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Article

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Synthesis, Structure and Tandem Mass spectrometric Characterization of the Diastereomers of Quinic Acid

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ABSTRACT

(-)-Quinic acid possess eight possible stereoisomers, which occur both naturally and as products of thermal food processing. In this contribution, we have selectively synthesized four isomers namely, *epi*-quinic acid, *muco*-quinic acid, *cis*-quinic acid and *scyllo*-quinic acid in order to develop a tandem LC-MS method identifying all stereoisomeric quinic acids. Four derivatives have been unambiguously characterized by single crystal X-ray crystallography. The missing diastereomers of quinic acid were obtained by non-selective isomerization of (-)-quinic acid using acetic acid/conc. H_2SO_4 allowing chromatographic separation and assignment of all diastereomers of quinic acid. We report for the first time that a full set of stereoisomers are reliably distinguishable on the basis of their tandem mass spectrometric fragment spectra as well as their elution order. A rational for characteristic fragmentation mechanisms is proposed. In this study, we also observed that *muco*-quinic acid , *scyllo*-quinic acid and *epi*-quinic acid are present in hydrolyzed Guatemala roasted coffee sample as possible products of roasting.

INTRODUCTION:

(-)-Quinic acid **1** is distributed naturally in a variety of plant materials ranging from coffee to cinchona bark to tobacco leaves and cranberries in its free form or in the form of its depsides, chlorogenic acids. It was first isolated in 1790 and was given an empirical formula of $C_7H_{12}O_6$ in 1838.[1] In a single coffee bean, up to 4.0 mg free (-) quinic acid is found.[2] In Colombian Arabica green coffee, up to 7.0 g/kg of quinic acid is present, which increases up to 10.0 g/kg upon roasting.[3] Quinic acid provides characteristic astringent taste to the beverage, hence it is also used as a flavor enhancer in certain beverages.[4] The esters of the 3*R*, 4*S*, 5*R* isomers of the quinic acid, also referred to as chlorogenic acids, constitute the most important quinic acid derivatives in nature and in our diet.[5,6] For quinic acid diastereomers the carbon numbering is retained with respect to the parent compound.[7]

Quinic acid possesses eight possible stereoisomers: four *meso* forms and two pairs of enantiomers (Figure 1). Formally the diastereomers **2-6** can be derived from the parent compound (-)-quinic acid **1** by inversion of one or two of the four stereogenic centres as demonstrated in Figure 1. As reported by Kuhnert and co-workers, 80 different chlorogenic acid derivatives have been identified in green coffee beans.[7] After roasting and brewing, this number is increased to a minimum of 120 derivatives identified on the basis of the presence of characteristic fragment ions corresponding to quinic acid and quinic acid lactones in MS^n chromatograms.[8,9] Products of epimerization of Chlorogenic acids for example *muco*-caffeoyl and feruloyl esters were reported in roasted coffee formed at elevated temperatures.[8] After ingestion of the foods containing quinic acid esters, metabolism in humans may also give rise to the esters of the diastereomers of quinic acid.[8,10] This fact supports the assumption that the roasting or food processing in general facilitates the isomerization at the stereogenic centers in 3*R*, 4*S*, 5*R* esters of the quinic acid. [11,12]

A number of esters of the diastereomers of quinic acid have been previously reported in the literature as plant secondary metabolites. For example, in *Lactuca indica* L., *Asimina triloba* and *Aster scaber* 3,5-Dicaffeoyl-*muco*-quinic acid was identified. In *Asimina triloba* 3-caffeoyl-*muco*-quinic acid was also identified. [13-15] In *Chrysanthemum morifolium* 3,5-dicaffeoyl-*epi*-quinic acid and 1,3-dicaffeoyl-*epi*-quinic acid was identified.[16] 3,5-dicaffeoyl-*epi*-quinic acid esters were also reported in *Ilex kudingcha*.[17] These *muco*, *epi* and *scyllo* esters of diastereomers of quinic acid are reported to show important biological activities like, hepatoprotectivity, antioxidant activity and anti HIV-1 integrase activity.[13-17] Considering the fact that regiosomers as well as the esters of the diastereomers of quinic acids are readily

distinguishable by their fragmentation pattern in tandem MS experiments, it is very important to acquire authentic synthetic standards with defined stereochemistry by organic synthesis. Development of the tandem mass spectrometric methods for the identification of diastereomers of quinic and chlorogenic acids will enable us to identify novel chlorogenic acid derivatives present in the biological samples even in very low concentrations. Since, these biological samples emerge either biosynthetically, through man-induced processes such as, roasting, brewing, cooking etc. or as products of metabolism, their mass spectrometric study will help to improve understanding of the changes in the chlorogenic acid profile through biosynthetic processes in great detail.

MATERIALS AND METHODS

Chemicals

All the chemicals (analytical grade) were purchased from Sigma-Aldrich (Taufkirchen, Germany) and were used without further purification.

LC/MS^n

The LC equipment (Agillent 1100 series, Karlsruhe, Germany) comprised a binary pump, an auto sampler with a 100 μL loop, and a DAD detector with a light-pipe flow cell (recording at 254 and 320 nm and scanning from 200 to 600 nm). This was interfaced with an HCT Ultra ion-trap mass spectrometer fitted with an ESI source (Bruker Daltonics HCT Ultra, Bremen, Germany) operating in full scan, auto MS^n mode to obtain fragment ion *m/z*. As necessary, MS^2, MS^3, and MS^4 fragment-targeted experiments were performed to focus only on compounds producing a parent ion at *m/z* 191, 173. Tandem mass spectra were acquired in Manual-MS^n mode using fixed collision energy. The fragmentation amplitude was set to 0.75 V. Also, direct injection experiments targeting the fragments in MS^2, MS^3, and MS^4 were performed on all diastereomers of quinic acid keeping the fragmentation amplitude constant at 1.0 volts. MS operating conditions (negative mode) was optimized using (-)-quinic acid with a capillary temperature of 365 ^{o}C, a dry gas flow rate of 10 L/min, and a nebulizer pressure of 10 psi.

HPLC

Separation was achieved on a 250mm × 4.6 mm i.d. column containing diphenyl 5 μm and 5mm × 4.6 mm i.d. guard column of the same material (Varian, Darmstadt, Germany). The elution solvent was water: formic acid (1000:0.05 v/v) delivered at a total flow rate of 800 μL/min by 30 min isocratically.

NMR

^{1}H NMR and ^{13}C NMR spectra were acquired on a JEOL ECX-400 spectrometer operating at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR at room temperature using a 5 mm probe. The chemical shifts (δ) are reported in parts per million and were referenced to Tetramethylsilane (TMS at 0 ppm). The coupling constants (*J*) are quoted in hertz. The following abbreviations are used: s, singlet; d, doublet; t, triplet; m, multiplet; bs, broad signal; bd, broad doublet; dd, doublet of doublets, ddd, doublet of doublet of doublets.

Synthesis of the mixture of the epimers of (-)-quinic acid

The mixture of the epimers of (-)-quinic acid was obtained non-selectively by the process previously described by Maier and co-workers.[6]

Synthesis of the *epi*-quinic acid (2)

Methyl quinate was prepared by refluxing quinic acid **1** (5g, 26.02 mmol) with MeOH (100 mL) and Amberlite IR120 acidic resin (5g) for 12 h. The reaction mixture was then filtered and concentrated in *vacuo*. The product was obtained in more than 95% yield and the purity of the product was confirmed by NMR. It was then subjected to selective silyl protection on C_3 and C_5 of the methyl quinate by *tert*-butyldimethylsilyl chloride (TBDMSCl).[18]

Methyl quinate (2650 mg, 12.86 mmol) was stirred with triethylamine (5 mL) in anhydrous dimethylformamide (26 mL) in an inert atmosphere and to this solution, *tert*-butyldimethylsilyl chloride (5034 mg, 33.436 mmol) was added and the mixture was stirred for 2 h at 0 ^{0}C and 16 h at room temperature. EtOAc was added and the residue was filtered. Concentrated filtrate was purified using flash chromatography (gradient eluent: 20% EtOAc in petroleum ether) to afford white crystalline methyl 3,5-Di-*O*-(*tert*-butyldimethylsilyl)quinate **7** in 75% yield, which was confirmed by single crystal X-ray diffraction have free C-4 on quinic acid skeleton. Compound 7 was used as a precursor for

the synthesis of *epi*-quinic acid **2** and *scyllo*-quinic acid **5** as shown in the supplementary information.

Dess-Martin periodinane (390 mg, 0.92 mmol) was added to the solution of compound **7** (200 mg, 0.46 mmol) dissolved in dichloromethane (15 mL) at room temperature. Reaction was stirred overnight and diluted with Et_2O. Then to this 20 ml of a 1:1 saturated mixture of $Na_2S_2O_3$ and $NaHCO_3$ solution was added until the reaction became clear. Organic layer was collected and the aqueous layer was extracted with EtOAc (3× 20 mL). Combined organic layers were collected, dried and concentrated in *vacuo*. Crude product was subjected to flash chromatography (eluent: 24% EtOAc in petroleum ether), which afforded methyl 3,5-Di-*O*-(*tert*-butyldimethylsilyl)-4-oxoquinate **12** in the form of sticky colorless solid in 87%. ^{13}C NMR (100 MHz, $CDCl_3$): 206.51, 173.15, 75.98, 75.33, 69.81, 52.94, 46.71, 41.22, 25.79, 25.61, 18.45, 17.88, -4.73, -5.04, -5.25, -5.30.

