Investigation of Isomeric Transformations of Chlorogenic Acid in Buffers and Biological Matrixes by Ultraperformance Liquid Chromatography Coupled with Hybrid Quadrupole/Ion Mobility/Orthogonal Acceleration Time-of-Flight Mass Spectrometry

Cen Xie,[†] Kate Yu,[‡,§] Dafang Zhong,[†] Tao Yuan,[†] Fei Ye,[†] Joseph Andy Jarrell,[‡] Alan Millar,[‡] and Xiaoyan Chen*,[†]

[†]Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 501 Haike Road, Shanghai 201203, PR China
[‡]Waters Corporation, 34 Maple Street, Milford, Massachusetts 01757, United States

Ⓢ *Supporting Information*

ABSTRACT: Ultraperformance liquid chromatography coupled with hybrid quadrupole/ion mobility/orthogonal acceleration time-of-flight (*oa*-TOF) mass spectrometry (UPLC-IM-MS) was used to study the isomeric transformations of *trans*-5-caffeoylquinic acid, an extremely active compound present in multiple vegetables, fruits, and beverages. The UPLC/*oa*-TOF MS results proved that in phosphate buffer (pH 7.4), plasma, or urine sample, *trans*-5-caffeoylquinic acid first isomerizes to *trans*-4-caffeoylquinic acid and then to *trans*-3-caffeoylquinic acid by intramolecular acyl migration. When exposed to UV light, *trans*-3-, -4-, and -5-caffeoylquinic acids undergo *cis/trans* isomerization to form *cis* isomers. The isomerization was solely dependent on the pH of the matrix, as well as the incubation temperature, and was independent of metabolic enzymes. UPLC-IM-MS results revealed that a reversible *cis/trans* isomerization of caffeoylquinic acids could also be induced by the electric field in an electrospray source. Thus, understanding the possible role of electric field-induced isomerization of caffeoylquinic acids may help lessen the confusion between gas phase phenomena and liquid state chemistry when applying IM-MS analysis. The comprehensive understanding of caffeoylquinic acid isomerization transformations is crucial for the appropriate handling of samples and interpretation of experimental data.

KEYWORDS: chlorogenic acid, acyl migration, *cis/trans* isomerization, ultraperformance liquid chromatography coupled with hybrid quadrupole/time-of-flight mass spectrometry, ion mobility

■ INTRODUCTION

Chlorogenic acids are an ester family formed by *trans*-cinnamic acids and D-(−)-quinic acid. Chlorogenic acids are numerous and ubiquitous in the plant kingdom. They are present in vegetables and fruits, as well as in beverages prepared from plants, such as coffee, tea, and wine.[1] Among the chlorogenic acids studied thus far, *trans*-5-caffeoylquinic acid (**4**, Figure 1, referring specifically to chlorogenic acid) is the most important due to its pharmacological properties (antioxidative, anti-inflammatory, analgesic, antipyretic, and anticarcinogenic), as well as its high content in plants.[2−4] Soluble coffee powder (2 g) has been reported to consist of 50−150 mg of *trans*-5-caffeoylquinic acid.[5,6]

trans-5-Caffeoylquinic acid is an extremely active compound. When treated with tetramethylammonium hydroxide at room[7] or high heating temperatures,[8,9] *trans*-5-caffeoylquinic acid isomerizes to *trans*-3-caffeoylquinic acid (**2**, Figure 1) and *trans*-4-caffeoylquinic acid (**5**, Figure 1). In the early stages of in vitro metabolism studies of *trans*-5-caffeoylquinic acid, we have also observed isomerization phenomena from microsomal incubation samples that could not be explained by common theories of metabolism. An in-depth study of *trans*-5-caffeoylquinic acid pharmacokinetics and metabolism can only be carried out with a comprehensive understanding of *trans*-5-caffeoylquinic acid

isomerization mechanisms, especially under physiological conditions.

Given that isomers have the same elemental composition, distinguishing them by their intact parent ion masses in mass spectrometry is difficult. The discrimination of some nonenantiomers using fragment ions generated by collision-induced dissociation in tandem mass spectrometry (MS/MS) is possible.[10] However, MS/MS data can often be ambiguous when multiple components are dissociated simultaneously. For this reason, chromatographic separation prior to mass spectrometric detection is crucial for isomer identification. Isomeric molecules, however, are usually very difficult to separate.

Ultraperformance liquid chromatography (UPLC) is the latest advancement in chromatographic analysis. Compared with high performance LC (HPLC), UPLC has a significantly improved chromatographic performance that results in high peak resolution, increased separation speed, and enhanced sensitivity. This facilitates the separation of isomeric compounds.

Another useful tool for separating isomers is ion mobility spectrometry (IMS). IMS separates ionic species as they drift
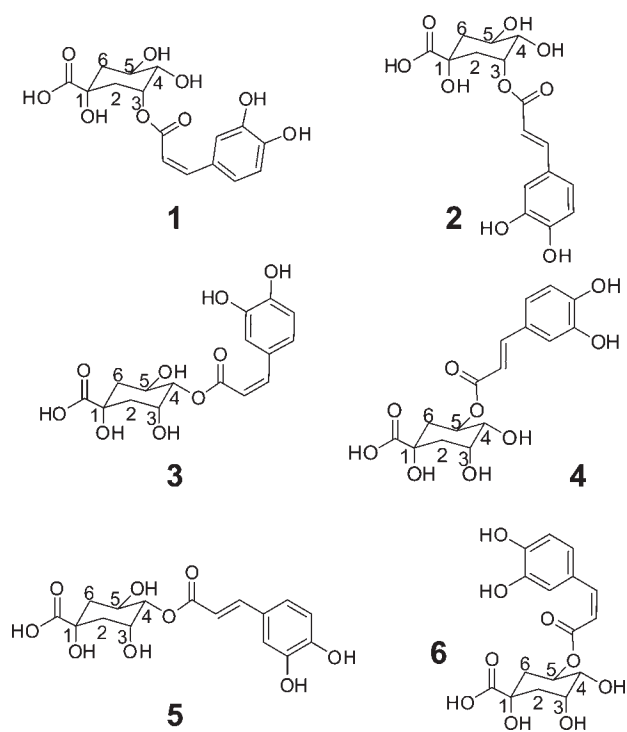
Figure 1. Molecular structures of *trans*-5-caffeoylquinic acid and its transformation products: **1**, *cis*-3-caffeoylquinic acid; **2**, *trans*-3-caffeoyl-quinic acid; **3**, *cis*-4-caffeoylquinic acid; **4**, *trans*-5-caffeoylquinic acid; **5**, *trans*-4-caffeoylquinic acid; **6**, *cis*-5-caffeoylquinic acid.

through a gas phase under the influence of an electric field. The rate of ion drift depends on the mass of the ion, its particular charge state, and its average rotational cross-section.[11] IMS makes possible the separation of different ions with the same masses, as long as they have different charge states or rotational cross-sections. Some investigators have demonstrated that IMS is capable of resolving many types of sequence-, positional-, structural-, and stereoisomers, which could not be distinguished from each other by the masses of precursor ions and fragment ions from mass spectrometry.[12−14] The main advantages of IMS are speed, selectivity, and simplicity of the separation process. In recent years, IMS technologies have been incorporated into mass spectrometry. The results obtained usually feature higher signal-to-noise ratios, isomer/isobar/conformer separation, and charge state identification. This incorporation also makes the routine utility of IMS much more feasible.[15] IMS has commonly been combined with several mass spectrometer analyzers, including hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometers (*oa*-TOF MS). Ion mobility-mass spectrometry (IM-MS) offers the possibility of distinguishing isomers even when they are coeluting in LC separations. It has been reported that IM-MS has been used to separate isomeric peptides and carbohydrates.[16,17]

In the present work, UPLC-IM-MS was used to investigate the isomeric transformations of *trans*-5-caffeoylquinic acid in phosphate buffers and in biological matrixes. The results clearly indicate that more attention should be paid to the stability of *trans*-5-caffeoylquinic acid during sample collection, preparation, and storage. A solid foundation has been established for future metabolism studies of *trans*-5-caffeoylquinic acid both in vivo and in vitro.

■ MATERIALS AND METHODS

Reagents and Analytical Standard Materials. The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA): *trans*-5-caffeoylquinic acid, caffeic acid, leucine enkephalin, HPLC-grade methanol, acetonitrile, and formic acid. Analytical grade phosphoric acid, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, and hydrochloric acid were purchased from China National Medicines (Shanghai, China). Deionized water, whose resistivity was greater than 18 MΩ deionized, was obtained from a Milli-Q Gradient Ultrapure System (Millipore, Molsheim, France).

