suggestions from the anonymous reviewers were profoundly appreciated.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2012.03.029.

References

[1] V. Bankova, A. Dyulgerov, S. Popov, N. Marekov, Z. Naturforsch. [C] 42 (1987) 147–152.
[2] P. Onori, S. DeMorrow, E. Gaudio, A. Franchitto, R. Mancinelli, J. Venter, S. Kopriva, Y. Ueno, D. Alvaro, J. Savage, G. Alpini, H. Francis, Int. J. Cancer 125 (2009) 565–576.
[3] M. Watabe, K. Hishikawa, A. Takayanagi, N. Shimizu, T. Nakaki, J. Biol. Chem. 297 (2004) 6017–6026.
[4] H.F. Liao, Y.Y. Chen, J.J. Liu, M.L. Hsu, H.J. Shieh, H.J. Liao, C.J. Shieh, M.S. Shiao, Y.J. Chen, J. Agric. Food Chem. 51 (2003) 7907–7912.
[5] C. Chiao, A.M. Carothers, D. Grunberger, G. Solomon, G.A. Preston, J.C. Barrett, Cancer Res. 55 (1995) 3576–3583.
[6] H.J. Hwang, H.J. Park, H.J. Chung, H.Y. Min, E.J. Park, J.Y. Hong, S.K. Lee, J. Nutr. Biochem. 17 (2006) 356–362.
[7] M.R. Fesen, K.W. Kohn, F. Leteurtre, Y. Pommier, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 2399–2403.
[8] M.R. Fesen, Y. Pommier, F. Leteurtre, S. Hiroguchi, J. Yung, K.W. Kohn, Biochem. Pharmacol. 48 (1994) 595–608.
[9] N. Marquez, R. Sancho, A. Macho, M.A. Calzado, B.L. Fiebich, E. Munoz, J. Pharmacol. Exp. Ther. 308 (2004) 993–1001.
[10] M.M. Abdel-Latif, H.J. Windle, B.S. Homasany, K. Sabra, D. Kelleher, Br. J. Pharmacol. 146 (2005) 1139–1147.
[11] P. Michaluart, J.L. Masferrer, A.M. Carothers, K. Subbaramaiah, B.S. Zweifel, C. Koboldt, J.R. Mestre, D. Grunberger, P.G. Sacks, T. Tanabe, A.J. Dannenberg, Cancer Res. 59 (1999) 2347–2352.
[12] G. Scapagnini, R. Foresti, V. Calabrese, A.M. Giuffrida Stella, C.J. Green, R. Motterlini, Mol. Pharmacol. 3 (2002) 554–561.
[13] K. Natarajan, S. Singh, T.R. Burke, D. Grunberger, B.B. Aggarwal, Proc. Nati. Acad. Sci. U.S.A. 93 (1996) 9090–9095.
[14] G.F. Sud'ina, O.K. Mirzoeva, M.A. Pushkareva, G.A. Korshunova, N.V. Sumbatyan, S.D. Varfolomeev, FEBS Lett. 329 (1993) 21–24.
[15] J.H. Chen, C.T. Ho, J. Agric. Food Chem. 45 (1997) 2374–2378.
[16] S. Son, B.A. Lewis, J. Agric. Food Chem. 50 (2002) 468–472.
[17] L.Y. Hsu, C.F. Lin, W.C. Hsu, W.L. Hsu, T.C. Chang, Biol. Pharm. Bull. 28 (2005) 1211–1215.
[18] X.Y. Wang, S. Stavchansky, S.M. Kerwin, P.D. Bowman, Eur. J. Pharmacol. 635 (2010) 16–22.
[19] J. Yang, G.A. Marriner, X.Y. Wang, P.D. Bowman, S.M. Kerwin, S. Stavchansky, Bioorg. Med. Chem. 18 (2010) 5032–5038.
[20] A. Rastogi, J. Yang, X.Y. Wang, J. Bynum, S. Stavchansky, P.D. Bowman, FASEB J. 24 (2010), 760.2.
[21] J. Yang, S.M. Kerwin, X.Y. Wang, S. Stavchansky, J. Bynum, P.D. Bowman, FASEB J. 23 (2009), 937.8.
[22] Y.J. Chen, A.C. Huang, H.H. Chang, H.F. Liao, C.M. Jiang, L.Y. Lai, J.T. Chan, Y.Y. Chen, J. Chiang, J. Food Sci. 74 (2009) H162–H167.
[23] Y. Long, M. Han, J. Chen, X.Z. Tian, Q. Chen, R. Wang, Vasc. Pharmacol. 51 (2009) 78–83.
[24] C. Cicala, S. Morello, C. Iorio, R. Capasso, F. Borrelli, N. Mascolo, Life Sci. 73 (2003) 73–80.
[25] S.K. Jaganathan, M. Mandal, J. Biomed. Biotechnol. 2009 (2009) (Article ID 830616, 13 pages).