Compound **12** was subjected to reduction at C-4 by the action of $NaBH_4$ and L-selectride. Out of these two reduction procedures, reduction with $NaBH_4$ showed higher selectivity towards the formation of *epi*- derivative as compared to L-selectride. 177 mg of compound **12** (0.4074 mmol) was dissolved in ethanol and the reaction flask was immersed in an acetone bath. Liquid nitrogen was slowly added until the bath temperature reached -30 ^0C. $NaBH_4$ (23 mg, 0.6111 mmol) was added and the mixture was stirred for 40 min. Solvent was removed immediately under reduced pressure at 30 ^0C in a rotary evaporator. The residue was then extracted with water and EtOAc mixture three times. Organic layers were collected and dried over Na_2SO_4 and concentrated. ^{13}C NMR of the crude product confirmed reduction at C4 indicated by the loss of a peak at 206 ppm. The crude product was directly subjected to hydrolysis by 2M HCl and water without further purification. Product of the hydrolysis was diluted with water and extracted with water and EtOAc mixture thrice. Aqueous layers were collected and concentrated in *vacuo*. Resulting white product was used in HPLC-MS analysis.

Synthesis of the *muco*-quinic acid (3)

muco-Quinic acid **3** was obtained from the methyl TMB-*muco*-quinate (TMB Ley's tetramethoxybutane), which was synthesized by the procedure previously reported by Jaiswal *et al.*.[8,] Methyl TMB-*muco*-quinate (500 mg, 1.56 mmol) was subjected to hydrolysis by 70% aqueous trifluoroacetic acid for 1 hour. Resulting methyl *muco*-quinate was stirred with 2M KOH for 20 min followed by neutralization by Amberlite IR 120 acidic resin for 10 min. The mixture was filtered and concentrated under reduced pressure. The resulting yellowish solid

was analyzed by NMR. ^{1}H NMR (400 MHz, D_2O): 1.73 (dd, 1H, H_{6ax}, $^{3}J_{HH}$ 12.4), 1.75 (dd, 1H, H_{2ax}, $^{3}J_{HH}$ 12.4), 1.86 (dd, 1H, H_{6eq}, $^{2}J_{HH}$ 4.1, $^{3}J_{HH}$ 11.2), 1.89 (dd, 1H, H_{2eq}, $^{2}J_{HH}$ 4.6, $^{3}J_{HH}$ 11.4), 3.21 (t, 1H, H_4, $^{3}J_{HH}$ 9.6), 3.62 (ddd, 2H, $^{2}J_{HH}$ 4.6, H_3 and H_5, $^{3}J_{HH}$ 11.9, 9.1). ^{13}C NMR (100 MHz, D_2O): 180.9, 79.5, 74.7, 69.8, 69.7, 40.5, 40.4

Synthesis of the *cis*-quinic acid (4)

3,4-*O*-Cyclohexylidene-1,5-quinide **13** was synthesized by adding quantities of 10.00 g (52.04 mmol) of quinic acid and 200 mg (1.05 mmol) of *p*-toluenesulfonic acid monohydrate (PTSA·H_2O) to 100 mL of cyclohexanone to give a white suspension. The reaction was then refluxed for 24 h to give a yellow solution which was cooled to 50 ºC and neutralized with a solution of NaOEt (71.5 mg) in EtOH (5 mL) to give a yellow clear solution. The solvents were removed under reduced pressure and to the resulting yellow viscous liquid a volume of 100 mL of EtOAc was added. The organic phase was washed with 50 mL of H_2O and the aqueous phase was back-extracted with 30 mL EtOAc. The combined organic layers were washed with a half-saturated $NaHCO_3$ solution, dried on Na_2SO_4, filtered and evaporated. The resulting yellow viscous liquid was recrystallized from a 1:1 n-heptane:EtOAc solution to afford white crystals of compound **13** (9.26 g, 36.43 mmol, 70%, mp. 172-174 °C); ^{1}H-NMR ($CDCl_3$): δ_H 4.73 (dd, 1H, *J* = 5.6, 2.8 Hz), 4.48 (td, 1H, *J* = 6.8, 2.8 Hz), 4.29 (ddd, 1H, *J* = 6.4, 2.8, 1.4 Hz), 2.66 (d, 1H, *J* = 11.9 Hz), 2.38-2.31 (ddd, 1H, *J* = 14.7, 7.8, 2.3 Hz), 2.31-2.25 (ddt, 1H, *J* = 11.9, 6.4, 2.3 Hz), 2.17 (dd, 1H, *J* = 14.7, 3.2 Hz), 1.73-1.68 (m, 2H), 1.67-1.60 (m, 2H), 1.57-1.51 (m, 4H), 1.43-1.36 (m, 2H); ^{13}C-NMR ($CDCl_3$): δ_C 178.9 (-COOR), 110.7 (C-1'), 76.1 (C-4), 71.8 (C-1), 71.6 (C-3), 71.2 (C-5), 38.6 (C-6), 37.0 (C-2), 34.5 (C-6'), 33.7 (C-2'), 25.1 (C-5'), 24.0 (C-3'), 23.6 (C-4').

3,4-*O*-Cyclohexylidene-1,5-quinide **13** (8.75 g, 34.41 mmol) was dissolved in 100 mL MeOH and a 21% solution NaOMe/MeOH was added (187 mg NaOMe). The clear solution was stirred overnight, the mixture was then quenched with glacial acetic acid (232 µL) and the volatile components were removed under vacuum. The resulting mixture was dissolved in EtOAc and washed 3 times (3x40 mL). The organic layer was dried over Na_2SO_4, filtered and the solvent was removed under low pressure. The crude product was purified by column chromatography on silica gel (20-50% EtOAc/petroleum ether) to give methyl 3,4-*O*-cyclohexylidene-quinate **14** as a white solid (5.87 g, 20.51 mmol, 60%). ^{1}H-NMR ($CDCl_3$): δ_H 4.46 (dt, 1H, *J* = 5.9, 3.7 Hz), 4.14-4.07 (m, 1H), 3.97 (t, 1H, *J* = 5.9 Hz), 3.79 (s, 3H), 2.26 (m, 1H), 2.25 (d, 1H, *J* = 4.1 Hz), 2.08 (ddd, 1H, *J* = 13.4, 4.1, 3.0 Hz), 1.86 (dd, 1H, 13.7, 11.0 Hz), 1.74-1.68 (m, 2H), 1.68-1.52 (m, 6H), 1.44-1.33 (m, 2H); ^{13}C-NMR ($CDCl_3$):

δ_C 175.6 (-*C*OOCH$_3$), 110.1 (C-1'), 79.4 (C-4), 74.1 (C-1), 73.1 (C-3), 68.7 (C-5), 53.0 (-CH$_3$), 39.0 (C-6), 38.0 (C-2), 34.9 (C-6'), 34.8 (C-2'), 25.0 (C-5'), 24.1 (C-3'), 23.7 (C-4').

To a suspension of Dess-Martin periodinane (2.96 g, 6.99 mmol) in anhydrous CH_2Cl_2 (65 mL) compound **14** (1.82 g, 6.36 mmol) was added. The reaction mixture was stirred at room temperature for 18 h, was then diluted with Et_2O (100 mL) and 50 mL of a saturated aqueous $Na_2S_2O_3$ and 50 mL of a saturated $NaHCO_3$ solution (100 mL) were added. The mixture was stirred until the solids were dissolved (20 min). The aqueous layer was extracted with Et_2O (2× 50 mL) and the combined organic layers were dried over Na_2SO_4, filtered and concentrated in vacuum. Product methyl 3,4-*O*-cyclohexylidene-5-oxoquinate **15** (1.81 g, 6.36 mmol, 100%) was used for the next step without further purification. ^{1}H-NMR ($CDCl_3$): δ_H 4.72 (m, 1H), 4.41 (d, 1H, *J* = 5.5 Hz), 3.81 (s, 3H), 2.89 (d, 1H, *J* = 14.2 Hz), 2.80 (dd, 1H, *J* = 14.7, 2.3 Hz), 2.56 (t, 1H, *J* = 2.3 Hz), 2.55 (d, 1H, *J* = 4.1 Hz), 1.74-1.64 (m, 2H), 1.64-1.56 (m, 4H), 1.56-1.47 (m, 2H), 1.43-1.32 (m, 2H); ^{13}C-NMR ($CDCl_3$): δ_C 204.4 (C-5), 173.0 (-*C*OOCH$_3$), 111.7 (C-1'), 78.2 (C-4), 76.9 (C-1), 75.9 (C-3), 53.5 (-CH$_3$), 49.1 (C-6), 37.0 (C-2), 35.3 (C-6'), 34.7 (C-2'), 24.9 (C-5'), 23.9 (C-3'), 23.8 (C-4').