Preparation and Isolation of *trans*-3-Caffeoylquinic Acid and *trans*-4-Caffeoylquinic Acid. *trans*-5-Caffeoylquinic acid (100 mg) was incubated in 2 mL of 100 mM phosphate buffer (pH 7.4) at 37 °C for 7 h. The reaction was terminated by adding formic acid to around pH 2. The reaction mixture was then purified with an LC-6AD semipreparative HPLC system (Shimadzu Corp., Kyoto, Japan) with a 250 mm × 10 mm i.d., Pursuit XRs 5 Diphenyl column (Varian Corp., Palo Alto, CA, USA). The mobile phase was composed of methanol, water, and formic acid (13.5:86.5:0.5, v/v/v), and the flow rate was 3.0 mL/min. About 11.2 mg of *trans*-3-caffeoylquinic acid and 21.6 mg of *trans*-4-caffeoylquinic acid were obtained. The purified *trans*-3- and 4-caffeoylquinic acids were characterized with one- and two-dimensional NMR spectroscopy, including ^1H NMR and $^1H-^1H$ correlation spectroscopy (400 MHz, in CD_3OD). The chemical shifts and correlations were compared to a reference sample of *trans*-5-caffeoylquinic acid. The elemental analysis of *trans*-3- and 4-caffeoylquinic acids was achieved on HRMS(ES−): calcd for $C_{16}H_{17}O_9$, *m/z* 353.0873 $[M − H]^−$; found, *m/z* 353.0879 (*trans*-3-caffeoylquinic acid) and *m/z* 353.0877 (*trans*-4-caffeoylquinic acid).

Incubations for Isomerization. To investigate its isomerization in phosphate buffer, *trans*-5-caffeoylquinic acid (500 μM) was prepared in 100 mM phosphate buffer of pH 7.4. The solution was dimidiated and kept in a water bath at 37 °C for 2 h. One portion of the solution was placed under a ZF-1 Triple-purpose UV Analyzer and was irradiated at 254 nm (Capy, 30 W; color filter, 200 × 50 mm) during incubation. The other portion was kept under nonactinic light. The reactions were terminated by acidification, adding 0.1 M hydrochloric acid to approximately pH 2.

To investigate the isomerization of *trans*-5-caffeoylquinic acid in biological samples, four male Sprague−Dawley (SD) rats (200−250 kg) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). Fresh rat plasma and urine were collected prior to the experiments. An aliquot of 500 μM *trans*-5-caffeoylquinic acid in rat plasma or urine was incubated at 37 °C for 8 h in a dark area. Reactions were terminated by acidification, adding 0.1 M hydrochloric acid to approximately pH 2.

ACQUITY UPLC-IM-MS Conditions. Chromatographic separation of isomers and degradation products was achieved using an ACQUITY UPLC system (Waters Corp., Milford, MA, USA) on a 2.1 mm × 100 mm i.d., 1.8 μm, ACQUITY UPLC HSS T3 column (Waters Corp.). Mobile phase A was water with 0.1% formic acid, and mobile phase B was acetonitrile. The gradient started from 1% B and held for 1 min, followed by a 7 min linear gradient from 1% to 50% B. The gradient was then stepped to 100% B at 7 min and held for 1 min and was finally stepped to 1% B to equilibrate the column for 3 min. The total LC run time was 12 min, the column temperature was maintained at 45 °C, and the flow rate was 0.6 mL/min.

The MS detection was provided by a Synapt G2 HDMS (Waters Corp., Milford, MA, USA). The negative ion electrospray mode was used for data collection. The desolvation gas was nitrogen, and the collision gas was argon. The carrier gas for IMS was nitrogen. The data acquisition range was *m/z* 50−900. The source temperature was 120 °C, and the desolvation temperature was 550 °C. The cone voltage was 20 V.
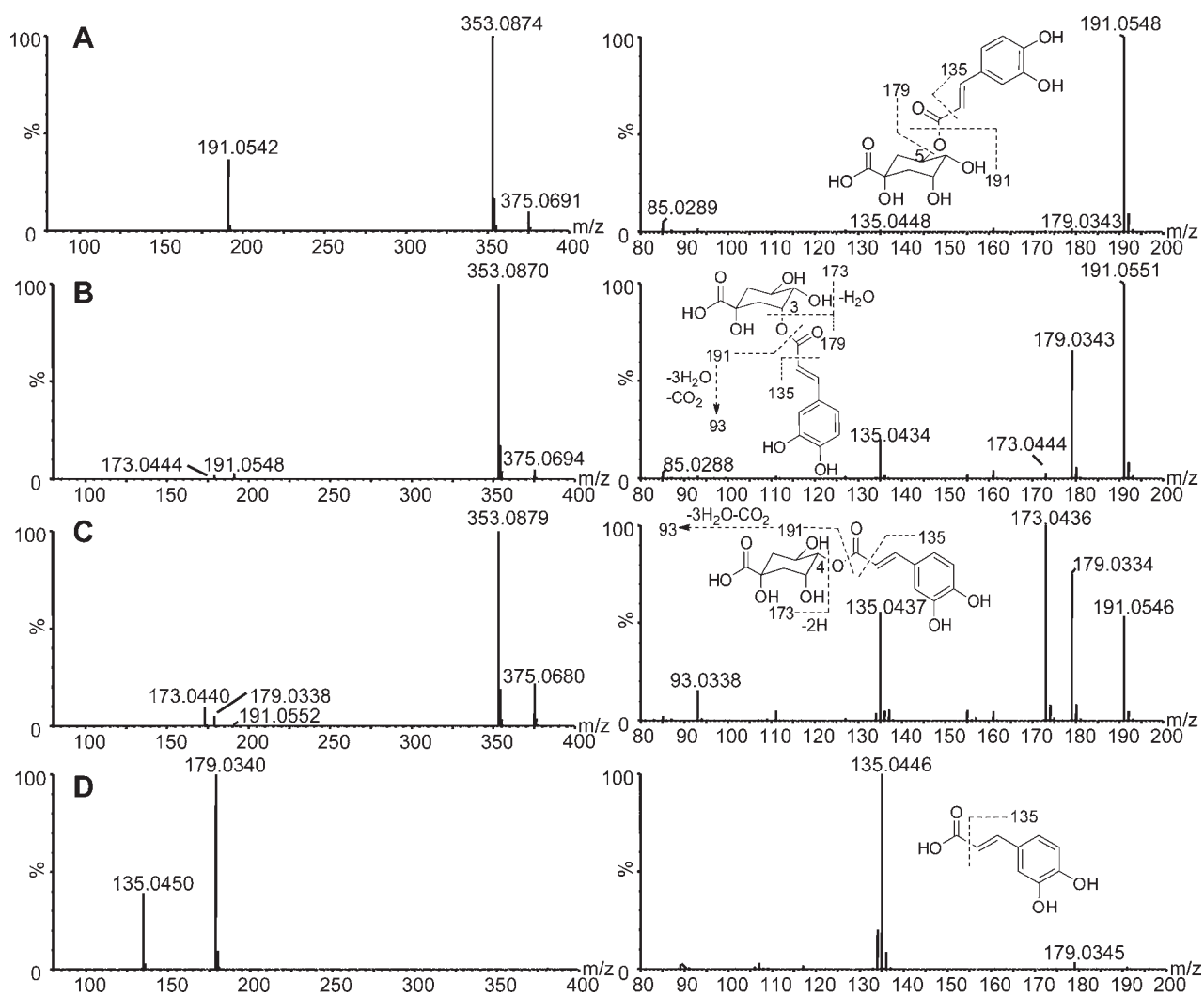
Figure 2. *oa*-TOF mass spectra of standard compounds: (A) *trans*-5-caffeoylquinic acid, (B) *trans*-3-caffeoylquinic acid, (C) *trans*-4-caffeoylquinic acid, and (D) caffeic acid. Left column: under low collision energy (trap at 4 eV and transfer at 2 eV). Right column: under high collision energy (trap ramped from 8−12 eV and transfer set at 5 eV).

The lock mass compound used was leucine enkephalin with a reference mass at m/z 554.2615.