[26] N. Celli, L.K. Dragani, S. Murzilli, T. Pagliani, A. Poggi, J. Agric. Food Chem. 55 (2007) 3398–3407.
[27] I. Gülçin, Toxicology 217 (2006) 213–220.
[28] J.L. Ruiz-Barba, A. Garrido-Fernandez, R. Jimenez-Diaz, Lett. Appl. Microbiol. 12 (1991) 65–68.
[29] G. Tunçel, C. Nergiz, Lett. Appl. Microbiol. 17 (1993) 300–302.
[30] T.W. Chung, S.K. Moon, Y.C. Chang, J.H. Ko, Y.C. Lee, G. Cho, S.H. Kim, J.G. Kim, C.H. Kim, FASEB J. 18 (2004) 1670–1681.
[31] M.T. Huang, R.C. Smart, C.Q. Wong, A.H. Conney, Cancer Res. 48 (1988) 5941–5946.
[32] A.S. Cho, S.M. Jeon, M.J. Kim, J. Yeo, K.I. Seo, M.S. Choi, M.K. Lee, Food Chem. Toxicol. 48 (2010) 937–943.
[33] G.C. Ceschel, P. Maffei, A. Sforzini, S. Lombardi Borgia, A. Yasin, C. Ronchi, Fitoterapia 73 (Suppl. 1) (2002) S44–S52.
[34] X.Y. Wang, P.D. Bowman, S.M. Kerwin, S. Stavchansky, Biomed. Chromatogr. 21 (2007) 343–350.
[35] P. Del Boccio, D. Rotilio, J. Sep. Sci. 27 (2004) 619–623.
[36] N. Celli, B. Mariani, L.K. Dragani, S. Murzilli, C. Rossi, D. Rotilio, J. Chromatogr. B 810 (2004) 129–136.
[37] X.Y. Wang, J.H. Pang, R.A. Newman, S.M. Kerwin, P.D. Bowman, S. Stavchansky, J. Chromatogr. B 867 (2008) 138–143.
[38] A. Canini, D. Alesiani, G. D'Arcangelob, P. Tagliatesta, J. Food Compos. Anal. 20 (2007) 584–590.
[39] B. Boros, S. Jakabova, A. Dornyei, G. Horvath, Z. Pluhar, F. Kilar, A. Felinger, J. Chromatogr. A 1217 (2010) 7972–7980.
[40] H.W. Im, B.S. Suh, S.U. Lee, N. Kozukue, M.O. Kameyama, C.E. Levin, M. Friedman, J. Agric. Food Chem. 56 (2008) 3341–3349.
[41] S.G. Liao, L.J. Zhang, C.B. Li, Y.Y. Lan, A.M. Wang, Y. Huang, L. Zhen, X.Z. Fu, W. Zhou, X.L. Qi, Z.Z. Guan, Y.L. Wang, Rapid Commun. Mass Spectrom. 24 (2010) 2533–2541.
[42] S.J. Wang, Z.Q. Zhang, Y.H. Zhao, J.X. Ruan, J.L. Li, Rapid Commun. Mass Spectrom. 20 (2006) 2303–2308.
[43] Z.C. Zhang, M. Xu, S.F. Sun, X. Qiao, B.R. Wang, J. Han, D.A. Guo, J. Chromatogr. B 871 (2008) 7–14.
[44] Y. Matsui, S. Nakamura, N. Kondou, Y. Takasu, R. Ochiai, Y. Masukawa, J. Chromatogr. B 858 (2007) 96–105.
[45] P.A. Guy, M. Renouf, D. Barron, C. Cavin, F. Dionisi, S. Kochhar, S. Rezzi, G. Williamson, H. Steiling, J. Chromatogr. B 877 (2009) 3965–3974.
[46] X.C. Li, C. Yu, Y.B. Cai, G.Y. Liu, J.Y. Jia, Y.P. Wang, J. Chromatogr. B 820 (2005) 41–47.
[47] K. Azuma, K. Ippoushi, M. Nakayama, H. Ito, H. Higashio, J. Terao, J. Agric. Food Chem. 48 (2000) 5496–5500.
[48] S.M. Wittemer, M. Veit, J. Chromatogr. B 793 (2003) 367–375.
[49] M. Nardini, E. Cirillo, F. Natella, C. Scaccini, J. Agric. Food Chem. 50 (2002) 5735–5741.
[50] H.F. Wang, G.J. Provan, K. Helliwell, Food Chem. 87 (2004) 307–311.
[51] M.R. Olthof, P.C. Hollman, M.N. Buijsman, J.M. van Amelsvoort, M.B. Katan, J. Nutr. 133 (2003) 1806–1814.
[52] Food and Drug Administration, Guidance for Industry—Bioanalytical Method Validation, FDA, New York, 2001.
[53] J.G. Zhang, M.W. Fariss, Biochem. Pharmacol. 63 (2002) 751–754.
[54] A.M. Tan, W. Jin, F. Deng, S. Hussain, A. Musuku, R. Massé, J. Chromatogr. B 877 (2009) 3673–3680.