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Trans-crocin 4 is not hydrolyzed to crocetin following i.p. administration in mice, while it shows penetration through the blood brain barrier

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ABSTRACT

A novel, fit-for-purpose, highly sensitive, analytical UPLC-PDA methodology was developed and fully validated, according to ICH, FDA and EMA guidelines, for the rapid and accurate quantification of *trans*-crocin 4 (TC4) and crocetin (CRC) in mice plasma and brain after *i.p.* administration. A PDA based methodology shows a wider applicability as it is cost effective and can be easily and seamlessly adopted by the pharma industry. The separation of the analytes was performed on a C18 Hypersil Gold column with 2.5 min run time, employing the internal standard (ISTD) methodology. The two methods were successfully applied for the determination of CRC and TC4 in mouse plasma and brain after *i.p.* administration of TC4 (50 mg/kg) in a time range of 0–240 min. Due to the selection of *i.p.* administration route, the first-pass metabolism and/or gastric hydrolysis were bypassed, a fact that enhanced the bioavailability of TC4. Furthermore, TC4 was found to be capable of crossing the Blood Brain Barrier (BBB) and build up levels in the mouse brain, regardless of its highly hydrophilic character. CRC was not detected in any plasma or brain sample, although it has been reported that TC4 quickly hydrolyzes to CRC after *p.o.* administration. Therefore *i.p.* administration could be used in the case of TC4 for the accurate determination of its biological role. Overall, the developed methodology offers important information about the bioavailability of TC4 in mouse plasma and for the first time, demonstrates the ability of TC4 to penetrate the BBB and localize inside the brain.

1. Introduction

Saffron, the dried stigmas of the flower *Crocus sativus* L. is considered to be among the most expensive spices in the world. *Crocus sativus* L., a stemless perennial herb of the Iridaceae family which is native to Greece and Southwest Asia, was first cultivated in Greece for its' red stigmas (style branches). Saffron is mainly used as a traditional herbal medicine but also as a flavoring and food coloring agent in everyday life. Chemical analysis of *Crocus sativus* L. *stigmas* has shown the presence of a wide variety of different constituents including carotenoids (e.g., a- and b-carotene), mono- and bis-esters of crocetin, picrocrocin and safranal [1]. Picrocrocin, a colorless glycoside, is the β − d − glucoside of hydroxysafranal (4 − hydroxy − 2,6,6 − trimethyl − 1 − cyclohexenhe − 1 − carboxaldehyde) and its mainly responsible for saffron's well − known bitterness. Safranal on the other hand, is the principal substance of saffron's aroma [2]. The main bioactive saffron constituents are the crocins, which are mono − and bis − esters of crocetin (apocarotenoid) with glucose, gentiobiose and/or gentiotriose [3]. Depending on the type and amount of sugar(s) conjugated with crocetin as well as the space-configuration, there are many different crocins produced i.e. *cis/trans* − crocin − 2 (CC2/TC2), *cis/trans* − crocin − 3 (CC3/TC3), *cis/trans* − crocin − 4 (CC4/TC4) while TC4 is found to be the most abundant crocin in all saffron species studied so far [3].

Several studies indicate that saffron's constituents have been proven to be effective against a wide range of common disorders including coronary artery disease, [4-6] stomach disorders, hypertension [7], learning and memory impairment [8], dysmenorrhea and premenstrual syndrome (PMS) [9]. Furthermore, saffron or its main constituents have shown remarkable activity against some neurodegenerative diseases that nowadays affect significant percentages of the general population such as Alzheimer's Disease (AD) [10–12], Parkinson's Disease (PD) [13], depression [14] and schizophrenia [15]. Finally, saffron exhibits dose − dependent inhibitory response on breast cancer cells [16]. Due to

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its plethora of pharmacological properties saffron is not only regarded to be a precious spice but also a highly valuable and health-promoting herb [17].

TC4 is of raising interest due to its' promising pharmacological properties as well as its' nontoxic character [18, 19]. Interestingly *i.p.* administration of crocin up to 3 g/kg in mice did not show any mortality after 24 and 48 h. Therefore TC4 has been considered to be a practically low-toxic substance [20]. Anti-oxidant and anti-inflammatory activity of TC4 suggest its therapeutic potential against various nervous system disorders [8]. It has been shown that TC4 enhances the sexual activity of male rats [21], exhibits antidepressant effects in rodents [22], prevents oxidative stress in the hippocampus contributing to the prevention of deficits in spatial learning and memory [23] and also enhances the learning ability and improves memory [24]. However, recent studies have demonstrated that TC4 also possesses anti-AD and anti-PD activity as it has shown significant inhibitory effect on the fibrillation of apo-alpha-lactalbumin (a-alpha-LA), under amyloidogenic conditions [25] and also on beta-amyloid (Aβ) fibrillogenesis [12]. CRC, the main metabolite of TC4, has been shown to inhibit Aβ fibrillization and attributed to the stabilization of Aβ oligomers [26], enhanced the angiogenesis in rats [27] and it is considered to be a potent antitumor agent as it has been shown to act as scavenger of free radicals [28]. Furthermore, CRC has shown neuroprotective effects against brain injuries as it inhibited apoptosis at early stages of the injury as well as promoted the angiogenesis step [27].

However despite the broad bioactivity of crocins, there are only few studies focused on the pharmacokinetic properties of TC4 and CRC while most of them include plasma and/or urine [29–34]. Many of the methods reported in previous studies did not exhibit adequate sensitivity for the quantitation of crocin in biological samples, and only its metabolite, CRC, could be detected and assessed. Despite the fact that LC-MS/MS methodologies developed are slightly more sensitive [33], the incorporation of the PDA detector offers a wider linear range as well as wider laboratory applicability because of its lower cost. Therefore, there is a need to develop a low-cost, fast and sensitive methodology in order to assess the bioavailability of crocin and its metabolites in brain and plasma.

Moreover, several factors contribute to a compound's bioavailability such as the administration route, its lipophilicity, sex, age, genotype, hormonal status etc. of the dosed organism as well as the individuality of each subject etc. [35–37]. So far there has been no other study describing the bioavailability of TC4 and CRC after *i.p.* administration in mice plasma, as well as the bioavailability of these compounds in mice brain. Given that saffron extracts have shown neuroprotective effects, it is of a great importance to discover the correlation between plasma and brain circulation levels following TC4 administration. In this study, we describe the development of two fully validated UPLC analytical methodologies for the simultaneous quantification of TC4 and CRC in both mice plasma and brain. This is a rapid, robust and fully automated procedure, with a simple sample pretreatment and a high sample turnover with only 2.5 min run time.