For the TOF experiments, data were acquired in the MS^E mode in which two separate scan functions were programmed for the MS acquisition method. One scan function was set at low collision energy (trap at 4 eV and transfer at 2 eV), and the other scan function was set at high collision energy (trap ramped from 8−12 eV and transfer set at 5 eV). The mass spectrometer switched rapidly between these two functions during data acquisition. As a result, information on intact precursor ions and on fragment ions was obtained from a single LC injection.

For the IMS experiments, the traveling wave (T-wave) ion mobility cell was operated at a wave velocity of 800 m/s and wave amplitude of 40 V. The helium gas flow and the nitrogen IMS carrier gas flow were both set at 80 mL/min.

For IMS/*oa*-TOF full scan experiments, the collision energies (CE) for both the trap and transfer T-wave cells were set at low values so that intact $[M − H]^-$ ions were detected. For IMS/*oa*-TOF Time Aligned Parallel (TAP) fragmentation experiments, the trap T-wave cell was set at a low collision energy value to allow intact $[M − H]^-$ ions to enter the IMS drift cell. Upon exiting the IMS drift cell, these ions were drift time-separated if they had different ion mobilities. These drift time-separated ions then entered the transfer T-wave cell (set at a high collision energy) and caused fragmentation within the transfer T-wave. As a result, the fragment ions detected by the TOF detector were drift time-aligned to their respective precursor ions.

Degradation Kinetic Measurement of *trans*-5-Caffeoyl-quinic Acid in Phosphate Buffers, Organic Solvents, and Biological Fluids. The isomerization and degradation profiles of *trans*-5-caffeoylquinic acid in phosphate buffers were evaluated. The *trans*-5-caffeoylquinic acid solutions (500 μM) were prepared in 100 mM phosphate buffers within pH 3.0−9.0. The solutions were incubated at 20 and 37 °C. Aliquots were removed at different time points (0, 0.33, 1, 2, 4, 6, 8, 9.5, and 24 h). The reaction was terminated by adding 0.1 M hydrochloric acid to around pH 2.

The *trans*-3-, -4-, and -5-caffeoylquinic acid solutions were prepared in phosphate buffers of pH 3, 5, 7, and 9. The solutions were incubated at 37 °C for 2 h. The reactions were terminated by 0.1 M hydrochloric acid. The area normalization method was used to determine the percentages of *trans*-5-caffeoylquinic acid and its isomers and to estimate the mutual conversion rates.

The stability of *trans*-5-caffeoylquinic acid (500 μM) in methanol and acetonitrile was investigated under the following conditions: 24 h at 37 °C, one week at 20 °C (ambient temperature), and one month at 4 °C. All solutions were kept in a dark area.
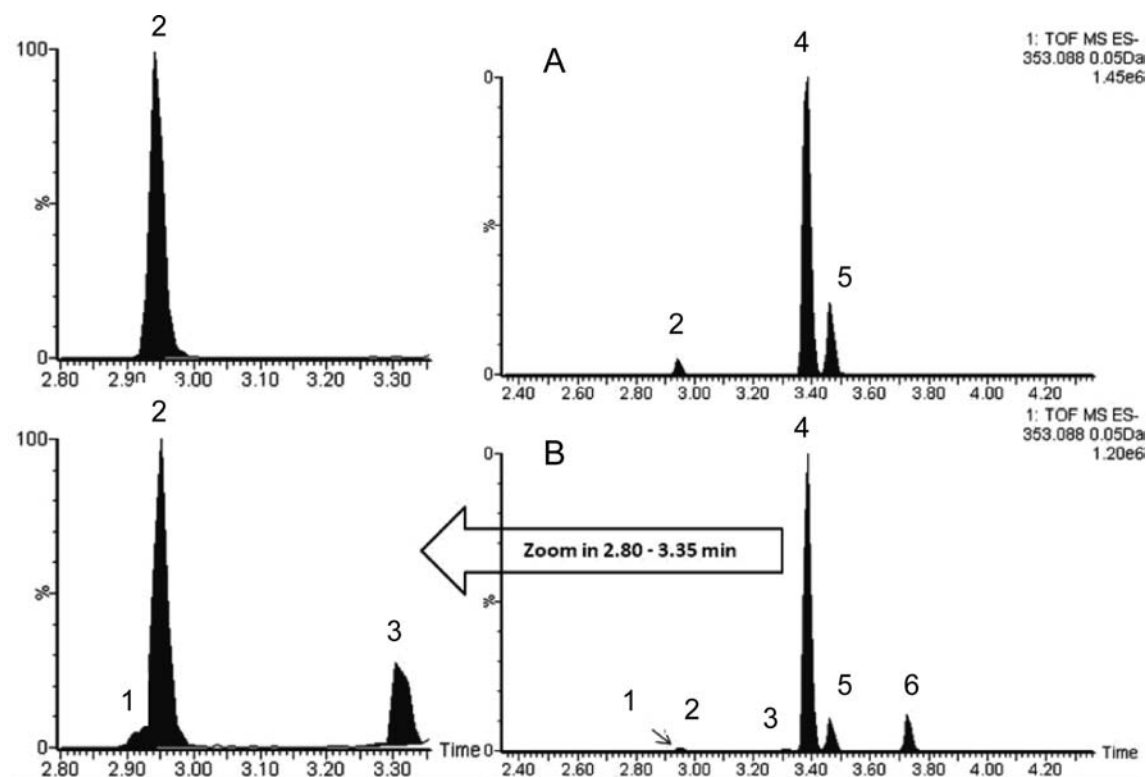
Figure 3. Extracted ion chromatograms of caffeoylquinic acids ($[M - H]^-$ 353.088) following incubations of *trans*-5-caffeoylquinic acid in pH 7.4 phosphate buffers for 2 h: (A) without UV irradiation and (B) with UV irradiation. Peak numbers correspond with compound numbers in Figure 1.

The isomerization and degradation profiles of *trans*-5-caffeoylquinic acid in rat plasma and urine samples were also evaluated. *trans*-5-Caffeoylquinic acid (500 μM) in rat plasma and urine was incubated at 37 °C in a dark area, and 100 μL of each solution was removed at different time points (0, 0.25, 0.5, 1.33, 1, 2, 4, 6, 8, 9.5, and 24 h). The reaction was stopped by adding 200 μL of acetonitrile (acidified to about pH 2 with formic acid).

Each set of the above experiments was conducted in triplicate. The product solutions were frozen at −80 °C pending further analysis. For the kinetic study, the method was analyzed for intra- and interday precision and accuracy. Lower limits of quantitation and quality control samples used six replicate preparations of acidified plasma (or buffered aqueous solution of pH 2.0) on three separate days at four concentration levels. The concentrations were 5, 20, 100, and 400 μM for *trans*-5-caffeoylquinic acid, while the concentrations were 0.5, 2, 20, and 160 μM for *trans*-3-caffeoylquinic acid and *trans*-4-caffeoylquinic acid. The coefficients of variation were below 15%. The degradation curves of 5-caffeoylquinic acid were established based on the value of the remaining drug (the initial relative concentration of 5-caffeoylquinic acid was presumed to be 100).

■ RESULTS

Analyses of Authentic Compounds by *oa*-TOF MS. A comprehensive understanding of fragmentation behaviors of the authentic compounds under the *oa*-TOF MS conditions is necessary for data interpretation. In the negative electrospray mode, the TOF MS full-scan spectra obtained at low collision energies for *trans*-3-, -4-, and -5-caffeoylquinic acids exhibited the deprotonated molecule $[M - H]^-$ at m/z 353.087.

Under high CEs, all three caffeoylquinic acid isomers displayed the same diagnostic fragment ions at m/z 191.053, 179.033,

173.044, 135.043, and 93.034 (Figures 2A−C). Cleavage of an ester bond gave the [quinic acid − H]$^-$ ion at m/z 191.053. Cleavage of the C−O bond adjacent to the ester yielded the [caffeic acid − H]$^-$ ion at m/z 179.033 and the [quinic acid − H_2O − H]$^-$ ion at m/z 173.044. The ion at m/z 135.043 was formed by a neutral loss of CO_2 (44 Da) from the [caffeic acid − H]$^-$ ion. The ion at m/z 93.034 was assigned as [phenol − H]$^-$ resulting from the quinic acid moiety after the successive loss of water molecules and CO_2.