To obtain methyl 3,4-*O*-cyclohexylidene-*epi*-quinate **16**, compound **15** (1.53 g, 5.33 mmol) was dissolved in a 1:1 mixture (v/v) MeOH/THF (100 mL) and was cooled to -30 ˚C with an acetone/liquid nitrogen bath. $NaBH_4$ (222 mg, 5.86 mmol) was added and the mixture was stirred at -30 ˚C for 1 h. The solvents were removed in vacuum and the residue was extracted three times with a water/EtOAc mixture. The organic layers were dried over Na_2SO_4, filtered and the solvent was removed under reduced pressure. The product was purified by column chromatography (40% EtOAc/petroleum ether). ^{1}H-NMR ($CDCl_3$): δ_H 4.52 (dt, 1H, *J* = 7.6, 5.0 Hz), 4.30 (dd, 1H, *J* = 7.6, 4.1 Hz), 3.90 (dt, 1H, *J* = 9.6, 4.1 Hz), 3.78 (s, 3H), 2.18-2.06 (m, 3H), 2.02 (dd, 1H, *J* = 14.2, 10.1 Hz), 1.75 (m, 2H), 1.69-1.59 (m, 4H), 1.59-1.51 (m, 2H), 1.44-1.35 (m, 2H); ^{13}C-NMR ($CDCl_3$): δ_C 175.4 (-*C*OOCH$_3$), 110.0 (C-1'), 73.8 (C-4), 73.4 (C-1), 72.5 (C-3), 66.1 (C-5), 53.0 (-CH$_3$), 38.1 (C-6), 36.4 (C-2), 35.6 (C-6'), 34.0 (C-2'), 25.2 (C-5'), 24.1 (C-3'), 23.7 (C-4').

Crystals of **4** suitable for single crystal X-ray diffraction were obtained in an NMR tube containing demethylated **16** dissolved in $CDCl_3$ by removal of the acid-labile cyclohexylidene protection promoted by the trace amounts of HCl present in the deuterated solvent. ^{1}H-NMR (D_2O): δ_H 3.76 (br, 1H), 3.67 (t, 1H, *J* = 3.2 Hz), 3.64 (t, 1H, *J* = 3.2 Hz), 1.97 (dd, 2H, J = 12.4, 4.1 Hz), 1.62 (t, 2H, *J* = 12.4 Hz); ^{13}C-NMR (D_2O): δ_C 177.1 (-*C*OOCH$_3$), 72.4 (C-4), 71.1 (C-1), 66.9 (C-3, C-5), 35.9 (C-2, C-6).

Synthesis of the *scyllo*-quinic acid (5)

Methyl 3,5-Di-*O*-(*tert*-butyldimethylsilyl)quinate (**7**) (500 mg, 1.15 mmol) was mixed and stirred with 12 mL of anhydrous pyridine and cooled to 0 ^{0}C. Methanesulfonyl chloride was added drop wise to the mixture. Reaction was stirred at room temperature for overnight. 10 mL $NaHCO_3$ added and after stirring for 10 min, aqueous phase was washed two times with Et_2O. Organic layers were collected, dried over Na_2SO_4 and concentrated. Crude product was subjected to flash chromatography (eluent: 22% EtOAc in petroleum ether), which afforded methyl 3,5-di-*O*-(*tert*-butyldimethylsilyl)-4-*O*-methanesulfonylquinate **8** in 95% yield. ^{1}H NMR (400 MHz, $CDCl_3$): 0.06 (s, 3H, MeSi), 0.08 (s, 3H, MeSi), 0.11 (s, 3H, MeSi), 0.12 (s, 3H, MeSi), 0.86 (s, 9H, Me_3CSi), 0.88 (s, 9H, Me_3CSi), 1.94 (dd, 1H, H_{6ax}, $^2J_{HH}$ 13.74, $^3J_{HH}$ 10.07, Hz), 2.00-2.10 (m, 1H, H_{2ax}), 2.12-2.31 (m, 2H, $H_{6eq,}$ H_{2eq}), 3.04 (s, 3H, MeS), 3.77 (s, 3H, -OMe), 4.25 (dd, 1H, H_4, $^3J_{HH}$ 8.7, $^2J_{HH}$ 2.3 Hz), 4.34 (m, 1H, H_5) and 4.57 (m, 1H, H_3).

Compound **8** (520 mg, 1.014 mmol) was dissolved in 5 mL anhydrous dimethylformamide and to this was added cesium fluoride (790 mg, 5.2 mmol). The mixture was stirred for 30 min and cinnamic acid (770 mg, 5.2 mmol) was added. The reaction was heated to 90 ^{0}C and stirred for 36 h. DMF was removed in vacuum and the obtained crude product was subjected to column chromatography (eluent: 45% EtOAc, 55% petroleum ether). One fraction collected from the column chromatography confirmed the formation of the desired product in the NMR analysis and formed a crystal in the NMR tube suitable for single crystal X-ray diffraction.

The mixture of compound **9** and compound **10** was subjected to hydrolysis through the same procedure as described earlier. Product of the hydrolysis was diluted with water and extracted with water and EtOAc mixture three times. Aqueous layers were collected and concentrated in *vacuo*. Resulting white product was used in HPLC/MS analysis.

Hydrolysis of the chlorogenic acids in roasted coffee

10 grams of finely grounded roasted Arabica coffee from Guatemala was boiled in distilled water for 1 hour. The mixture was then filtered and the water in the filtrate was removed under reduced pressure. Resulting residue was subjected to base hydrolysis by treatment of 25 mL 2M NaOH for 2 h. The reaction was neutralized by addition of 2M HCl. The mixture was diluted with 25 mL water and the aqueous layer was extracted with EtOAc (3 × 20 mL). The aqueous layers were collected and concentrated in *vacuo*. Resulting yellowish product was used directly in HPLC-MS analysis.

Synthesis of the methyl esters of *epi*-, *muco*-, *cis*-, *scyllo*-quinic acids and (-)-quinic acid

As described earlier, methyl quinates of all the synthesized diastereomers except the methyl *cis*-quinate **17** were prepared by refluxing respective diastereomers in MeOH with equal amount of Amberlite IR120 acidic resin for 12 h. The reaction mixture was then filtered and concentrated in *vacuo*. The resulting product was used directly in HPLC/MS analysis in case of methyl *epi*-quinate, methyl *scyllo*-quinate and methyl *muco*-quinate. Crystals of methyl *cis*-quinate **17** suitable for single crystal X-ray diffreaction (Figure 4) were obtained in an NMR tube containing methyl 3,4-*O*-cyclohexylidene-*epi*-quinate **16** dissolved in $CDCl_3$ by removal of the acid-labile cyclohexylidene protection promoted by the trace amounts of HCl present in the deuterated solvent. The methyl quinate of (-)-quinic acid was confirmed by the NMR and then utilized in HPLC/MS.

X-ray crystallography

Crystals were mounted on a Hampton cryoloop in light oil for data collection at 100 K. Indexing and data collection were performed on a Bruker D8 SMART APEX II CCD diffracto-meter with κ geometry and Mo Kα radiation (graphite mono- chromator, λ = 0.71073 Å; Karlsruhe, Germany). Data integration was performed using SAINT. Routine Lorentz and polarization corrections were applied. The SHELX package was used for structure solution and refinement. Refinements were full-matrix least-squares against F^2 using all data. In the final refinement, all non-hydrogen atoms were refined anisotropically and hydrogen atoms were either found directly and refined isotropically or placed in calculated positions. Crystallographic data (CIF files) are available at www.ccdc.cam.ac.uk.

RESULTS AND DISCUSSIONS

Synthesis of quinic acid diastereomers

Several methods for the synthesis of the *epi*-quinic acid **2** have been reported, [18] exploiting formation of a 1, 5-quinide followed by selective protection of C-3-OH by silyl protecting group and subsequent inversion of configuration at C_4 by oxidation-reduction sequences. This approach was found to be unreliable in our hands, since silyl-protection was observed to be non-selective and separation low yielding. In the present work, once the methodology to obtain **7** in pure form was developed, the ambiguity in the selective protection was eliminated in order to leave C-4-OH free for further treatment (Figure 2). Methyl 3,5-di-*O*-(*tert*-

butyldimethylsilyl) quinate **7** served as a precursor for both of the diastereomers, *epi*-quinic acid **2** and *scyllo*-quinic acid **5**. Of these, diastereomer **2** was obtained by oxidation-reduction pathway and **5** was synthesized by incorporating S_N2 reaction strategy, using cinnamic acid as a nucleophile leading to inversion of configuration at C_4. However, in fact the actual product isolated, was found to have rearranged to yield 3-*O*-cinnamoyl-1,4-*scyllo*-quinide **9** possibly due to the traces of acid present in the work-up procedure. 3-*O*-Cinnamoyl-1, 4-*scyllo*-quinide **9** and 3-*O*-cinnamoyl-1,5-quinide **10** were co-crystallized and were obtained in very low yield. Hence, attempts towards the isolation of compound **9** were avoided and the crystals were directly hydrolyzed to obtain a 9: 1 mixture of *scyllo*-quinic acid **5** and quinic acid as judged by NMR and LC-MS data.