Although there is a more sensitive methodology appearing in the literature [33], the currently developed methodology shows comparably low detection limits (2 vs 10 ng/mL of plasma). Nevertheless, the UPLC-PDA methodology for plasma is fit-for-purpose, as it exhibits a broader linear range (10–6000 ng/mL), whereas the levels determined by the bioavailability study are well higher than the LLOQ. Preliminary pilot studies concerning the concentration levels of TC4 in plasma showed the circulation of relatively high levels (> 400 ng/mL in plasma) indicating that a method's sensitivity in the low ng/mL area is not actually required. Furthermore, a PDA based methodology receives wider applicability as it is cost effective and can be easily and seamlessly adopted by the pharma industry. Considering the administration route, an *i.p.* administration methodology has been adopted in order to bypass the first-pass metabolism and/or gastric hydrolysis and gain a more holistic bioavailability profile of the substance (elimination of the liver-induced metabolism as well as exposure of TC4 in the low pH of the stomach) [38]. Hence, this study demonstrates for the first time the absence of hydrolysis of TC4 to CRC in plasma after *i.p.* administration, and provides preliminary evidence on the ability of TC4 to penetrate the Blood Brain Barrier (BBB) and localize inside the brain.

2. Materials and methods

2.1. Study design

The mice study was conducted under medical supervision at National and Kapodistrian University of Athens Medical School. Twelve (12) male mice were treated with 50 mg/kg pure TC4 (dissolved in 0.9% aqueous NaCl) through *i.p.* administration. Plasma samples and the whole brain of the mice were collected at predefined time points (0, 15, 30, 60, 120 and 240 min after administration). The protocol was approved by the ethical committee of the National and Kapodistrian University of Athens and was conducted according to the ICH-GCP guidelines (ICH GCP, 1996). The study received a permit from the Veterinary Directorate of the Prefecture of Athens (Approval #: 478/2014) according to the Greek legislation conforming to the 2010/53/EU Council Directive.

2.2. Chemicals and analytical reagents

The analytical reference standard of TC4 was isolated from *Crocus sativus* stigmas following a procedure previously developed and described in our laboratory [39]. Plant material *C. sativus* dried stigmas (saffron) was kindly provided by the Cooperative De Safran (Krokos Kozanis, West Macedonia, Greece). The purity of TC4 was found to be > 95% by HPLC-PDA using the continuous peak purity approach and its structure was verified by ^{1}H Nuclear Magnetic Resonance (NMR) spectroscopy. Crocetin (CRC) was produced after saponification of TC4 with aq. sodium hydroxide (10% w/v) at 60 °C for 4 h. The solution was then acidified with phosphoric acid and the yielded precipitate was washed with water. CRC was recrystallized from dimethylformamide [17] and its purity was better than 95% (HPLC). The internal standard (ISTD) 4-nitro-aniline (Fig. 1) was purchased from Sigma-Aldrich ® (Darmstad, Germany). All solvents were of LC-MS grade. Acetonitrile, methanol and water, were purchased from Avantor ® (Gliwice, Poland) whereas trifluoroacetic acid (TFA) was purchased from Sigma-Aldrich ®. Blank mice serum and brain for the validation of the PK studies were obtained from healthy animals (wild type mice) under written consent.

2.3. Stock solutions, calibrators and QCs preparation

Stock solutions of TC4, CRC and ISTD were prepared at a concentration of 1 mg/mL in methanol and stored at dark place at −20 °C. Working solutions were prepared in a daily basis by diluting appropriate volumes of the stock solutions in methanol in order to achieve the following concentration levels: 100, 10, 1 μg/mL and 100 ng/mL for both TC4 and CRC in the same initial solution and 100 μg/mL for the ISTD. The TC4 and CRC calibration curve in mouse plasma was constructed in the dynamic range of 10–6000 ng/mL (10, 25, 50, 100, 250, 500, 1000, 2000, 4000, 6000 ng/mL) while the concentration of the ISTD was kept at 100 μg/mL. In order to proceed to the validation of the described methodology, a different set of solutions were used as Quality Control (QC) samples in concentrations 30, 80, 3000, 4600 ng/mL (LQC1, LQC2, MQC and HQC respectively). For the construction of the calibration curve in mice brain the dynamic range was set from 0.05–5 ng/mg (0.05, 0.125, 0.25, 0.5, 1.25, 2.5, 4, 5 ng/mg) while the concentration of the ISTD was also kept at 100 μg/mL. The concentrations of the QC samples were 0.2, 0.35, 3, 3.5 ng/mg (LQC1, LQC2, MQC and HQC respectively).

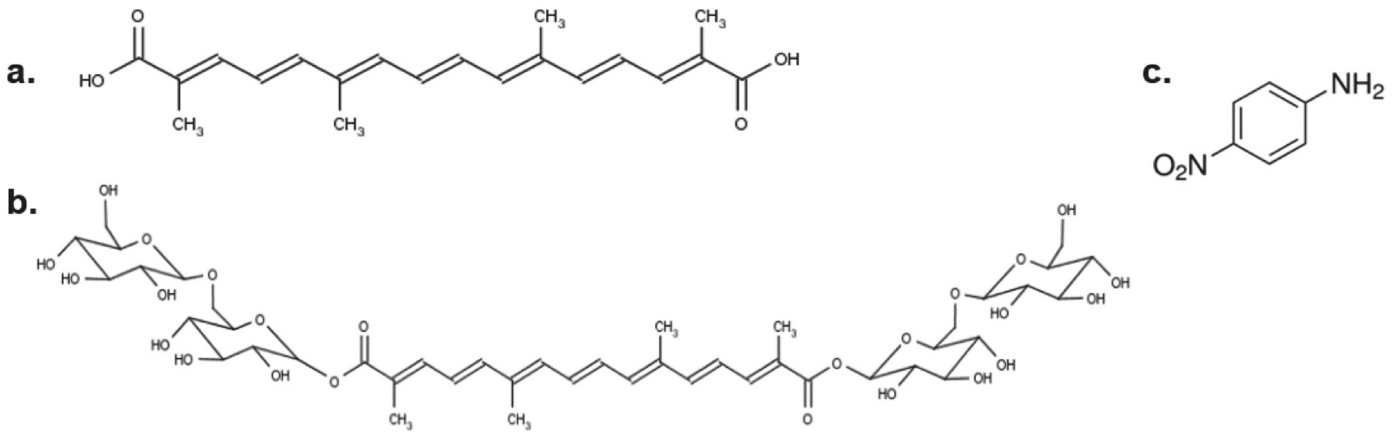


Fig. 1. Chemical structures of (a.) crocetin (CRC) (b.) trans-crocin 4 (TC4) and (c.) ISTD (4-nitro-aniline).