Even though all three *trans*-caffeoylquinic acids (3, 4, and 5) showed the same fragmentation pathways at the same collision energy, the relative abundances of these diagnostic fragment ions were different.[18] Both *trans*-5-caffeoylquinic acid and *trans*-3-caffeoylquinic acid produced the same base peak ion at m/z 191.054. However, the signal intensity of the ion at m/z 179.033 was clearly different (higher for *trans*-3-caffeoylquinic acid and lower for *trans*-5-caffeoylquinic acid). In contrast, the base peak ion for *trans*-4-caffeoylquinic acid was m/z 173.044, which is characteristic for the isomer with caffeic acid substituted at position 4.

The fragmentation of the authentic compound caffeic acid was also investigated. Under negative electrospray, caffeic acid showed a deprotonated molecule $[M - H]^-$ at m/z 179.034 (Figure 2D). The characteristic product ion at m/z 135.045 was formed as a result of a neutral loss of CO_2.

Fragmentation appears to be a favored process for *trans*-caffeoylquinic acids given that major fragment ions were observed even in low CE scans for all three *trans*-caffeoylquinic acid isomers (*trans*-3-caffeoylquinic acid, *trans*-4-caffeoylquinic, acid and *trans*-5-caffeoylquinic acid) and for caffeic acid.

UPLC/*oa*-TOF MS Analyses of *trans*-5-Caffeoylquinic Acid Isomerization in Phosphate Buffer Incubations. Figure 3

Table 1. Summarized IM-MS Results for *cis*-3-Caffeoylquinic Acid (**1**), *trans*-3-Caffeoylquinic Acid (**2**), *cis*-4-Caffeoylquinic Acid (**3**), *trans*-4-Caffeoylquinic Acid (**5**), *cis*-5-Caffeoylquinic Acid (**6**), and *trans*-5-Caffeoylquinic Acid (**4**)

isomer	theoretical exact mass (m/z)	measured mass (m/z)	mass error (ppm)	fragment (m/z)	D_t (bin)	calculated mobility $(m^{-2} V s)$	calculated collision cross-section $(Å^2)$
1	353.0873	353.0863	−2.8	191.057 (100%), 179.038, 173.048 (~70%), 135.044	73	2288.3	123.4
2	353.0873	353.0862	−3.1	191.054 (100%), 179.033, 173.042 (~70%), 135.043	75	2219.9	119.7
3	353.0873	353.0861	−3.4	191.054, 179.032 (~80%), 173.043 (100%), 135.044	75	2163.8	116.6
4	353.0873	353.0874	0.3	191.054 (100%), 179.031, 173.043, 135.045	85	2428.2	130.9
5	353.0873	353.0875	0.6	191.054, 179.033 (~80%), 173.044 (100%), 135.043	86	2401.2	129.4
6	353.0873	353.0877	1.1	191.054 (100%), 179.035, 173.044, 135.043	74	2143.6	115.5

shows the extracted ion chromatograms (XICs) at m/z 353.087 obtained from two solutions. Figure 3A is from the *trans*-5-caffeoylquinic acid solution incubated in pH 7.4 phosphate buffer without UV light exposure. Figure 3B is from the *trans*-5-caffeoylquinic acid solution incubated in pH 7.4 phosphate buffer under UV light.

Compared with the fresh *trans*-5-caffeoylquinic acid standard solution (control sample), two additional *trans*-5-caffeoylquinic acid isomers were observed in Figure 3A (**2** and **5**), and five additional *trans*-5-caffeoylquinic acid isomers were observed in Figure 3B (**1**−**3**, **5**, and **6**). As discussed below, the structures of these isomers were determined by comparing with the standards (purchased and synthesized) and by referencing a literature report.[19]

The XIC at m/z 353.088 ($[M − H]^-$) shows six peaks at a low CE scan (Figure 3B). The peaks eluted at 2.95, 3.46, and 3.39 min are identified as *trans*-3-caffeoylquinic acid (**2**), *trans*-4-caffeoylquinic acid (**5**), and *trans*-5-caffeoylquinic acid (**4**), respectively. Additional three peaks eluted at 2.93, 3.30, and 3.73 min show similar fragmentation patterns with *trans*-3-caffeoylquinic acid, *trans*-4-caffeoylquinic acid, and *trans*-5-caffeoylquinic acid, respectively (Table 1). On the basis of a literature report,[19] these three peaks are tentatively proposed to be *cis*-3-caffeoylquinic acid (**1**), *cis*-4-caffeoylquinic acid (**3**), and *cis*-5-caffeoylquinic acid (**6**), respectively.

Compared with the control sample, only *trans*-3-caffeoylquinic acid (**2**), *trans*-4-caffeoylquinic acid (**5**), and *trans*-5-caffeoylquinic acid (**4**) were observed in the pH 7.4 phosphate buffer without UV irradiation (Figure 3A). This indicates that the *cis/trans* isomerization of the 5-caffeoylquinic acid C=C double bond was UV-induced, whereas the formation of *trans*-5-caffeoylquinic acid positional isomers was independent of UV exposure.

UPLC/*oa*-TOF MS Analyses of *trans*-5-Caffeoylquinic Acid Isomerization in Rat Plasma and Urine Incubations. The *trans*-conformation positional isomers, *trans*-3-caffeoylquinic acid (**2**) and *trans*-4-caffeoylquinic acid (**5**), were observed when *trans*-5-caffeoylquinic acid was incubated with fresh rat plasma and urine under nonactinic light. On the basis of the UPLC/UV chromatogram, the peak ratio of *trans*-3-caffeoylquinic acid (**2**), *trans*-4-caffeoylquinic acid (**5**), and *trans*-5-caffeoylquinic acid (**4**) was about 2:2.5:5 after 8 h of incubation. In addition, a degradation product with a deprotonated molecule $[M − H]^-$ at m/z 179.034, whose peak area was less than 5% of *trans*-5-caffeoylquinic acid, was detected. This product was confirmed as caffeic acid by comparison with the authentic compound. These results indicate that caffeic acid may be released from 5-caffeoylquinic acid by esterase hydrolysis in biological fluids.

UPLC-IM-MS Analyses of *trans*-5-Caffeoylquinic Acid Isomerization in Phosphate Buffer Incubations. In the UPLC-

IM-MS experiments, the ion mobility drift time (D_t; bin) of all caffeoylquinic acids (**1**−**6**) were determined in addition to the chromatographic retention time (R_t; min) and mass-to-charge information (m/z). This four-dimensional UPLC-IM-MS result can be studied in Drift Scope with three possible axis options ($m/z/D_t$, D_t/R_t, and $m/z/R_t$). Figure 4 shows an example of one option, drift time versus retention time (D_t/R_t), using the extracted ion at m/z 353.088.

Figure 4 shows clear drift time differences among *trans*-3-, -4-, and -5- caffeoylquinic acids. *trans*-3-Caffeoylquinic acid showed the fastest drift time (74 bins), whereas *trans*-4- and -5-caffeoylquinic acids showed drift times at 86 and 85 bins, respectively. Both the *cis*- and *trans*-3-caffeoylquinic acid isomers had similar drift times and showed a single mobility peak on the drift time axis at each of their respective retention times. However, each of the other four isomers (*cis*-4-caffeoylquinic acid, *trans*-4-caffeoylquinic acid, *cis*-5-caffeoylquinic acid, and *trans*-5-caffeoylquinic acid) showed two distinct peaks on the drift time axis at each of their respective retention times. These two peaks indicate that each of these four isomers existed in two steady states as they traveled through the ion mobility drift cell.

Degradation Kinetics of *trans*-3-, -4-, and -5-Caffeoylquinic Acids in Different Matrixes. The isomerization and degradation profiles of *trans*-3-, -4-, and -5-caffeoylquinic acids were separately evaluated in phosphate buffers of different pH values (pH 3, 5, 7, and 9). A pH-dependent process was observed. In acidic conditions (pH ≤ 5), these caffeoylquinic acid isomers were all found to be stable. As the buffer pH increased, their stabilities decreased, and positional isomerization was observed. The conversion rates are listed in Table 2.

In the phosphate buffer of pH 7.4, the half-lives of *trans*-5-caffeoylquinic acid were 99.0 h (20 °C) and 11.0 h (37 °C). This indicated that temperature strongly affects the stability of *trans*-5-caffeoylquinic acid. A low temperature certainly helps to stabilize *trans*-5-caffeoylquinic acid. The degradation rate constants (k_d) and half-lives ($t_{1/2}$) of *trans*-5-caffeoylquinic acid at different pH (7.4 and 9) and temperatures (20 and 37 °C) are summarized in Table 3.