Cis-Quinic acid **4** was obtained starting from 1,3-cyclohexylidene protected quinide [19] **13** (Figure 3)**.** Opening of the lactone ring using methoxide furnished methylester **14**, which was subsequently oxidized to ketone **15** using the Dess-Martin periodinane reagent. Diastereoselective reduction using $NaBH_4$ yielded a 4:1 mixture of diastereomers, from, which methyl ester **17** could be obtained in pure form by crystallization. Hydrolysis yielded the target compound **4** in poor yield, however in crystalline form suitable for single crystal X-ray diffraction studies, hence confirming its structure.

Discussion of single crystal X-ray structures

For five synthetic intermediates crystals suitable for single crystal X-ray diffraction could be obtained. The resulting X-ray structures confirm the relative stereochemistry of the quinic acid scaffold as diastereomers of quinic acid and therefore support the relative stereochemistry of the final quinic acid diastereomer products. In the crystal of *cis*-quinic acid **4** (Figure 4), we observed two molecules in the asymmetric unit representing the same structure. Compounds 3-*O*-cinnamoyl-1,4-*scyllo*-quinide **9** and 3-*O*-cinnamoyl-1,5-quinide **10** co-crystallized along with one molecule of chloroform in one asymmetric unit. The crystal structure of 3,5-di-*O*-(*tert*-butyldimethylsilyl)quinate **7** showed conformational disorder in one of the silyl groups (Si1A and Si1B), both conformations are shown in Figure 4. The disorder was modeled and showed a value of 50% for each conformation. Table 2 illustrates the crystal data and structure refinement for compounds methyl *cis*-quinate **17**, *cis*-quinic acid **4**, 3-*O*-cinnamoyl-1,4-*scyllo*-quinide(**9**), 3-*O*-cinnamoyl-1,5-quinide **10**, 3,4-*O*-Cyclohexylidene-1,5-quinide (**13**) and methyl 3,5-Di-*O*-(*tert*-butyldimethylsilyl)quinate **7**.

335 **LC-MS analysis of quinic acid diastereomers**

336 With authentic standards in hand for four quinic acid diastereoismers from this work and
337 *muco* quinic acid from previous work,[8] a HPLC method was developed allowing separation
338 of quinic acid diastereomers for future food analysis and tandem MS investigations. All
339 quinic acid diastereomers can be readily detected in negative ion mode as pseudomolecular
340 ions ([M-H], $C_7H_{11}O_6$) at *m/z* 191. 0550 +/- 0.0003 using high resolution MS or at 191.1 using
341 an ESI ion trap mass spectrometer. Using Maier's protocol for unselective isomerization using
342 concentrated acid [6] the missing isomer *neo*-quinic acid **6** could be tentatively assigned by
343 arguments of exclusion. Observation the retention times of all the diastereomers, shows that
344 they elute very close to each other . The elution order can be given as, **1**>**5**>**2**>**3**=**4**. Where **1**
345 (quinic acid) elutes at 3.9 min and **3**/**4** *muco*- and/or *cis*-quinic acid elute at 3.4 minutes (Table
346 1). Although diastereomers **3** and **4** partially co-elute at the same retention time, their tandem
347 MS shows clear distinction as can be seen in Figure 7 and 8. All tandem MS data for quinic
348 acid diastereomers **1**-5 with the precursor ion at *m/z* 191.0 are shown in Figures 5-9.

349 An analysis of a roasted and subsequently hydrolyzed Arabica coffee extract shows that in an
350 extracted ion chromatogram at *m/z* 191 four diastereomers of quinic acid can be observed and
351 assigned (Figure 10) The identified compounds are *epi*-quinic acid **2**, *muco*-quinic acid **3** and
352 *cis*-quinic acid **4** along with quinic acid **1**. Following an earlier report we assume that these
353 compounds are formed at elevated temperature during coffee roasting, since they are absent
354 from the corresponding green coffee bean material. [6, 20-23] It is surprising to note that quinic
355 acid secondary alcohols are prone to epimerization under thermal conditions, whereas
356 structurally related carbohydrates are not. [24-26]

357 **Tandem mass spectrometry of quinic acid diastereomers**

358 Recent advances in tandem mass spectrometry have shown that certain classes of isomeric
359 compounds can be reliably distinguished by tandem mass spectrometry based on significant
360 differences in their tandem mass spectra. In particular regioisomers have been shown to
361 display characteristic fragmentation mechanisms allowing a reliable assignment of structure.
362 [27-29] Much less is known about differences in fragment spectra of sets of diastereomers with
363 the compounds available here allowing a proof of principle study that diastereomeric
364 structures can be distinguished and assigned based on their tandem MS spectra.

365 Fragment spectra were obtained in negative ion mode using collision induced dissociation
366 (CID) using an ion trap mass spectrometer. MS^2, MS^3 and in some cases MS^4 spectra were

recorded. The MS^2 fragmentation all of the diastereomers was investigated under constant collision energy in the LC-MS runs at 0.75 volts (see Table 1). Optimum value for the collision energy was obtained by taking (-)-quinic acid **1** as a standard. All diastereomers show characteristic fragmentations including neutral loss of 46 Da ($-CH_2O_2$) and sequential neutral loss of 18 Da ($-H_2O$). Comparison of MS^n spctra of fragment ions with MS^2 spectra of reference compounds the structure of the fragment ions resulting from water loss can differ leading either to olefinic fragment ions or to epoxide fragment ions. The diastereomers differ however significantly in the intensity of fragment ions and the order of fragmentation events in MS^n.

Diastereomers **2** and **5** elute very close to each other and they fragment almost identically in MS^2, however, in MS^3, *scyllo*-quinic acid **5** produces a base peak at *m/z* 93 (C_6H_5O) whereas *epi*-quinic acid **2** gives a base peak at *m/z* 111 ($C_6H_7O_2$) in MS^3. In another case, (-)-quinic acid **1** and *muco*-quinic acid **3** show similar fragmentation patterns giving base peaks at *m/z* 127 ($C_6H_7O_3$) and *m/z* 109 ($C_6H_5O_2$) in MS^2 and MS^3 respectively but, they differ in the retention times with compound **3** eluting 0.5 min earlier than compound **1**. In another case distinction between diastereomers **1** and **3** on the basis of their tandem MS, fragmentation had to be carried out in MS^4.

As shown in scheme **A** in Figure 11, (-)-quinic acid (**1**) with a pseudomolecular ion at *m/z* 191.1 ($C_7H_{11}O_6$) as precursor ion undergoes concomitant dehydration and deformylation to produce a base peak at *m/z* 127 ($C_6H_7O_3$) corresponding to fragment **Q1** in MS^2. Loss of 46 Da and 18 Da takes place simultaneously as no secondary peak at *m/z* 145, corresponding to deformylated quinic acid can be detected. Interestingly the carboxyl moiety on C-1 is formally fragmented as formic acid reminiscent of an ester elimination or Tchugaev elimination in mechanistic organic chemistry. Presumably dehydration takes place in between C-2 and C-3 due to the fact that the proton on C-2 and hydroxyl group on C-3 share an *anti*-periplanar arrangement. The precursor ion at *m/z* 127 further fragments in MS^3 to give base peak at *m/z* 109, which could suggest the presence of dihydroxybenzene-type structure. However, since C-4-OH and C-5-OH do not possess the *anti*-periplanar arrangement between either protons and hydroxyl groups required for stereoelectronic reasons for an E_2 type elimination a different pathway is taken. Comparison of MS^2 spectra of all three dihydroxybenzenes (1,3-, 1,4- and 1,2-$C_6H_6O_2$) shows that none of them is identical to the MS^4 spectrum of **1** with *m/z* 127 as precursor ion (see Figure S5 in supplementary information). The fragmentation of an epoxide such as cyclohexene oxide in MS^2 gives only a

peak at *m/z* 81 with a neutral loss of 16 Da reminiscent of the MS^4 of (-)-quinic acid **1**. Hence an epoxide fragment ion **Q3** is proposed as product of fragmentation of **Q1**.

Scheme **B** in **Figure 11** shows the proposed fragmentation pathway for *epi*-quinic acid **2**. The fragmentation can be explained if elimination of water through an E_2 route takes place between C-1 and C-2 to give *epi*-shikimic acid with a base peak at *m/z* 173 ($C_7H_9O_5$) (**Q3**). Indeed the MS^3 with *m/z* 173 as precursor ion is almost identical to the MS^2 spectrum of shikimic acid.[29] A direct infusion experiment shows that *m/z* 111 ($C_6H_7O_2$) is a primary peak in the MS^3 of *epi*-quinic acid (**2**) (Figure 11). It can be explained through the assumption of the presence of **Q4** arising through the dehydration in between C-4 and C-5, which possess the required *anti*-periplanar arrangement of a proton and –OH group as can be seen in the Figure 11.