2.4. Instrumentation

For the quantitation study, UPLC-PDA analysis was performed on an ACQUITY UPLC® System. The core system comprises of a Binary Solvent Manager, a Sample Manager with an integral Column Heater and a PDA Detector (180–550 nm). Centrifugation of the serum samples was performed in a Mikro 200R centrifuge (Hettich Lab Technology, Germany). For the instrument control and the data processing the Waters® Empower™ 3.0 software suite has been employed using the QuanLynx data manager for all the quantification procedures employed. When needed, the SPSS 22.0 statistics software and R statistical language have also been used.

2.5. Sample pretreatment

2.5.1. Serum samples

Serum standards were prepared for UPLC-PDA analysis by direct protein precipitation with acetonitrile:methanol (ACN:MeOH) 2:1 (v/v). A 50 µL aliquot of plasma spiked with 5 µL of 100 µg/mL ISTD was treated with 150 µL ACN:MeOH 2:1 leading to the precipitation of the plasma proteins. This procedure was followed by a vortexing step for 30 s. and the mixture was centrifuged at 12000 rpm for 10 min. Subsequently, 150 µL of the supernatant were transferred into an 220 µL insert and were instantly analyzed as described below.

2.5.2. Brain samples

Brain standards were prepared in a blank brain homogenate which was prepared by homogenization of brain in methanol (10 mg brain in 500 µL MeOH). Brain homogenates were spiked with appropriate quantities of TC4 and CRC stock solution and 5 µL of 100 µg/mL ISTD and followed by a vortexing and centrifugation step. Subsequently, 150 µL of the supernatant were transferred into an insert and were instantly analyzed as described below.

2.6. Chromatographic conditions

All analyses were performed on an Acquity UPLC system (Waters Corp., Milford, MA, USA) comprised of a Binary Solvent Manager, a sample manager and a PDA detector. An RP-C18 Hypersil Gold column (50 × 2.1 mm, 1.9 µm; Supelco, Darmstadt, Germany) preceded by a precolumn (Waters Van- Guard 5 mm × 2.1 mm, 1.7 µm) of the same packing material and an on-line filter were used for the chromatographic separation of TC4, CRC and ISTD. A flow rate of 0.5 mL/min has been used throughout all chromatographic experiments. The mobile phase consisted of 0.01% aq. TFA (% v/v - solvent A) and acetonitrile (solvent B). A gradient elution method with a total run time of 2.5 min including the column equilibration time was used as follows: initial conditions 95% A: 5%B, 0 to 0.7 min, 40%A: 60% B; 0.7 to 1.1 min, 15%A: 85% B; 1.1 to 1.3 min, 0%A: 100% B; 1.30 to 1.50 min, 0%A: 100% B; 1.5 to 1.9 min, 60%A: 40% B; 1.9 to 2.1 min, 95%A: 5% B; 2.1 to 2.5 min, 95%A: 5% B. The column temperature maintained stable at 40 °C throughout all experiments while the autosampler tray temperature was set at 4 °C avoiding direct light exposure as crocins are photosensitive and could be easily degraded when exposed to light. The injection volume was 5 µL.

2.7. Validation of the bioanalytical method

Assay validation was performed according to the ICH (http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf), FDA (https://www.fda.gov/downloads/drugs/ guidancecomplianceregulatoryinformation/guidances/ucm368107.pdf) and EMA (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf) guidelines for bioanalytical methods validation employing the ISTD methodology. All responses were measured as the ratio between the peak areas of the compounds of interest (TC4, CRC) and the ISTD. The assays were validated by evaluating the specificity, selectivity, calibration model, precision and accuracy (intra- and inter-day), lower limit of quantification (LLOQ), recovery from spiked mice plasma and brain samples, carry over-effect, stability (short- and long-term, autosampler and freeze–thaw), robustness, sample dilution and incurred sample reanalysis.

2.7.1. Selectivity and specificity

Selectivity was studied by comparing chromatograms of six blank substance-free mice plasma and brain samples from six subjects with blank samples spiked with TC4, CRC and ISTD. All plasma and brain samples were subjected to the above described pretreatment procedure and were further analyzed by the aforementioned methodology in order to test for potential interferences or co-elution of the compounds of interest and endogenous plasma substances. Selectivity was tested in different concentrations as well as the lower limit of quantification (LLOQ).

2.7.2. Calibration curves – regression model

In order to estimate the validation parameters, three different types of calibration curves were constructed (in solvent, in pre-spiked and post-spiked plasma and brain samples). For plasma analysis, this procedure included analysis of the calibration standards at ten concentration levels ranging from 10 to 6000 ng/mL (n = 10) and calculating the analyte/ ISTD ratio. Curve fitting was achieved by non-linear regression analysis employing a quadratic model with $1/x^2$ weighting factor for TC4 and CRC. The method demonstrated acceptable accuracy

for the concentration range studied, as the back calculated concentrations of the calibration standards did not exceed ± 15% of the nominal value. Pre- and post-spiked serum samples were prepared for UPLC analysis by direct protein precipitation as described above. For brain analysis, the dynamic range was set from 0.05–5 ng/mg (n = 8) followed by calculation of the analyte/ISTD ratio. Curve fitting was achieved by non-linear regression analysis employing a quadratic model with $1/x^2$ weighting factor for both TC4 and CRC. The method demonstrated acceptable accuracy for the concentration range studied where the back calculated concentrations of the calibration standards were within ± 15% of the nominal value.

2.7.3. Carry-over study

The potential carry-over effect has been evaluated by injecting blank samples after the injection of the Upper Limit of Quantification (ULOQ).

2.7.4. Incurred sample reanalysis

All the samples have been reanalyzed with the aforementioned methodology.

2.7.5. Stability

Stability testing (assessed according to EMA, FDA, ICH procedures) was applied in order to evaluate the stability of the analytes during sample treatment, after long-term (frozen at −80 °C) and short-term (bench-top at room temperature) storage, after three freeze and thaw cycles and during the analytical process. All stability tests in plasma and brain have been performed at three spiked levels for TC4 and CRC (n = 5) simultaneously [i.e. HQC = 4600 ng/mL, LQC1 = 80 ng/mL and LQC2 = 30 ng/mL for plasma and HQC = 3.5 ng/mg, LQC1 = 0.35 ng/mg and LQC2 = 0.2 ng/mg for brain]. Short-term stability has been evaluated by reinjecting spiked samples that have been left for 6 h at room temperature away from artificial light in an effort to simulate the precise working conditions. Long-term stability has been estimated by storing spiked samples at −80 °C for 1 month and re-injecting them, whereas the freeze-and-thaw stability has been evaluated by analyzing the spiked samples after three consecutive cycles of freezing and thawing at room temperature. Autosampler stability has been evaluated after reanalyzing spiked samples that have remained in the autosampler's tray for 12 h at 5 °C.