The isomerization and degradation profile of *trans*-5-caffeoylquinic acid in rat plasma and urine was found to be similar to the findings in the pH 7.4 phosphate buffer experiment. The calculated half-lives were ~10 h in the biological fluids, slightly shorter than that in the phosphate buffer (11.0 h).

trans-5-Caffeoylquinic acid was also stable in straight acetonitrile or methanol for at least 24 h at 37 °C, one week at 20 °C (ambient temperature), and one month at 4 °C. A slight concentration increase of the *trans*-5-caffeoylquinic acid solution was observed, which was possibly caused by solvent evaporation. When *trans*-5-caffeoylquinic acid was incubated in straight
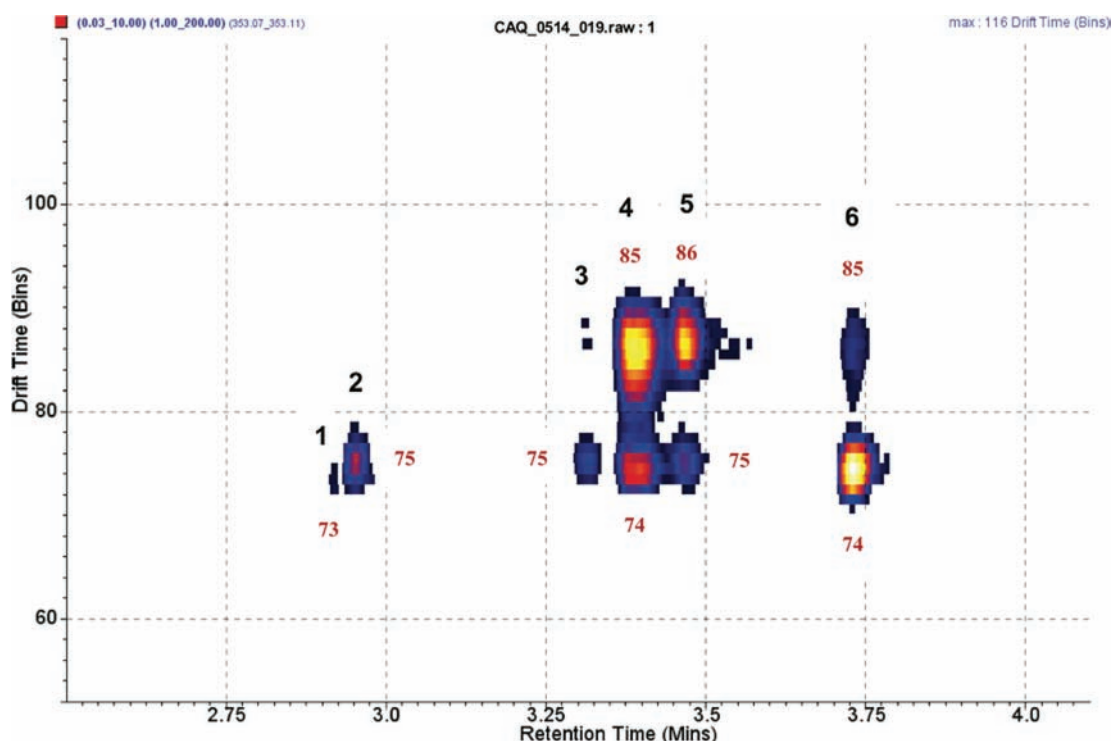
Figure 4. Drift scope results (expressed in D_t/R_t) for the extracted ion at m/z 353.088, $[M - H]^-$, of *trans*-5-caffeoylquinic acid incubated in pH 7.4 phosphate buffer with UV irradiation. The drift time (D_t) unit shown here is bin, and the retention time (R_t) is min. The black label (**1**−**6**) indicates the peaks correlated with *cis*-3-caffeoylquinic acid (**1**), *trans*-3-caffeoylquinic acid (**2**), *cis*-4-caffeoylquinic acid (**3**), *trans*-5-caffeoylquinic acid (**4**), *trans*-4-caffeoylquinic acid (**5**), and *cis*-5-caffeoylquinic acid (**6**). The red numbers labeled are the corresponding drift times for the peaks shown.

Table 2. Interconversions of *trans*-3-Caffeoylquinic Acid, *trans*-4-Caffeoylquinic Acid, and *trans*-5-Caffeoylquinic Acid in Phosphate Buffers at 37 °C for 2 h

treated isomer	pH	yield of isomeric product (%)		
		3-	4-	5-
3-	3	100	0	0
3-	5	100	0	0
3-	7	83.1	16.5	0.5
3-	9	47.6	35.0	17.6
4-	3	1.2	98.1	0.7
4-	5	0.6	98.9	0.5
4-	7	25.6	68.9	5.4
4-	9	42.3	33.8	23.9
5-	3	0.2	0.6	99.1
5-	5	0.2	0.3	99.5
5-	7	2.0	7.0	91.0
5-	9	26.2	31.3	42.5

Table 3. Pseudofirst-Order Degradation Rate Constants and Half-Lives for *trans*-5-Caffeoylquinic Acid

constant	temp (°C)	PBS		rat fluids	
		pH 7.4	pH 9	plasma	urine
k_d (h^{-1})	20	0.007	0.662		
	37	0.063	1.113	0.068	0.069
$t_{1/2}$ (h)	20	99.0	1.05		
	37	11.0	0.623	10.2	10

acid and *trans*-4-caffeoylquinic acid when treated with a strong base, tetramethylammonium hydroxide, at room temperature.[7] Meanwhile, high extraction temperature (125−225 °C) causes *trans*-5-caffeoylquinic acid positional isomerization in aqueous solutions[8,9] as well. Such acyl migration was also found in cinnamoylquinic acid and hydroxycinnamoylquinic acid, structural analogues of caffeoylquinic acid, under basic and heating (90 °C) conditions.[20,21] However, the isomerization mechanisms remain considerably debatable, especially under physiological conditions, which is crucial for correctly interpreting the pharmacokinetic and metabolic data of caffeoylquinic acids. In the present study, the stability of *trans*-5-caffeoylquinic acid in phosphate buffers of different pH and in biological fluids was evaluated. The results indicated that *trans*-5-caffeoylquinic acid is extremely active, and its major degradation pathway is the isomerization process. Two isomerization behaviors were observed for *trans*-5-caffeoylquinic acid: positional and *cis*/*trans* isomerization. The former depended on the solution pH and temperature, and the latter was UV-induced.
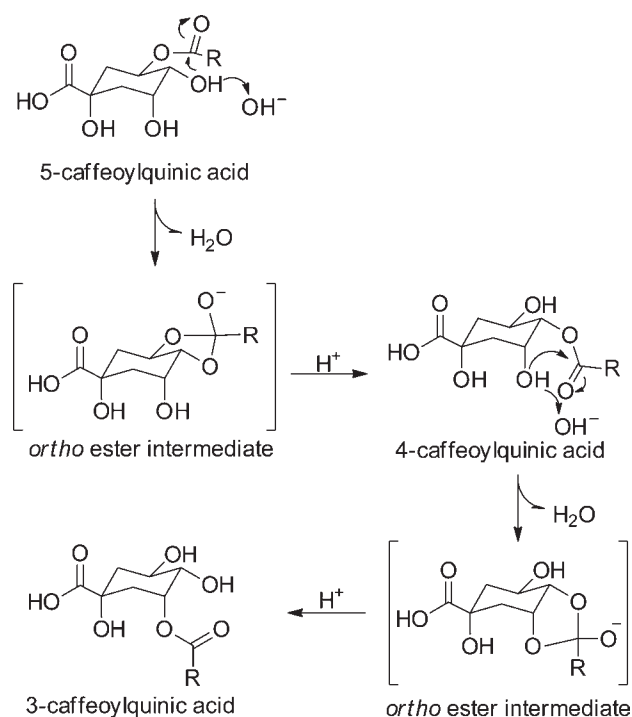
methanol, a trace amount of methyl 5-caffeoylquinate was detected. When *trans*-5-caffeoylquinic acid was incubated in a mixture of methanol and water at 37 °C for 1 h, it was converted into *trans*-3- and -4-caffeoylquinic acids.