The suggested fragmentation of *muco*-quinic acid **3** is shown in scheme **C**. The carboxyl group on C-1 is cleaved as a formic acid and the dehydration takes place in between C-2 and C-3 due to anti-periplanar arrangement similar to the fragmentation in **1** to give base peak in MS^3 at *m/z* 127 (**Q5**). As shown in the scheme, when the cyclohexane ring is inverted, an *anti*-periplanar geometry between C-2-H and C-3-OH arises suitable for dehydration between C-2 and C-3. As can be observed in the direct infusion experiment in Figure 13, *m/z* 109 fragments further to give *m/z* 81.2 exactly similar to the MS^4 of (-)-quinic acid (**1**). This means that the fragments **Q2** and **Q6** are fragment ions of identical structure. Diastereomers **1** and **3** can be distinguished in MS^2 of **3**, as a secondary peak at *m/z* 145 was observed, which signifies the decarboxylated molecule (Figure 10). In the structural arrangement of the diastereomer **3**, absence of the hydrogen bonding between 1,3-diaxially positioned oxygen anion on C1 and hydrogen on C-3-OH does not facilitate the elimination of C-3-OH as observed in case of (-)-quinic acid **1**.

MS^2 of the *cis*-quinic acid **4** gives base peak at *m/z* 93 along with the secondary peaks at *m/z* 173 and *m/z* 111. Base peak at *m/z* 93 suggests that decarboxylation and three dehydration processes had taken place simultaneously to obtain the stable aromatic phenolate fragment ion **Q9**. Unfortunately, phenol as an external standard and *m/z* 93 in compound **4** do not fragment further as observed in direct infusion experiments, even at high collision energies. However mechanistic arguments show that the phenolate structure of **Q9** is reasonable if not expected, since in intermediate **Q7** all C-OH moieties show an *anti*-periplanar arrangement with **Q8** at *m/z* 111 as a secondary peak and reaction intermediate in MS^2.

scyllo-Quinic acid **5** shows unique fragmentation pattern if compared to the other diastereomers in present work. In MS^2 it shows the pattern similar to the MS^2 of the *epi*-quinic acid **2** and in MS^3, *m/z* 173 directly fragments into *m/z* 93 similar to the MS^2 of the *cis*-quinic acid **4** as can be seen in Figure 10. It is unclear at this point of time how the fragmentation scheme in case of **5** can be explained mechanistically.

In conclusion, we have selectively synthesized all the diastereomers of the quinic acid except for the *neo*-quinic acid **6** to prove that they can be chromatographically resolved and can be identified by their characteristic fragmentation behavior in tandem MS spectra. This study also illustrates the importance of the tandem mass spectrometry in the area of identification and confirmation of the stereochemistry of comparatively small molecules like the diastereomers of the quinic acid. It is also worth mentioning that it is the first time the crystal structures of 3-*O*-cinnamoyl-1,4-*scyllo*-quinide **9** , 3-*O*-cinnamoyl-1,5-quinide **10**, *cis*-quinic acid **4**, methyl *cis*-quinate **17** and 3,4-*O*-Cyclohexylidene-1,5-quinide **13** have been reported (Figure 4). The fragmentation mechanisms are explained on the basis of the conformational differences between the diastereomers of the quinic acid. Presence of the diastereomers like **3**, **5** and **2** in hydrolyzed roasted coffee sample proves the existence of selected diastereomers of chlorogenic acids in roasted coffee. However, it still remains unclear if the epimerization occurs after the esterification with cinnamoyl functionality or the cinnamoyl group esterifies with epimerized quinic acid during the food processing.

Supplementary informations are available containing crystallographic data, further synthetic details and chromatograms and spectral data for all quinic acid diasteromers discussed.

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TABLE LEGEND

Table 1. MS^2 data of quinic acid diastereomers in negative ion mode at 75% collision energy

FIGURE LEGEND

Figure 1. Stereoisomers of quinic acid

Figure 2. Reaction scheme for obtaining *scyllo*-quinic acid **5** and *epi*-quinic acid **2**

Figure 3: Reaction scheme for the synthesis of *cis*-quinic acid **4**

Figure 4: Single crystal X-ray structures of *cis*-quinic acid (**4**) , 3,4-*O*-Cyclohexylidene-1,5-quinide (**13**), 3-*O*-cinnamoyl-1,4-*scyllo*-quinide (**9**)**,** 3-*O*-cinnamoyl-1,5-quinide (**10**), methyl *cis*-quinate (**17**), methyl 3,5-Di-*O*-(*tert*-butyldimethylsilyl)quinate (**7**) Conformer **A** and of methyl 3,5-Di-*O*-(*tert*-butyldimethylsilyl)quinate (**7**) Conformer **B.**

Figure 5: MS^2 and MS^3 spectra of (-) quinic acid **1** in negative ion mode

Figure 6: MS^2 and MS^3 spectra of *epi*- quinic acid **2** in negative ion mode

Figure 7: MS^2 and MS^3 spectra of *muco*-quinic acid **3** in negative ion mode

Figure 8: MS^2 spectrum of *cis*- quinic acid **4** in negative ion mode

Figure 9: MS^2 and MS^3 spectra of *scyllo*- quinic acid **5** in negative ion mode

Figure 10: Extracted ion chromatogram in negative ion mode of methanolic roasted coffee bean extract at *m/z* 191.1 showing quinic acid diastereomers (for chromtaograms of individual quinic acids diastereomers please ssee supplementary information)

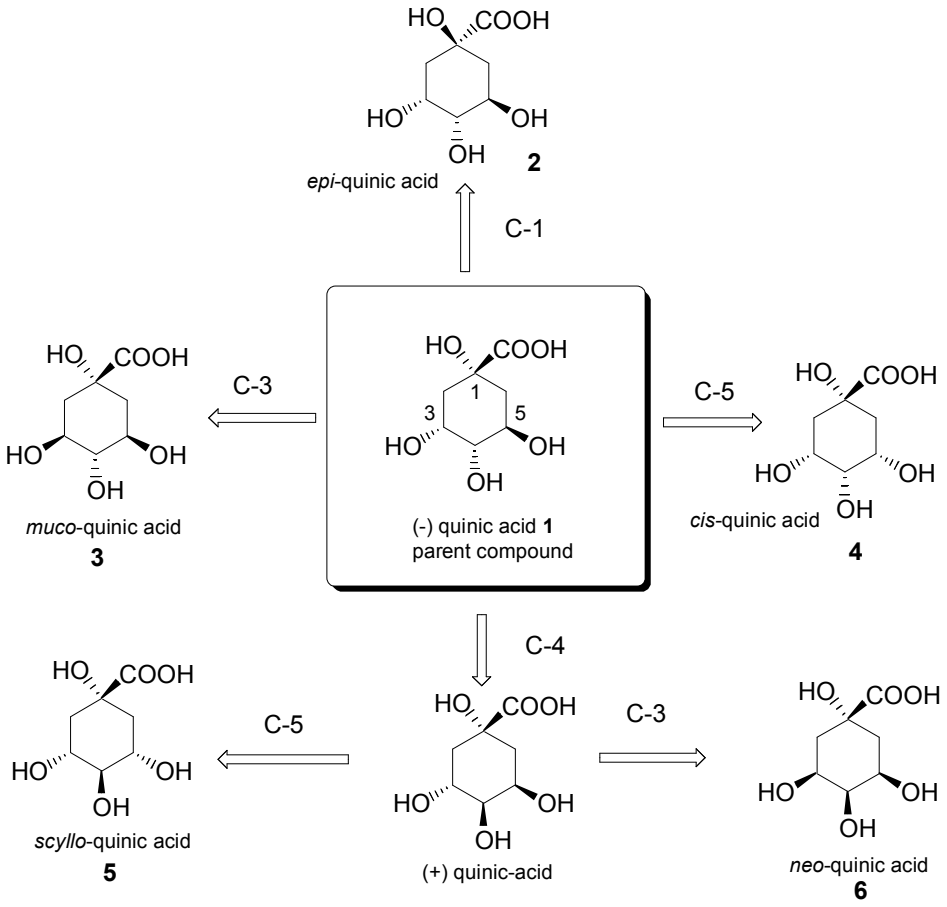
Figure 11: Proposed fragmentation scheme of quinic acids diastereomers in negative ion mode

Tables

Table 1. Chromatographic and MS^2 data of quinic acid diastereomers **1-5** in negative ion mode at 75% collision energy

Compound No.	Retention Time [min]	MS^2 (m/z 191.0 as precursor ion)								
		Base peak	Secondary peak							
		m/z	*m/z*	int%	*m/z*	int%	*m/z*	int%	*m/z*	int%
1	3.9	127	173	82	85	56	93	51	111	23
2	3.6	173	127	86	145	30	85	24	111	17
3	3.4	127	173	64	85	45	109	20	145	19
4	3.4	93	173	37	111	34	155	7	61	5
5	4.2	173	127	91	85	50	111	48	93	46