2.7.6. Precision and accuracy (intra-day and inter-day)

Five replicates (n = 5) of each QC sample (LQC, MQC, HQC, were repeatedly analyzed in order to evaluate the intra- and inter-day precision and accuracy expressed as relative standard deviation (%) (RSD %) and relative error (%) (RE%) respectively. The inter-day precision and accuracy were assessed by analyzing five sets including all QC samples on three consecutive days.

2.7.7. Lower limit of quantification (LLOQ)

LLOQ defined as the lowest TC4 and CRC concentration of the calibration curve that could be determined with %RSD, %RE < 20% (n = 5) and signal-to-noise ratio (S/N) at least 5. Therefore, five blank samples were prepared and the (S/N) was found to be > 5 which has been determined by comparison between the measured signals (heights) from samples spiked at the LLOQ levels and those of blank plasma samples leading to the determination of the minimum concentration that the analytes can be reliably quantified.

2.7.8. Robustness

In order to evaluate the method's robustness, three experimental conditions (the flow rate of the mobile phase, the column temperature and the pH of the mobile phase) were altered by ± 5% compared to the established values. The LQC1, LQC2 and HQC were analyzed in five replicates by altering one parameter per time followed by calculation of the chromatographic peak area and retention time according to the previously described methodology. The results were expressed as % RSD.

2.7.9. Dilution integrity

For the demonstration of the ability to dilute and analyze samples that contain TC4 and CRC above ULOQ, a set of 2-fold and 10-fold spiked plasma compared to the ULOQ i.e. 12,000 and 60,000 ng/ mL were analyzed. The spiked samples were diluted with appropriate volumes of blank drug-free human plasma, processed according to the sample pretreatment procedure and analyzed by the developed methodology. For this reason, matched samples in human and mouse plasma have been prepared and analyzed. It has been demonstrated that the interchange of mice plasma with human plasma to the dilution integrity study did not significantly affect the result as it has been shown that the specificity and selectivity have been remained unaltered under the experimental conditions employed in the current study (%RSD = 1.41). The substitution of mice plasma with human plasma has been rendered necessary due to the lack of adequate quantities of mice or rat plasma for the dilution integrity study. Regarding the brain dilution integrity study, it has not been performed, due to the lack of brain homogenate in appreciable amount. Nevertheless, this has been compensated by the fact that no sample has exhibited higher concentrations than the ULOQ of the corresponding calibration curve.

2.7.10. Plasma regression model

Non-linear regression analysis has been employed, using a quadratic model with $1/x^2$ weighting fitted to the TC4 and CRC calibration curves over the range of 10 to 6000 ng/mL (n = 5). The models produced, exhibited adequate fitting (correlation coefficient R^2 better than 0.995 for both TC4 and CRC, whereas the back-calculated values exhibited % RE lower than the 15% margin in all cases. The results show that the model is acceptable for the quantitation of the analytes in mice plasma.

The equation for the calibration curve used for the quantification of TC4 in the plasma samples is:

$$y = 1.860e-004(\pm 4.287e-005) + 5.277e-005(\pm 1.835e-006)*x + 3.998e-011(\pm 6.590e-010)*x^2 \text{ using } 1/x^2 \text{ weighting [mean}\pm\text{(SD)]}$$

The equation for the calibration curve used for the quantification of CRC in the plasma samples is:

$$y = 0.03273(\pm 3.532e-004) + 1.572e-004(\pm 1.512e-005)*x + 8.053e-010\,(\pm 5.429e-009)*x^2 \text{ using } 1/x^2 \text{ weighting [mean}\pm\text{(SD)]}$$

2.7.11. Brain regression model

The quadratic model with $1/x^2$ weighting that was fitted to the TC4 and CRC calibration curves in brain over the range of 0.05 to 5 ng/mg (n = 5) had a correlation coefficient R^2 better than 0.996 for both TC4 and CRC whereas the back calculated values did not exceed the 15% (RE%) margin in all cases. The equation for the calibration curve used for the quantification of TC4 in the brain samples is:

$$y = 3.160e-007(\pm 2.162e-005) + 2.092e-004(\pm 1.161e-005)*x + 6.023e-007\,(\pm 1.923e-007)*x^2 \text{ using } 1/x^2 \text{ weighting [mean}\pm\text{(SD)]}$$

The equation for the calibration curve used for the quantification of CRC in the brain samples is:

$$y = 3.423e-003(\pm 3.525e-004) + 1.132e-003(\pm 1.892e-004)*x + 5.557e-006\,(\pm 3.135e-006)*x^2 \text{ using } 1/x^2 \text{ weighting [mean}\pm\text{(SD)]}$$

3. Results and discussion

In every step of the validation procedure, all calculations were constructed by plotting the area ratios of TC4/ISTD and CRC/ISTD versus the added concentration of both substances.

3.1. Analytical method development and optimization of UPLC-PDA conditions

The chromatographic conditions employed for the determination of TC4 and CRC in mouse plasma and brain were thoroughly optimized by varying parameters such as the column chemistry (C18, C18-NPS, C8 and Hilic), column geometry (100 × 2.1 mm, 1.9 μm; 50 × 2.1 mm, 1.9 μm; 100 × 2.1 mm, 1.7 μm), mobile phase (organic modifiers and buffers), gradient elution type, column temperature and flow rate. The best results in terms of peak resolution and shape as well as speed of analysis have been achieved employing a Hypersil Gold (C18, 50 × 2.1 mm, 1.9 μm) reversed-phase column with acetonitrile as the organic modifier of the mobile phase. The addition of 0.01% TFA in the mobile phase, improved the analyte peak shape therefore it has been used throughout all analyses (sharpness, width, symmetry). Moreover, the total analysis time was limited to 2.5 min. (incl. Equilibration conditions) offering a rapid chromatographic methodology with adequate resolution (TC4 eluted at 0.91 min, ISTD at 1.02 min and CRC at 1.64 min with resolution $R_{TC4/ISTD} = 4$ and $R_{ISTD/CRC} = 6.15$). In order to enhance the separation of the compounds of interest as well as to maintain the life duration of the column, it was necessary to utilize a precolumn so as to absorb all the unwanted endogenous compounds of both plasma and brain that would hinder the experimental procedure. Given the fact that both TC4 and CRC are extremely colored compounds lying to the red region of the visible spectrum, the optimum λ_{max} chosen for the quantification of TC4 and CRC was 440 nm after careful observation with the PDA [3] whereas the λ_{max} for the ISTD exhibits λ_{max} at 373 nm. Thus, the developed UPLC-PDA methodology included 3 observation channels (440, 373, 220 nm) the last one being used to assess the methodology's performance for species that do not bear double bonds.