■ DISCUSSION

Several studies[7−9] have demonstrated the effect of pH and temperature on the transformation of *trans*-5-caffeoylquinic acid. *trans*-5-Caffeoylquinic acid may isomerize to *trans*-3-caffeoylquinic

11083

dx.doi.org/10.1021/jf203104k |*J. Agric. Food Chem.* 2011, 59, 11078–11087

Figure 5. Proposed acyl migration pathways of 5-caffeoylquinic acid.

trans-5-Caffeoylquinic acid was much more stable in an acid buffer solution or in 100% methanol or acetonitrile than in an alkaline buffer solution or in a methanol–water mixture. This suggests that hydroxide ions [OH⁻] are necessary for positional isomerization. The amount of *trans*-4-caffeoylquinic acid produced was far more than that of *trans*-3-caffeoylquinic acid during the initial *trans*-5-caffeoylquinic acid incubation. When equilibrium was reached, all three isomers had similar concentrations. We, therefore, propose that *trans*-5-caffeoylquinic acid is first isomerized to *trans*-4-caffeoylquinic acid and then to *trans*-3-caffeoylquinic acid, following a transesterification sequence of 5 → 4 → 3. Meanwhile, *trans*-3-caffeoylquinic acid was also readily converted into *trans*-4-caffeoylquinic acid first and then to *trans*-5-caffeoylquinic acid. Cinnamoylquinic acid is an analogue of caffeoylquinic acid. Hanson[20] used [^{14}C]-quinic acid to investigate the inter- or intramolecular acyl migration of cinnamoylquinic acid in basic solution. The radioactivity was undetectable in the ester fractions, and the entire recovered radioactivity was associated with the free quinic acid. Therefore, the base-catalyzed migration of cinnamoyl groups about the quinic acid core is not an intermolecular process. Accordingly, we assumed that transformation of caffeoylquinic acids followed an intramolecular *ortho*-acyl migration mechanism. The plausible process is speculated and shown in Figure 5.

In the presence of hydroxide ions, the carbonyl carbon of *trans*-5-caffeoylquinic acid was susceptible to attack by a nucleophile on the neighboring hydroxyl group of the quinic acid ring. This attack resulted in the internal transesterification of *trans*-5-caffeoylquinic acid. The caffeoyl moiety migrated from the C5-position to the 4- and 3-positions through *ortho*-cyclic ester intermediate structures, which further hydrolyzed into esters. Experiments in phosphate buffers were also carried out using a single *trans*-3-caffeoylquinic acid or a single *trans*-4-caffeoylquinic

acid as the incubating compound. The results indicated that they also followed the same acyl migration process.

The data in Table 2 demonstrate two phenomena. The first was that *trans*-4-caffeoylquinic acid converts into *trans*-3-caffeoylquinic acid more easily than into *trans*-5-caffeoylquinic acid. The second was that *trans*-3-caffeoylquinic acid is more resistant to acyl migration than *trans*-4- and -5-caffeoylquinic acids. In order to get a better comprehension of these processes, a computational molecular modeling method was utilized. The pK_a value of the carboxyl group of caffeoylquinic acids was approximately 3.2. Under the present incubation conditions (ca. pH 7.4), almost all the carboxyl groups of these isomers were dissociated. Therefore, the carboxyl group was deprotonated for modeling instead of the phenol group. The program Gaussian 03 was used with the density functional theory employing the Becke-3-Lee-Yang-Parr (B3LYP) hybrid function and the 6-31G(d,p) basis set. The minimum energy conformations for the anions of *trans*-3-caffeoylquinic acid, *trans*-4-caffeoylquinic acid, and *trans*-5-caffeoylquinic acid are displayed in Figure 6. In the *trans*-4-caffeoylquinic acid structure, C1–OH and C3–OH bore a 1,3-*syn*-diaxial arrangement. The hydrogen atom from C1–OH formed a hydrogen bond (length = 2.057 Å) with the oxygen from C3–OH. We speculate that this facilitates the dissociation of the hydrogen atom from C3–OH under basic condition thus forming a C3–O⁻ ion. This C3–O⁻ attacks the C4 carbonyl group to form an *ortho*-cyclic ester intermediate. As a result, *trans*-4-caffeoylquinic acid isomerizes to *trans*-3-caffeoylquinic acid more easily.

trans-3-Caffeoylquinic acid also had a characteristic 1,3-*syn*-diaxial arrangement such that C4–OH was on the same side of the C3 carbonyl group oxygen atom. This arrangement sterically hinders a C4–OH attack against the C3 carbonyl group and results in a lower transesterification. In contrast, the hydroxyl group was on the same side of the carbon atom (instead of the oxygen atom) for both *trans*-4- and -5-caffeoylquinic acids. This configuration presented less steric hindrance to nucleophilic attack and facilitated the formation of *ortho*-cyclic ester intermediates. This was the reason for the greater stability of *trans*-3-caffeoylquinic acid than both *trans*-4- and -5-caffeoylquinic acids.

In the ion mobility experiments, the drift times of *trans*-3-caffeoylquinic acid with *cis*-3-, -4-, and -5-caffeoylquinic acids were shorter than those of *trans*-4- and -5-caffeoylquinic acids. The theoretical collision cross-sectional values of these isomers were calculated using the trajectory method of the open source software MOBCAL based on their ground state conformations.[22] Molecular modeling by the Gaussian 03 program showed that the geometry of three *cis*-isomers and *trans*-3-caffeoylquinic acid was folded, that *trans*-5-caffeoylquinic acid had a minor folding geometry, and that *trans*-4-caffeoylquinic acid was completely linear (Figure 6). Thus, the three *cis*-isomers and *trans*-3-caffeoylquinic acid had smaller collision cross-sections than the other two isomers and also exhibited shorter drift times (Table 1).

When *trans*-3-, -4-, and -5-caffeoylquinic acids were analyzed separately by UPLC-IM-MS, each analyte produced a single chromatographic peak. However, the *trans*-4- and -5-caffeoylquinic acid LC peaks showed two distinct ion mobility peaks in the Drift Scope, whereas the *trans*-3-caffeoylquinic acid LC peak showed a single mobility peak. The drift times of the minor mobility peaks from *trans*-4- and -5-caffeoylquinic acids were similar to that from *trans*-3-caffeoylquinic acid. We, therefore, propose that both *trans*-5-caffeoylquinic acid and *trans*-4-caffeoylquinic acid
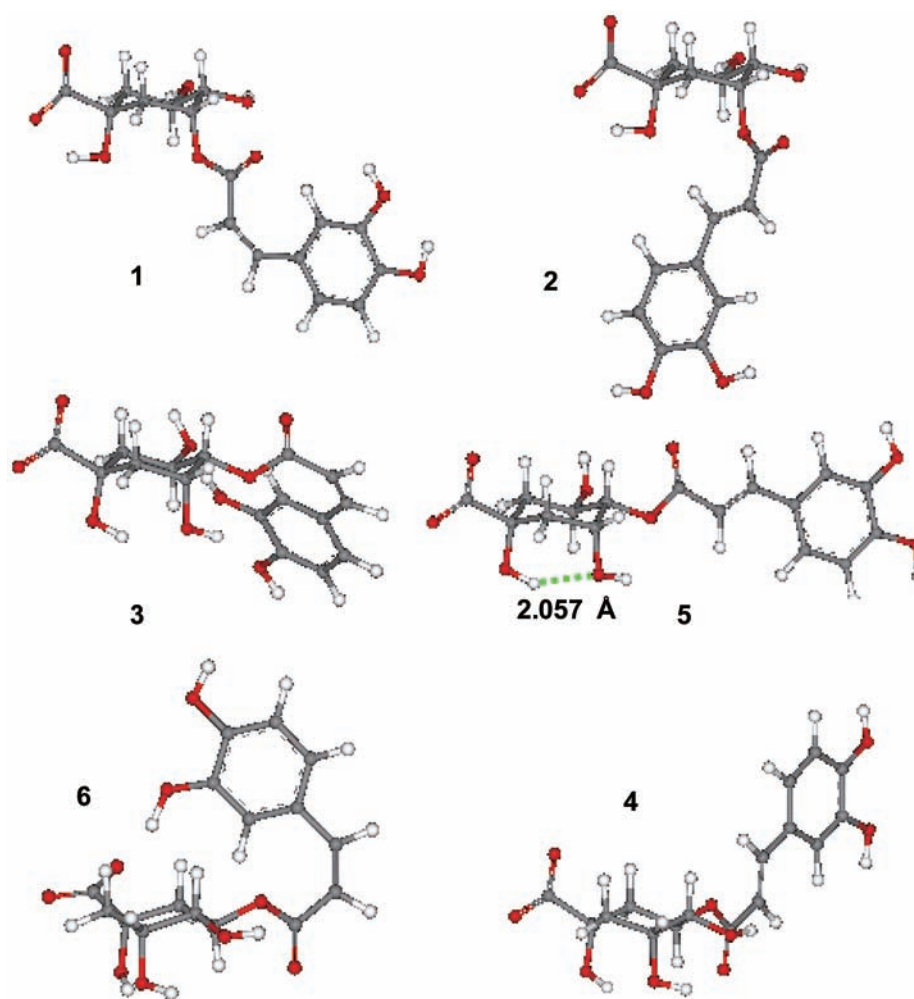
Figure 6. Optimized lowest energy structures of anionic *cis*-3-caffeoylquinic acid (**1**), *trans*-3-caffeoylquinic acid (**2**), *cis*-4-caffeoylquinic acid (**3**), *trans*-4-caffeoylquinic acid (**5**), *cis*-5-caffeoylquinic acid (**6**), and *trans*-5-caffeoylquinic acid (**4**) simulated by the program Gaussian 03.

undergo acyl migration to *trans*-3-caffeoylquinic acid in the gas phase.