Figures


epi-quinic acid
2
C-1
muco-quinic acid
3
C-3
(-) quinic acid 1
parent compound
C-5
cis-quinic acid
4
C-4
scyllo-quinic acid
5
C-5
(+) quinic-acid
C-3
neo-quinic acid
6

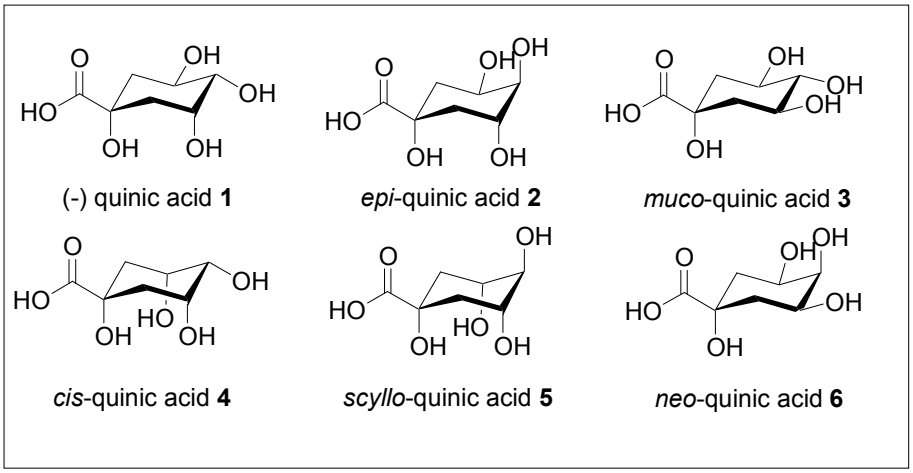
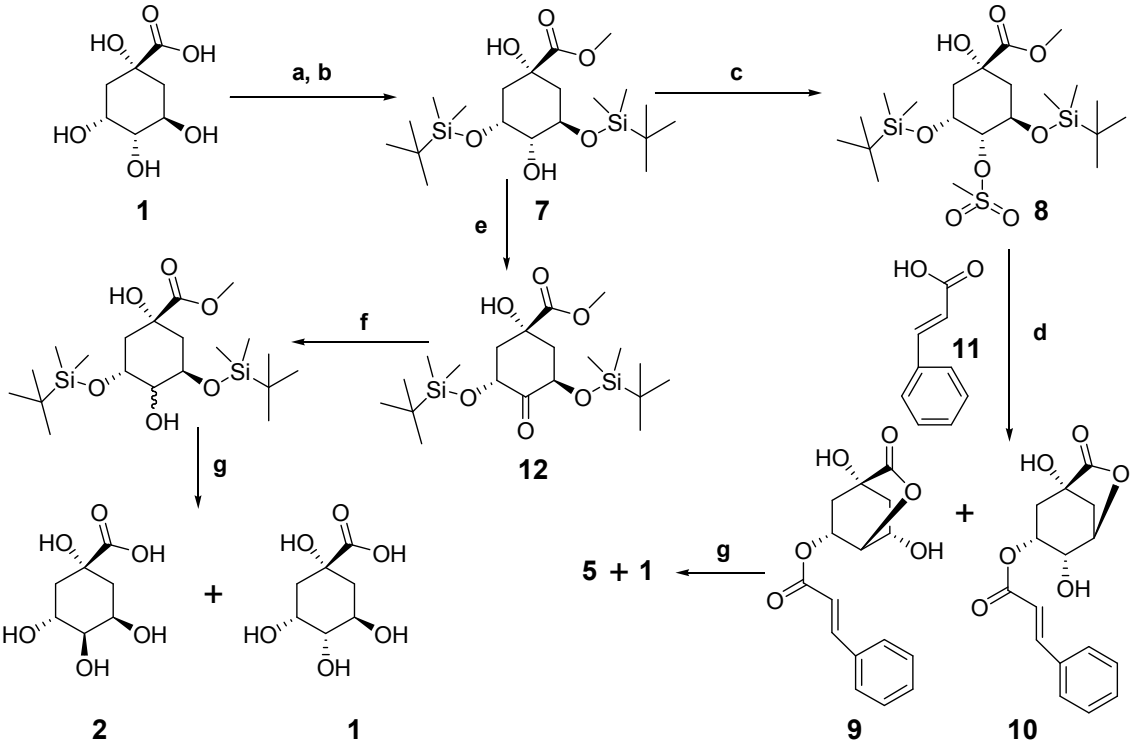
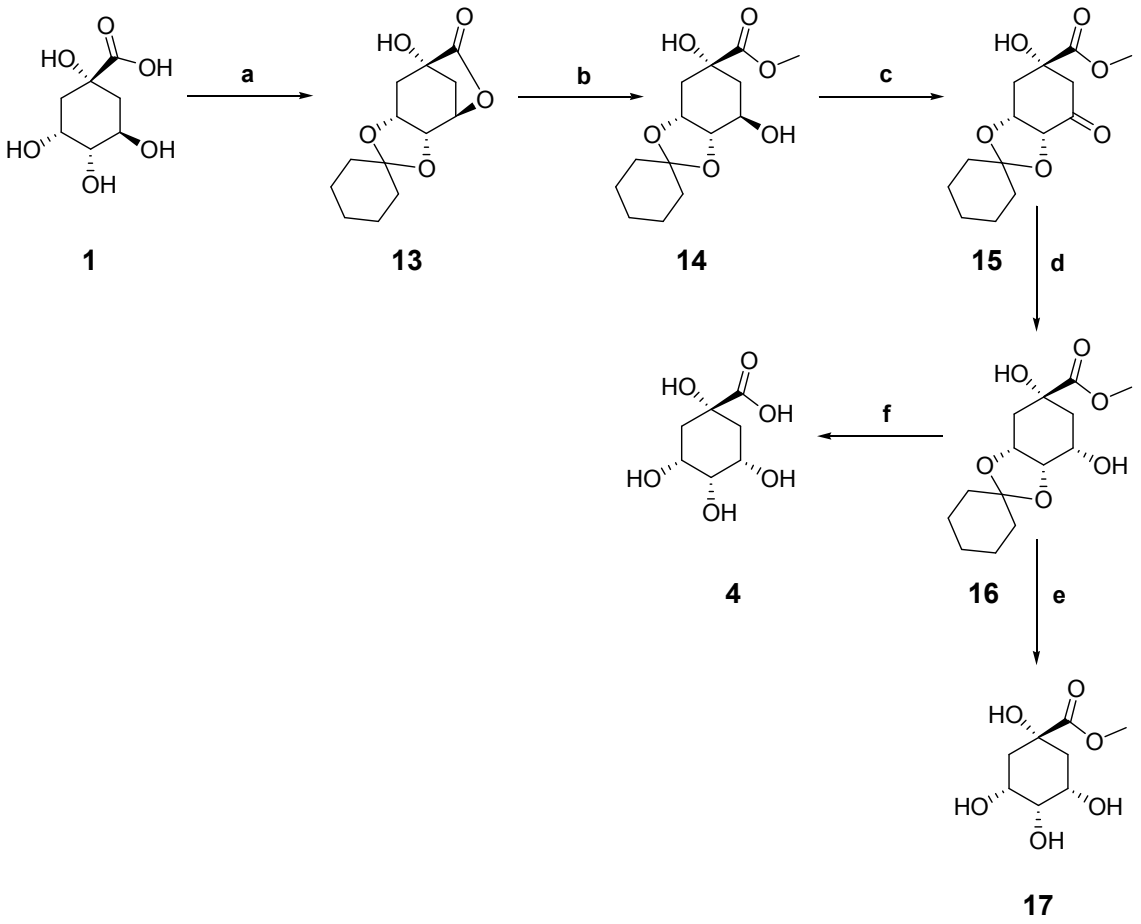

(-) quinic acid 1
epi-quinic acid 2
muco-quinic acid 3
cis-quinic acid 4
scyllo-quinic acid 5
neo-quinic acid 6

Figure 1. Stereochemical relationship of diastereomers of quinic acid (arrows indicating inversion at stereogenic centre) with trivial names and diastereomers of quinic acid **1-6** in chair conformation



Reagents and conditions: (a) MeOH, reflux, 12 h, Amberlite IR 120, 100%; (b) 2.6 equiv TBDMSCl, 2.6 equiv Et_3N, DMF 0 ^{0}C, 2 h and then to RT, 16 h 75%; (c) 1.5 equiv MsCl, Py, RT, overnight, 95%; (d) **11**, CsF, DMF, 90 ^{0}C, 24 h 2% **9** and **10**; (e) 2 equiv Dess-Martin periodinane, CH_2Cl_2 , overnight, RT, 87%; (f) 1.5 equiv $NaBH_4$, Ethanol, -30 ^{0}C, 40 min.; (g) 2M HCl, H_2O, 1 h.