3.2. Sample preparation and extraction protocol

In order to efficiently remove any unwanted impurities from plasma and brain samples, many sample pretreatment protocols were evaluated. The main criteria of acceptance for the sample pretreatment methodology were its efficiency, measured by the % recovery and the convenience of the employed procedure in terms of cost and time. Therefore, an SPE-based sample preparation procedure was rejected, as the protein precipitation employed in this study exhibited faster sample preparation with minimal cost and quantitative recovery efficiency as well. The quantitative recovery of the analytes along with the simplicity and rapidity of this sample pretreatment protocol, rendered the method compatible with high-throughput analysis of clinical samples.

For the sample preparation of the serum various solvents such as methanol, ethanol, ACN and mixtures of ACN and methanol in various ratios (2:1, 3:1, 1:1) were tested. After thorough examination of the analytes' recovery (%) in each case, acetonitrile:methanol 2:1 (v/v) mixture (at 0 °C) was chosen as extraction solvent system, as it ensured satisfactory protein precipitation, good extraction recovery and high reproducibility for both TC4 and CRC as well as the ISTD, without interfering with various co-eluting endogenous components.

In order to proceed to the sample preparation of the brain samples, a homogenization step was required. In accordance with the literature, a variety of protocols have been proposed to extract analytes from the brain [40]. According to the adopted protocol, 10 mg of brain are homogenized with 500 μL of a precipitation solvent. Water was rejected in favor of the organic solvents in order to shorten the evaporation step followed homogenization. Various solvents were tested and ice-cold methanol (0 °C) has been proven to be the most effective in terms of recovery. Furthermore, in an effort to simplify and accelerate the experimental procedure, homogenization was performed in the entire amount of brain needed for the set of experiments planned to occur during the day i.e. calibration curve and QCs with appropriate amount of precipitation solvent (500 μL acetonitrile for each 10 mg of brain) as it was proven that there was no difference between the peak areas deriving from samples spiked and then homogenized separately and samples that have been pre-homogenized and afterwards spiked (TC4: %RSD = 2.33 and %RE = −3.27 and CRC: %RSD = 2.57 and %RE = −1.52).

3.3. Quantification of trans-crocin 4

4-nitro-aniline was chosen as the ISTD because it meets all the necessary prerequisites for this selection i.e. it exhibited high and repeatable recovery, suitable chromatographic properties under the described experimental conditions, it has not been reported as an endogenous plasma and brain metabolite and had no interference with the compounds of interest (TC4 and CRC). Representative UPLC–PDA at λ = 440 nm (λ_{max} for TC4 and CRC) chromatograms of plasma and brain samples are shown in Figs. 2 and 3, whereas the corresponding chromatograms of plasma and brain samples at λ = 373 nm (λ_{max} for ISTD) are shown in Figs. S1 and S2 (Supplementary Part). Processed blank samples, exhibited no observable peaks for none of the three compounds.

3.4. Validation of the bioanalytical method

3.4.1. Specificity

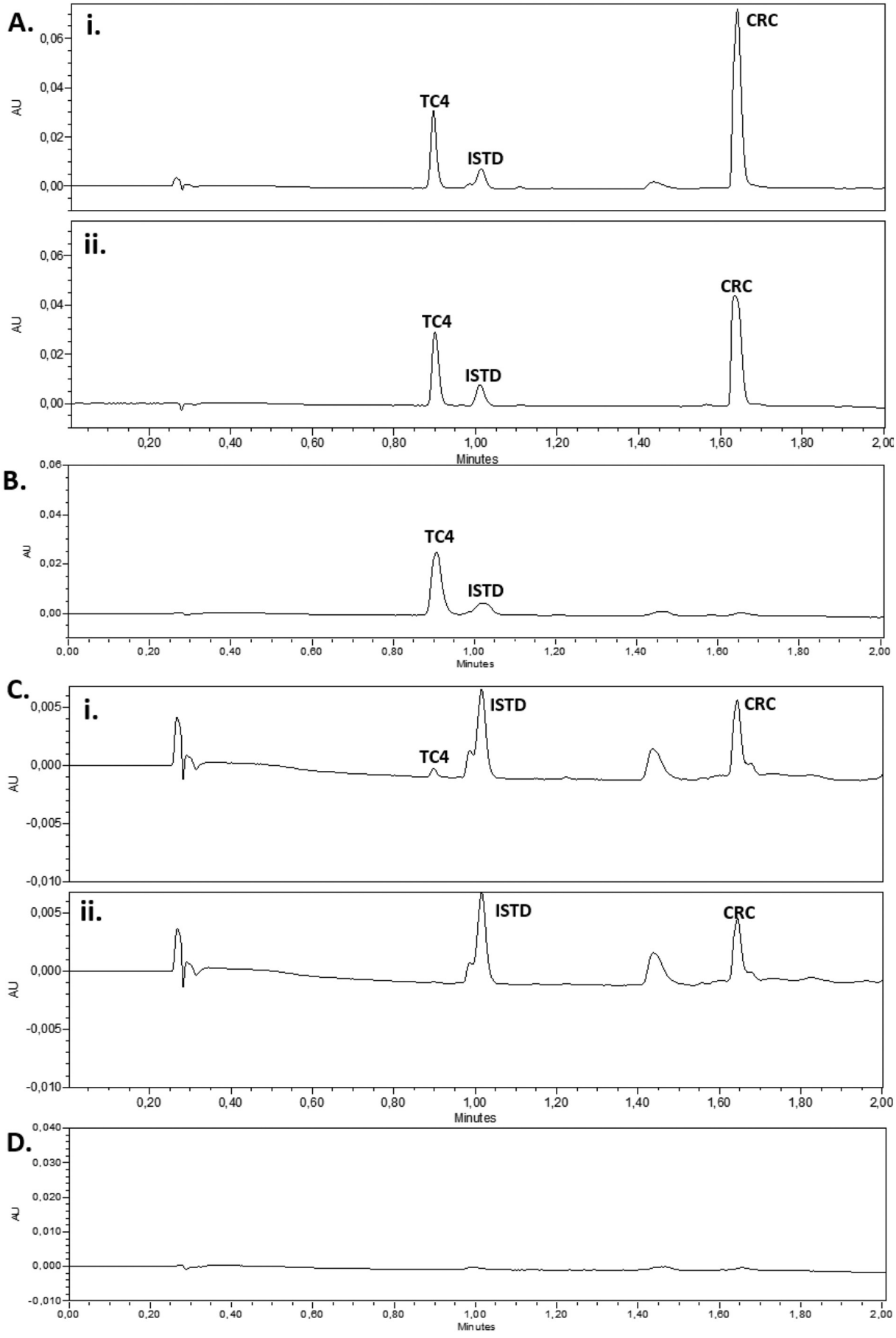
In order to evaluate the presence of potential endogenous metabolites that would interfere with the quantification of the analytes the specificity of the method has been evaluated. The gradient UPLC method developed, allowed the baseline separation of the analytes, without the presence of any interferences from endogenous plasma or brain compounds at the corresponding retention times of the analytes and ISTD. The addition of the ISTD has been made at the protein precipitation step in order to ensure that any potential interference the analytes might have with the plasma proteins, would not have any effect on the ratio between analyte/ISTD. This step, offers an additional level of confidence to the proposed methodology as it eliminates potential analytical errors.