However, the result obtained from the UPLC/IMS TAP fragmentation showed that the two ion mobility peaks of *trans*-4-caffeoylquinic acid produced identical fragment patterns (both *m/z* values and relative ratios among the fragment ions). This fragmentation pattern was clearly different from that of the *trans*-3-caffeoylquinic acid, indicating that the two steady states of *trans*-4-caffeoylquinic acid were not the result of positional isomers. Similar findings were obtained for *trans*-5-caffeoylquinic acid.

We also investigated the possible presence of an impurity in *trans*-5-caffeoylquinic acid, which was obtained from a commercial source. NMR analysis confirmed the purity of *trans*-5-caffeoylquinic acid. A chiral separation was also attempted, and only a single LC peak was observed. These data indicated that the minor mobility peak of *trans*-5-caffeoylquinic acid was improbably resulting from an impurity.

The reference solution containing *cis*-3-, -4-, and -5-caffeoylquinic acids was obtained by incubating *trans*-5-caffeoylquinic acid in pH 7.4 phosphate buffer with UV irradiation. The resultant solution was analyzed by IMS (Figure 4). *cis*-3-Caffeoylquinic acid (**1**) showed a single mobility peak, whose drift time was the same as that of *trans*-3-caffeoylquinic acid (**2**). *cis*-4-Caffeoylquinic

acid (**3**) and *cis*-5-caffeoylquinic acid (**6**) both exhibited two distinct mobility peaks, which were identical to the corresponding *trans*-isomers (**4** and **5**). Thus, we proposed that *cis*- and *trans*-isomers of 4-caffeoylquinic acid or 5-caffeoylquinic acids (**3**−**6**) all underwent *cis/trans* isomerization inside the instrument. As a result, the minor mobility peaks of *trans*-4- and -5-caffeoylquinic acids were attributed to the generation of *cis*-4- and -5-caffeoylquinic acids. The *cis*- and *trans*-isomers of 3-caffeoylquinic acid could not be separated in the IMS. Therefore, only one ion mobility peak was observed.

It has been reported that the C=C double bond can undergo a reversible isomerization between *trans*- and *cis*-isomers either by photoexcitation or by being subjected to strong electric fields.[23−25] For example, a potential difference of 2 V imposed across a separation of 6 Å is reported as sufficient to induce *cis/trans* isomerization in azobenzene.[23] Depending on the specific STM tip geometry used, this implies an electric field of 0.33 V/Å or less. Molecules on the surface of an electrosprayed droplet are exposed to electric fields of similar strength.[26,27] Thus, one possible explanation for our observations is that when isomers of 5-caffeoylquinic acid are introduced into a mass spectrometer by electrospray ionization, they undergo an electric field-induced transition of the C=C double bond between an extended (*trans*) and a compact (*cis*) configuration. Accordingly, *cis/trans* isomerization may have resulted from rotation around the C=C bond with

disruption of the double bond under $\pi-\pi^*$ excitation. *trans*-5-Caffeoylquinic acid and -4-caffeoylquinic acid could have been inverted from linear (*trans*) to orthogonal (*cis*) forms and vice versa, which could explain the two distinct peaks in the IMS result. On the basis of the above theory, *cis/trans* conversion may have also occurred in the 3-caffeoylquinic acids. However, because there was no drift time difference between these two distorted 3-caffeoylquinic acid conformations, only a single mobility peak was observed for each of the 3-caffeoylquinic acids with IMS.

The present experiments proved that caffeoylquinic acids undergo positional isomerization in aqueous solutions. Therefore, using aqueous methanol for caffeoylquinic acid extraction alters the authentic contents of different caffeoylquinic acids from plants. This makes it impossible to determine whether any of the isomers originated from the plant itself or were produced during the extraction procedure. Water should thus be avoided when extracting caffeoylquinic acids from plants.

There have been many pharmacokinetic and metabolism studies reported about *trans*-5-caffeoylquinic acid.[28,29] Nevertheless, none have addressed the fact that *trans*-5-caffeoylquinic acid isomers may be produced under physiological conditions in vivo or introduced by incorrect sample handling in vitro. In our experiment, the calculated half-lives in the biological fluids were slightly shorter than that in the phosphate buffer. We speculated that esterase in biological fluids possibly caused this slight decrease in degradation kinetics, as it could catalyze *trans*-5-caffeoylquinic acid hydrolysis to caffeic acid. However, acidic conditions (pH 3) and low temperatures (<4 °C) could stabilize *trans*-5-caffeoylquinic acid in biological samples for at least one month. Once the samples are obtained, the use of methanol should be avoided during deproteinization and extraction. This is because trace amounts of methyl 5-caffeoylquinate may be generated via the esterification of the quinic acid carboxyl group with methanol. Moreover, all extraction procedures should be carried out in the dark to prevent the *cis/trans* transformation of the C=C double bond.

IM-MS is an emerging technique for pharmaceutical analysis.[30] In this context, we found that the *cis/trans* isomerization of the C=C double bond of caffeoylquinic acids can be induced by an electrodynamic field in the IM-MS. Other compounds containing double bonds may also undergo *cis/trans* isomerization inside the instrument. These results may help prevent researchers from confusing gas phase phenomena with liquid state chemistry when performing IM-MS analysis.

From a practical point of view, *trans*-5-caffeoylquinic acid is extremely active and undergoes isomerization in both phosphate buffers and biological incubations. *trans*-5-Caffeoylquinic acid metabolites may be composed of a mixture of isomers in microsomal incubations or in vivo samples. Inappropriate sample extraction and handling may introduce transformation products that can be mistakenly treated as new components or metabolites or can be erroneously quantitated in the examined matrixes.

In this work, the isomerization transformations of *trans*-5-caffeoylquinic acid were explored using UPLC-IM-MS. When catalyzed by hydroxide ions, *trans*-5-caffeoylquinic acid undergoes *ortho*-acyl migration to form *trans*-4-caffeoylquinic acid and *trans*-3-caffeoylquinic acid. When exposed to UV light, *trans*-5-caffeoylquinic acid undergoes *cis/trans* isomerization to form the *cis*-5-caffeoylquinic acid isomer. Acyl positional isomers can be separated with IMS on the basis of their gas phase ion mobility differences, which are directly related to the structural conformation of the gas phase ions. We also found that under the influence

of the electric fields in an electrospray source *cis/trans* isomerization can also be induced. Given that the *cis/trans* isomers have different drift times in IMS, this *cis/trans* isomerization could be misinterpreted as the isomerization process of caffeoylquinic acids in solution. The present work demonstrates that UPLC-IM-MS provides a vanguard methodology for separating and discriminating isomeric caffeoylquinic acids.

ASSOCIATED CONTENT

S **Supporting Information.** [1H] NMR and [1H]−[1H] correlation spectroscopy spectra of *trans*-3-, 4-, and 5-caffeoylquinic acid in CD$_3$OD; the concentration−time curves of *trans*-5-caffeoylquinic acid degradation, as well as *trans*-3-caffeoylquinic acid and *trans*-4-caffeoylquinic acid generation in phosphate buffers at different pH values (3, 5, 7.4, and 9) and temperatures (20 and 37 °C); the concentration−time curves of *trans*-5-caffeoylquinic acid degradation, as well as *trans*-3-caffeoylquinic acid and *trans*-4-caffeoylquinic acid generation in rat plasma and urine at pH 3.0 and normal pH; UPLC/IMS TAP fragmentation results for *cis*- and *trans*-3-caffeoylquinic acid, 4-caffeoylquinic acid, and 5-caffeoylquinic acid. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author
*Tel/Fax: +86 21 50800738. E-mail: xychen@mail.shcnc.ac.cn.