Figure 2. Reaction scheme for obtaining *scyllo*-quinic acid (**5**) and epi-quinic acid (**2**)



Reagents and conditions: (a) cyclohexanone, PTSA·H_2O, reflux, 24 h; (b) 21% NaOMe/MeOH, MeOH, rt, overnight; (c) Dess-Martin periodinane, DCM, rt, 18 h; (d) $NaBH_4$, MeOH/THF (1:1), -30 ˚C, 1 h; (e) KOH, THF, rt, 45 min; (f) HCl (trace amounts in $CDCl_3$).

Figure 3: Reaction scheme for the synthesis of *cis*-quinic acid **4**

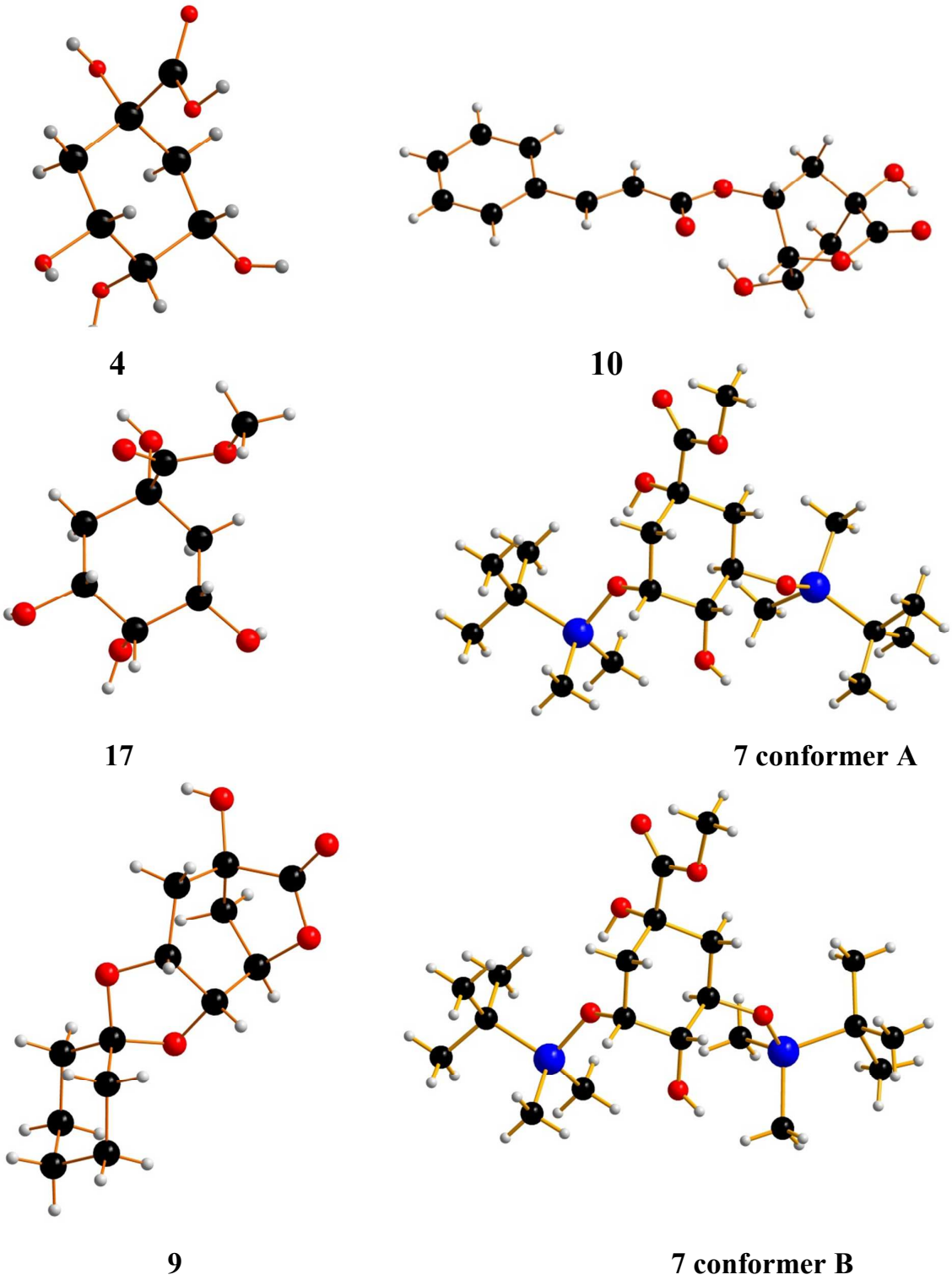


Figure 4: Single crystal X-ray structures of *cis*-quinic acid (**4**) , 3,4-*O*-Cyclohexylidene-1,5-quinide (**13**), 3-*O*-cinnamoyl-1,4-*scyllo*-quinide (**9**)**,** 3-*O*-cinnamoyl-1,5-quinide (**10**), methyl

cis-quinate (**17**), methyl 3,5-Di-*O*-(*tert*-butyldimethylsilyl)quinate (**7**) Conformer **A** and of methyl 3,5-Di-*O*-(*tert*-butyldimethylsilyl)quinate (**7**) Conformer **B.**