3.4.2. Regression model

Non-Linear regression analysis has been employed for the assessment of the calibration analysis for TC4 and CRC in both plasma and brain. The range used for both analytes in plasma was 10 to 6000 ng/mL (n = 10) whereas in brain, where lower concentrations were expected, the corresponding range has been set to be 0.05 to 5 ng/mg (n = 8).

3.4.3. Lower limit of quantification (LLOQ)

The lower limit of quantification (LLOQ) is the lowest analyte concentration of the calibration curve that could be determined with precision < 20% and accuracy between 80% and 120%, showing a S/N ratio better than 5. The LLOQ for each analyte was determined as the concentration of the analyte that led to a peak with a signal/noise ratio of at least 5 times the response compared to that of a blank sample. The LLOQ have been determined to be 25 ng/mL for TC4 and 10 ng/mL for CRC for plasma and 0.05 ng/mg for TC4 and CRC for brain while %RE and %RSD were less than %20 margin and the S/N was at least 5 times the signal of a blank sample. The data indicate that the sensitivity of the described methodologies for the analysis of TC4 and CRC in mice plasma and brain is acceptable.



(caption on next page)

Fig. 2. Representative UPLC–PDA (λ = 440 nm) chromatograms of: A. HQC sample spiked with TC4 and CRC (4600 ng/mL) and ISTD (100 μg/mL) (i) in mice plasma; (ii) in MeOH; B. Plasma sample from mouse treated with 50 mg TC4 (*i.p.*) collected 30 min after administration; **C.** LLOQ (i) TC4 (25 ng/mL); (ii) CRC (10 ng/mL) D. blank mice plasma. The t_R for TC4, CRC and ISTD are 0.91, 1.64 and 1.02 min, respectively.

3.4.4. Carry-over study

No carry-over effect was observed during the study as there was no chromatographical peak of the analytes following the injection of a blank plasma or brain sample.

3.4.5. Incurred sample reanalysis

In order to assess the validity of the results in successive days, the total number of samples was reanalyzed. The results demonstrated that the % difference between the two measurements was < 20% of their mean, indicating that the developed methodology can afford accurate data on the concentration of TC4 and CRC in plasma and brain.

3.4.6. Stability

The stability of the QC samples was compared to that of freshly prepared and instantly analyzed one while the %RSD and %RE values obtained were within the 15% margin. TC4 and CRC were found to be stable enough for at least 3 freeze and thaw cycles and also for the investigated time periods.

The %RSD values of short- and long-term stability of the QC plasma samples were found to be < 2.8% (short-term) and 7.9% (long-term) for TC4 and 0.9% (short-term) and 5.0% (long-term) for CRC respectively (Table S1 – Supplementary Part). Autosampler stability was found to be < 9.7% for TC4 and 9.3 for CRC whereas the %RSD values of the QC samples included in the 3 freeze and thaw cycles were no > 1.6% for TC4 and 1.4% for CRC. The %RSD values of short- and long-term stability of the QC brain samples were found to be < 5.4% (short-term) and 9.2% (long-term) for TC4 and 1.0% (short-term) and 7.2% (long-term) for CRC respectively. Autosampler stability was found to be < 5.8% for TC4 and 5.3% for CRC whereas the %RSD values of the QC samples included in the 3 freeze and thaw cycles were no > 9.2% for TC4 and 7.2% for CRC. Corresponding %RE values could be found in Table S1 – Supplementary Part. Our results show that the samples could remain for at least 30 days at −80 °C as no out-of-limit degradation can be observed.

3.4.7. Intra-day and inter-day precision and accuracy

Intra- (n = 5) and inter-day (n = 15) precision and accuracy were evaluated for both substances at the three QC levels (HQC, MQC, LQC1, LQC2) (Table 1). The intra-day precision in plasma did not exceed 6.7% (TC4) and 13.9% (CRC) whereas the inter-day did not exceed 6.1% (TC4) and 3.7% (CRC). On the other hand, the intra-day precision in brain did not exceed 9.5% (TC4) and 14.9% (CRC) whereas the inter-day did not exceed 8.8% (TC4) and 7.0% (CRC). At the LLOQ level, the accuracy, expressed as the relative percentage error (%RE) was found to be within ± 12.3% whereas the precision, expressed as the %relative standard deviation (%RSD) was found to be < 13.9% The above results demonstrate that the developed methodology offers acceptable reliability and accuracy for the most part.

3.4.8. Robustness

In order to certify that the developed analytical methodology is not affected by minor alterations to the experimental conditions the robustness of the method was extensively studied as described above. The results indicated that none of the changes performed caused an alteration of > 3.6% for all the parameters tested certifying that the developed methodology has sufficient robustness in order to allow quality control measurements. Hence, %RSD of the resolution between TC4, CRC and ISTD was estimated as < 3.6% after the application of the predefined robustness alterations, whereas changing the column temperature from 40 °C to 38 °C and 42 °C had no significant alteration to the resolution between peaks (%RSD < 1.9%) as well as to the sensitivity (%RSD 2.4%). Finally, changing the flow rate of the mobile phase from 0.5 mL/min to 0.475 and 0.525 mL/min the %RSD of the resolution between peaks did not exceed 3.6% and the sensitivity was < 1.4%. Furthermore, by altering the percentage of TFA in the stationary phase neither the peak area and shape nor the resolution between peaks were considerably affected.

3.5. Bioavailability of trans-crocin 4 in brain and plasma

The two proposed analytical methodologies were applied to the analysis of mice plasma samples and brain samples obtained after *i.p* administration of TC4. In particular, twelve (12) healthy, wild type (C57BL/6 J), male mice were treated with 50 mg/kg pure TC4 through *i.p.* administration. Plasma samples and the whole brain of the mice were collected by authorized personnel at predefined time points (0, 15, 30, 60, 120 and 240 min after administration). Thus, whole plasma samples were gently shaken and centrifuged at 4000 rpm for 10 min at 25 °C and after the collection of serum were stored immediately in EDTA containing vacutainers at −80 °C. The brains were immediately stored at −80 °C until analysis.