Author Contributions
§Co-first author.

ABBREVIATIONS USED

UPLC, ultraperformance liquid chromatography; *oa*-TOF MS, hybrid quadrupole/orthogonal acceleration time-of-flight mass spectrometry; IMS, ion mobility spectrometry; IM-MS, ion mobility mass spectrometry; SD, Sprague−Dawley; T-wave, traveling wave; CE, collision energy; TAP, Time Aligned Parallel; XIC, extracted ion chromatogram; D_t, drift time; R_t, retention time

REFERENCES

(1) Clifford, M. N. Chlorogenic acids and other cinnamates: Nature, occurrence, dietary burden, absorption and metabolism. *J. Sci. Food Agric.* **2000**, *80*, 1033–1043.

(2) Bouayed, J.; Rammal, H.; Dicko, A.; Younos, C.; Soulimani, R. Chlorogenic acid, a polyphenol from *Prunus domestica* (Mirabelle), with coupled anxiolytic and antioxidant effects. *J. Neurol. Sci.* **2007**, *262*, 77–84.

(3) dos Santos, M. D.; Almeida, M. C.; Lopes, N. P.; de Souza, G. E. Evaluation of the anti-inflammatory, analgesic and antipyretic activities of the natural polyphenol chlorogenic acid. *Biol. Pharm. Bull.* **2006**, *29*, 2236–2240.

(4) Challis, B. C.; Bartlett, C. D. Possible cocarcinogenic effects of coffee constituents. *Nature* **1975**, *254*, 532–533.

(5) Trugo, L. C.; Macrae, R. Chlorogenic acid composition of instant coffees. *Analyst* **1984**, *109*, 263–266.

(6) Ramirez-Martinez, J. R. Phenolic compounds in coffee pulp: Quantitative determination by HPLC. *J. Sci. Food Agric.* **1988**, *43*, 135–144.

(7) Clifford, M. N.; Kellard, B.; Birch, G. G. Characterisation of chlorogenic acids by simultaneous isomerisation and transesterification with tetramethylammonium hydroxide. *Food Chem.* **1989**, *33*, 115–123.

(8) Dawidowicz, A. L.; Typek, R. Thermal stability of 5-o-caffeoylquinic acid in aqueous solutions at different heating conditions. *J. Agric. Food Chem.* **2010**, *58*, 12578–12584.

(9) Zanoelo, E. F.; Benincá, C. Chemical kinetics of 5-O-caffeoylquinic acid in superheated steam: Effect of isomerization on Maté (*Ilex paraguarensis*) manufacturing. *J. Agric. Food Chem.* **2009**, *57*, 11564–11569.

(10) Clifford, M. N.; Marks, S.; Knight, S.; Kuhnert, N. Characterization by LC-MS^n of four new classes of *p*-coumaric acid-containing diacyl chlorogenic acids in green coffee beans. *J. Agric. Food Chem.* **2006**, *54*, 4095–4101.

(11) McCullough, B. J.; Kalapothakis, J.; Eastwood, H.; Kemper, P.; MacMillan, D.; Taylor, K.; Dorin, J.; Barran, P. E. Development of an ion mobility quadrupole time of flight mass spectrometer. *Anal. Chem.* **2008**, *80*, 6336–6344.

(12) Bagal, D.; Valliere-Douglass, J. F.; Balland, A.; Schnier, P. D. Resolving disulfide structural isoforms of IgG2 monoclonalantibodies by ion mobility mass spectrometry. *Anal. Chem.* **2010**, *82*, 6751–6755.

(13) Ashline, D. J.; Lapadula, A. J.; Liu, Y.-H.; Lin, M.; Grace, M.; Pramanik, B.; Reinhold, V. N. Carbohydrate structural isomers analyzed by sequential mass spectrometry. *Anal. Chem.* **2007**, *79*, 3830–3842.

(14) McCooeye, M.; Ding, L.; Gardner, G. J.; Fraser, C. A.; Lam, J.; Sturgeon, R. E.; Mester, Z. Separation and quantitation of the stereoisomers of ephedra alkaloids in natural health products using flow injection-electrospray ionization-high field asymmetric waveform ion mobility spectrometry-mass spectrometry. *Anal. Chem.* **2003**, *75*, 2538–2542.

(15) Kanu, A. B.; Dwivedi, P.; Tam, M.; Matz, L.; Hill, H. H. Ion mobility−mass spectrometry. *J. Mass Spectrom.* **2008**, *43*, 1–22.

(16) Dwivedi, P.; Bendiak, B.; Clowers, B. H.; Hill, H. H. Rapid resolution of carbohydrate isomers by electrospray ionization ambient pressure ion mobility spectrometry-time-of-flight mass spectrometry (ESI-APIMS-TOFMS). *J. Am. Soc. Mass Spectrom.* **2007**, *18*, 1163–1175.

(17) Srebalus Barnes, C. A.; Hilderbrand, A. E.; Valentine, S. J.; Clemmer, D. E. Resolving isomeric peptide mixtures: A combined HPLC/Ion Mobility-TOFMS analysis of a 4000-component combinatorial library. *Anal. Chem.* **2001**, *74*, 26–36.

(18) Clifford, M. N.; Johnston, K. L.; Knight, S.; Kuhnert, N. Hierarchical scheme for LC-MS^n identification of chlorogenic acids. *J. Agric. Food Chem.* **2003**, *51*, 2900–2911.

(19) Clifford, M. N.; Kirkpatrick, J.; Kuhnert, N.; Roozendaal, H.; Salgado, P. R. LC-MS^n analysis of the *cis* isomers of chlorogenic acids. *Food Chem.* **2008**, *106*, 379–385.

(20) Hanson, K. R. Chlorogenic acid biosynthesis, chemical synthesis and properties of the mono-O-cinnamoylquinic acids. *Biochemistry (Moscow)* **1965**, *4*, 2719–2731.

(21) Haslam, E.; Makinson, G. K.; Naumann, M. O.; Cunningham, J. Synthesis and properties of some hydroxycinnamoyl esters of quinic acid. *J. Chem. Soc.* **1964**, 2137–2146.

(22) MOBCAL: A Program to Calculate Mobilites and Collision Cross-Sections. http://www.indiana.edu/∼nano/software.html (accessed Sep 14, 2011).

(23) Tamai, N.; Miyasaka, H. Ultrafast dynamics of photochromic systems. *Chem. Rev.* **2000**, *100*, 1875–1890.

(24) Henzl, J.; Mehlhorn, M.; Gawronski, H.; Rieder, K. H.; Morgenstern, K. Reversible cis−trans isomerization of a single azobenzene molecule. *Angew. Chem., Int. Ed.* **2006**, *45*, 603–606.

(25) Alemani, M.; Peters, M. V.; Hecht, S.; Rieder, K.-H.; Moresco, F.; Grill, L. Electric field-induced isomerization of azobenzene by STM. *J. Am. Chem. Soc.* **2006**, *128*, 14446–14447.

(26) Loscertales, I. G.; Fernández de la Mora, J. Experiments on the kinetics of field evaporation of small ions from droplets. *J. Chem. Phys.* **1995**, *103*, 5041–5060.

(27) Clifford, M. N.; Knight, S.; Kuhnert, N. Discriminating between the six isomers of dicaffeoylquinic acid by LC-MS^n. *J. Agric. Food Chem.* **2005**, *53*, 3821–3832.

(28) Li, Q.; Sun, L. X.; Xu, L.; Jia, Y.; Wang, Z. W.; Shen, Z. D.; Bi, K. S. Determination and pharmacokinetic study of syringin and chlorogenic acid in rat plasma after administration of Aidi lyophilizer. *Biomed. Chromatogr.* **2006**, *20*, 1315–1320.

(29) Wang, S. J.; Zhang, Z. Q.; Zhao, Y. H.; Ruan, J. X.; Li, J. L. Simultaneous quantification of chlorogenic acid and caffeic acid in rat plasma after an intravenous administration of mailuoning injection using liquid chromatography/mass spectrometry. *Rapid Commun. Mass Spectrom.* **2006**, *20*, 2303–2308.

(30) O'Donnell, R. M.; Sun, X.; Harrington, P. d. B. Pharmaceutical applications of ion mobility spectrometry. *TrAC, Trends Anal. Chem.* **2008**, *27*, 44–53.