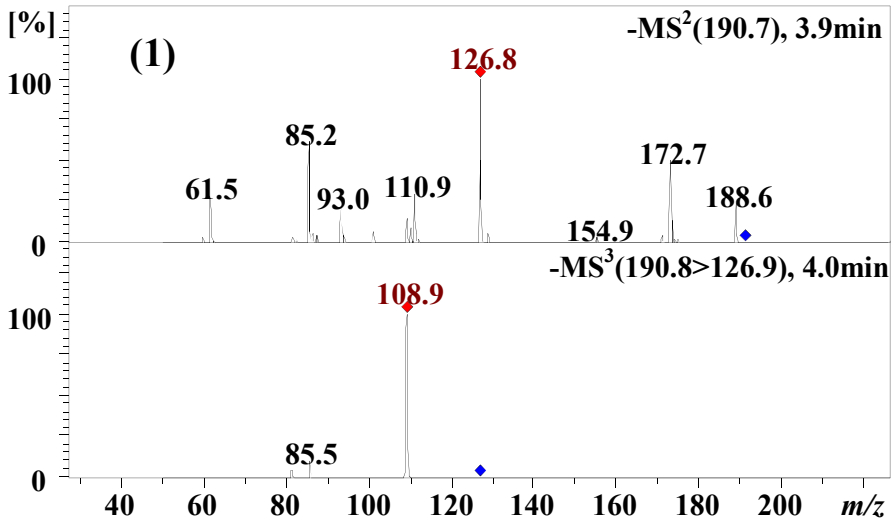


Figure 5: MS^2 and MS^3 spectra of (-) quinic acid **1** in negative ion mode

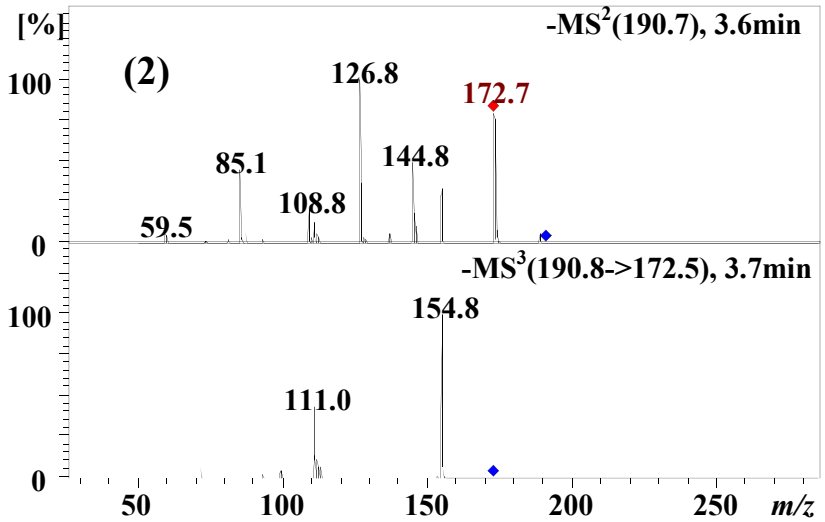


Figure 6: MS^2 and MS^3 spectra of *epi*- quinic acid **2** in negative ion mode

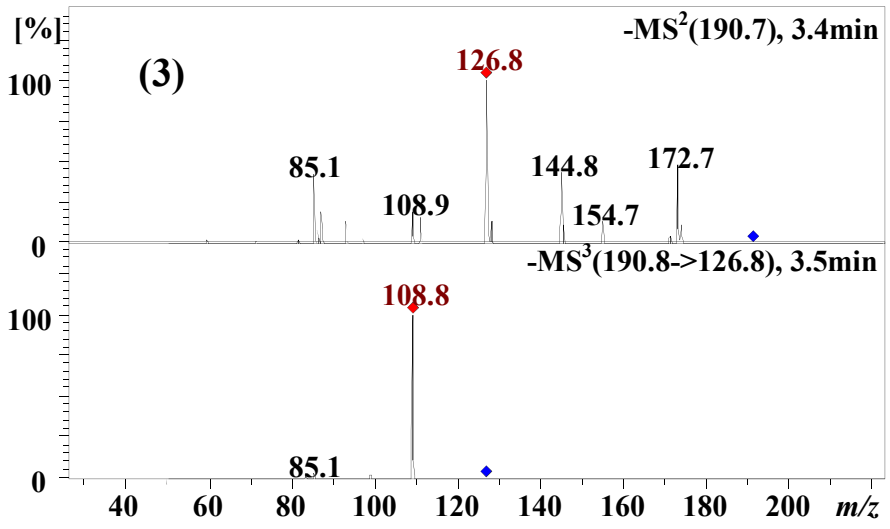


Figure 7: MS^2 and MS^3 spectra of *muco*-quinic acid **3** in negative ion mode

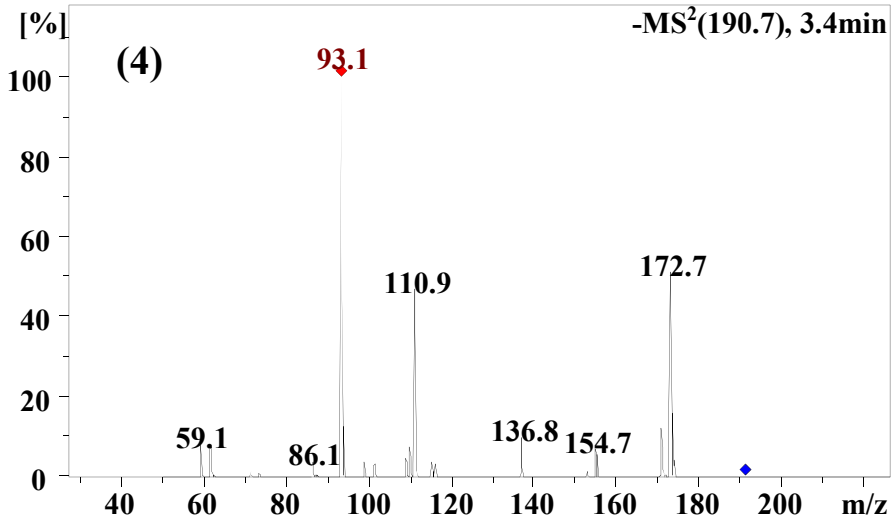


Figure 8: MS^2 spectrum of *cis*- quinic acid **4** in negative ion mode

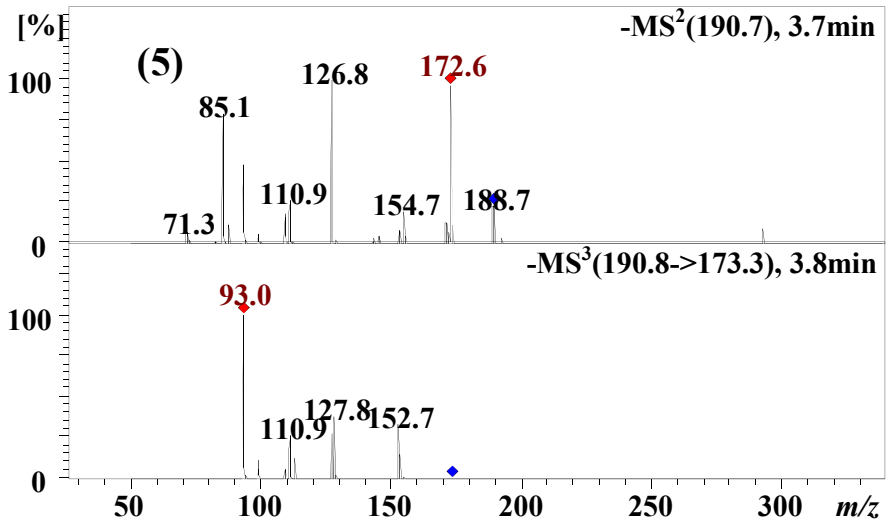


Figure 9: MS^2 and MS^3 spectra of *scyllo*- quinic acid **5** in negative ion mode

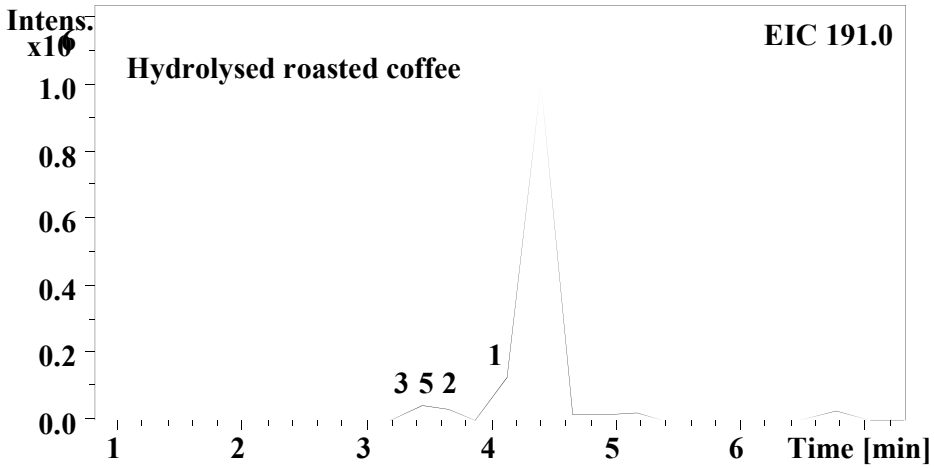


Figure 10: Extracted ion chromatogram in negative ion mode of methanolic roasted coffee bean extract at *m/z* 191.1 showing quinic acid diastereomers (for chromtaograms of individual quinic acids diastereomers please see supplementary information)

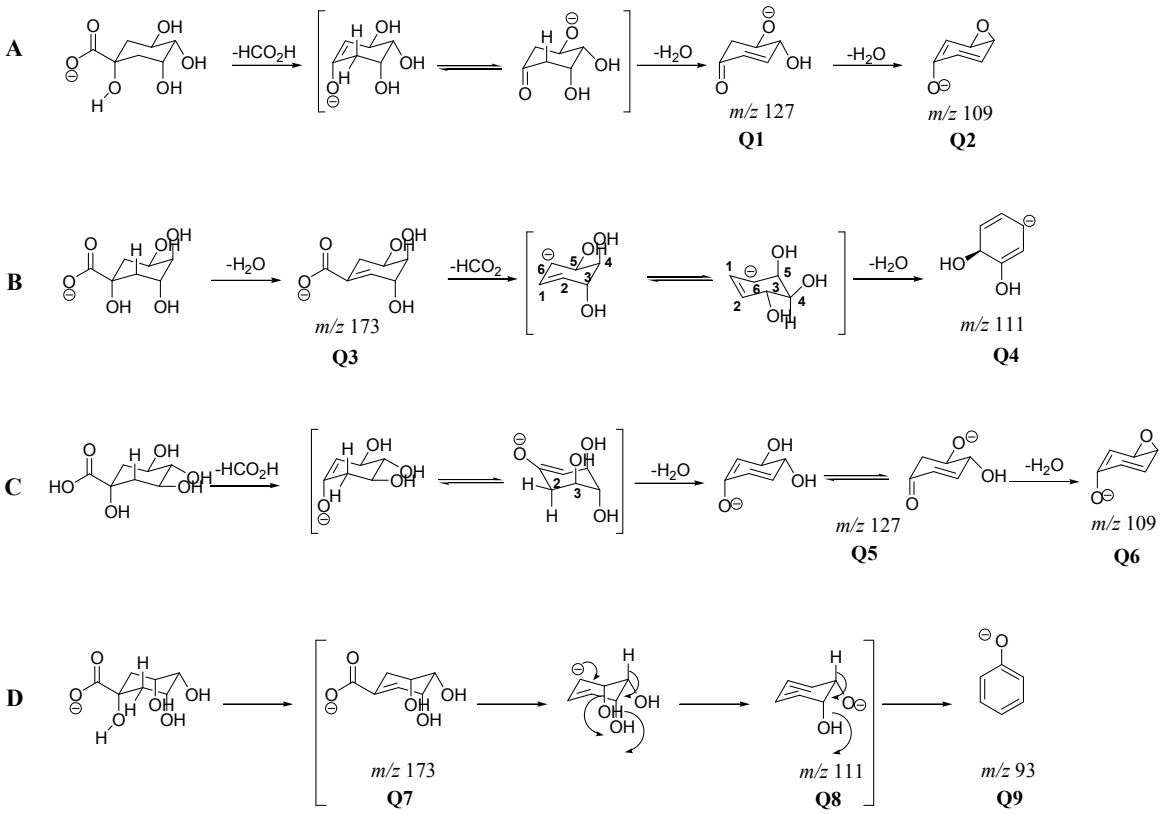


Figure 11: Proposed fragmentation scheme of quinic acids diastereomers in negative ion mode

TOC Graphic