3.5.1. Plasma

Experimentally determined TC4 plasma levels of the studied mice in the predefined time points are depicted in Fig. 4A. Given the fact that the *i.p.* administration route was selected in order to bypass the first-pass effect and/or its gastric hydrolysis, it is well demonstrated that TC4 was found to be in considerably high circulating levels i.e. 2600 ng/mL with a $t_{1/2}$ of 120 min. These results show high accumulation of crocin in plasma in accordance with the *i.v.* results obtained by Yue Zhang et al. [33] which exhibited ~ 6300 ng/mL showing a $t_{1/2}$ = 3.4 h. In that respect, we should point out that *i.p.* administration is resembling more the *p.o.* administration [38]. Nevertheless, it is noteworthy that CRC was not detected in any plasma sample following the *i.p.* administration as it is demonstrated in Fig. 2B. On the contrary, it has been reported that TC4 quickly hydrolyzes to CRC after *p.o.* administration [30, 32–34]. Therefore, in order to evaluate the distinct biological role of TC4, an *i.p.* administration scheme should be employed as any observed biological effects are not overlapped by the presence of CRC in plasma. Furthermore, the levels obtained by this administration route are capable of clearly evaluating its biological role and any possible toxicological side effects.

3.5.2. Brain

Experimentally determined TC4 levels in the brain of the studied mice in the predefined time points are shown in Fig. 4B. The results demonstrate the bioavailability of TC4 in mice brain in a time range of 0–240 min. It should be noted that some measured amount of TC4 in the brain tissue could be due to the blood perfusing the brain because capillary depletion prior to brain homogenates was not performed, as it has been previously noted by W.M. Pardridge and others [41–44]. It seems that there is a time phase delay between blood and brain peak concentrations suggesting that there is indeed BBB crossing and distribution in brain tissue. Therefore, we believe that this study provides preliminary evidence on TC4's ability to penetrate the BBB as it is demonstrated in Fig. 3B. Considering the hydrophilic nature of the TC4 molecule, this transport is probably achieved through an active receptor-mediated transport (RMT) process with the aid of protein carriers.

Our study shows that there are sustained levels of TC4 built up in the brain [i.e., **mean 24,6 (± 6,39) ng/20 mg** brain for 120 min], indicating its' potential role as a neuroactive agent. As a matter of fact,

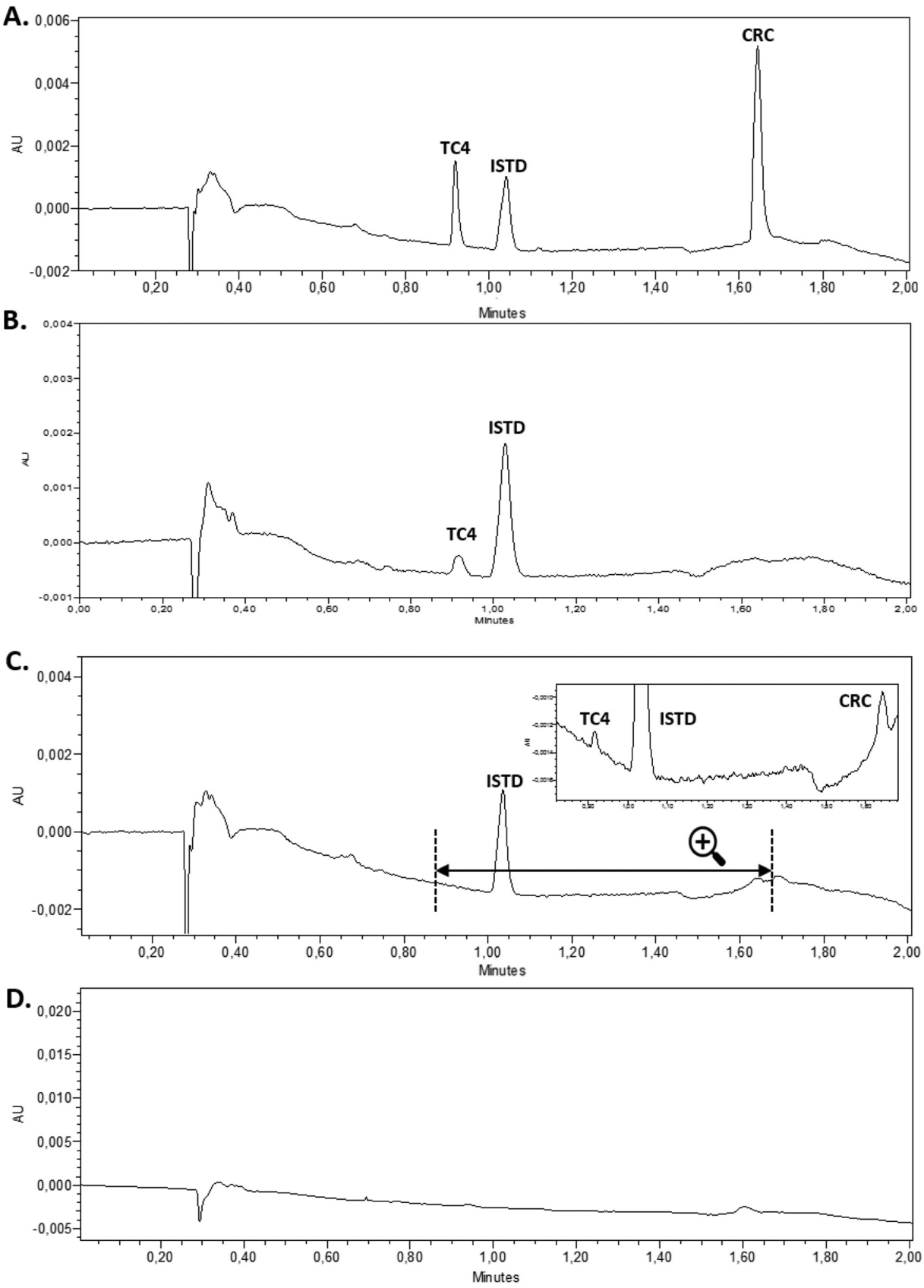


Fig. 3. Representative UPLC–PDA (λ = 440 nm) chromatograms of: A. HQC mice brain sample spiked with TC4 and CRC (3.5 ng/mg) and ISTD (100 μg/mL); B. Brain sample from mouse treated with 50 mg TC4 (*i.p.*) collected 30 min after administration; C. LLOQ of TC4 and CRC (0.05 ng/mg) D. blank mice brain. The t_R for TC4, CRC and ISTD are 0.91, 1.64 and 1.02 min, respectively.

Table 1
Precision and accuracy data (intra-day and inter-day) for TC4 and CRC in mice plasma and brain.

TC4 plasma		Intra day (n = 5)			Inter day (n = 15)		
QC level	Spiked concentration (ng/mL)	Mean concentration found (ng/mL)	RE%	RSD%	Mean concentration found (ng/mL)	RE%	RSD%
HQC	4600	4427	3.8	5.4	4750	−3.3	0.8
MQC	3000	2877	4.1	4.6	2886	3.8	1.3
LQC1	80	91	−14.3	5.1	76	4.5	6.1
LQC2	30	31	−2.2	6.7	26	12.1	4.1

CRC plasma		Intra day (n = 5)			Inter day (n = 15)		
QC level	Spiked concentration (ng/mL)	Mean concentration found (ng/mL)	RE%	RSD%	Mean concentration found (ng/mL)	RE%	RSD%
HQC	4600	4543	1.2	2.1	4719	−2.6	1.2
MQC	3000	2744	8.5	1.5	2859	4.7	1.3
LQC1	80	72	10.3	4.6	76	4.8	2.1
LQC2	30	26	13.5	13.9	29	3.9	3.7

TC4 brain		Intra day (n = 5)			Inter day (n = 15)		
QC level	Spiked concentration (ng/mg)	Mean concentration found (ng/mg)	RE%	RSD%	Mean concentration found (ng/mg)	RE%	RSD%
HQC	3.5	3.5	1.4	2.6	3.5	0.7	0.9
MQC	3	3.1	−4.6	4.4	3.0	0.3	1.5
LQC1	0.35	0.4	−6.3	7.6	0.3	12.3	2.9
LQC2	0.2	0.2	−2.9	9.5	0.2	5.1	8.8

CRC brain		Intra day (n = 5)			Inter day (n = 15)		
QC level	Spiked concentration (ng/mg)	Mean concentration found (ng/mg)	RE%	RSD%	Mean concentration found (ng/mg)	RE%	RSD%
HQC	3.5	3.5	0.1	1.4	3.4	4.1	0.6
MQC	3	3.1	−3.7	5.0	2.8	5.3	0.5
LQC1	0.35	0.4	−1.3	14.9	0.3	11.3	5.5
LQC2	0.2	0.2	−4.0	13.0	0.2	13.7	7.0

Fig. 4B shows that the levels of TC4 obtained in the brain have reached a nearly steady state from 15 to 120 min. Moreover, the fact that no circulating CRC levels have been found (Figs. 2B and 3B), may suggest that an alteration to the administration route (from *p.o.* to *i.p.*) could lead to the maintenance of TC4 circulating levels for a longer period of time allowing the application of its pharmacological activities in other organs such as the brain.

4. Conclusion

The main goal of the current study was to develop two UPLC-PDA analytical methodologies for the sensitive and simultaneous determination of *trans*-crocin-4 and CRC in mice plasma and brain, taking into account the reduced analytical cost and the ease of adoptability from the pharmaceutical industries. The developed methodologies featured short analysis time (2.5 min incl. equilibration) and excellent resolution between TC4, CRC and ISTD. Thus, the UPLC separation procedure has been evaluated as a method capable of allowing ultra-high resolution chromatographic runs in a significantly restricted analysis time. After full validation according to EMA, FDA and ICH guidelines, the developed methodology, due to its sensitivity, allows the determination and monitoring of low analyte concentrations, often observed after natural

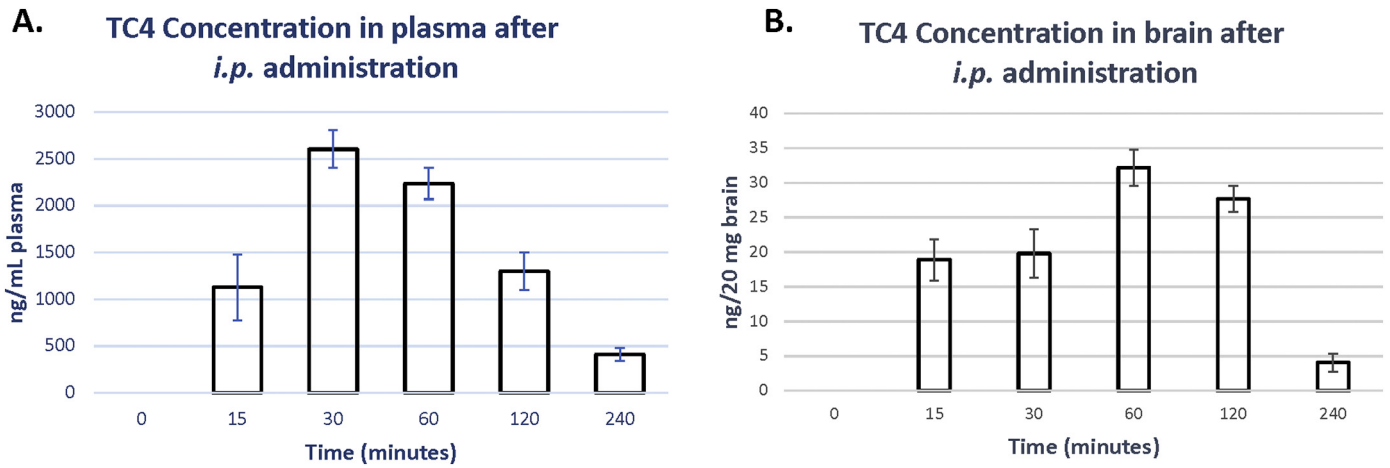


Fig. 4. Experimentally determined concentration levels of TC4 in A. mice plasma and B. mice brain samples after *i.p.* administration in the time range of 0–240 min after administration. Results are expressed as average values and the error bars represent the mean absolute difference.

products *i.p.* administration. This has allowed us to successfully apply the developed methodology to an exploratory quantitative study of TC4 and CRC in mouse plasma and brain. As far as the plasma is concerned, the bioavailability of TC4 in plasma was found to be in considerably high levels probably due to the application of *i.p.* administration where the first-pass metabolism and/or gastric hydrolysis were bypassed. In addition, this study shows the advantage of the *i.p.* administration, where detection of TC4 in mouse brains was observed for the first time, thus providing preliminary evidence on TC4's ability to penetrate the BBB, albeit its extremely hydrophilic character. In addition, no circulating CRC levels were detected in either plasma or brain samples. Finally, these results indicate that TC4 could serve as an active pharmaceutical ingredient, even though an extensive evaluation is needed in order to assess its efficacy and safety as a neuroprotective agent, as well as elucidate its mechanism of action.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fitote.2018.06.012.

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