Some Notes on the Chlorogenic Acids.
3. LC and LC–MS
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Contents

	Objectives	
	Summary	
	Referees	
1.	**Characterisation by Reversed-phase Liquid Chromatography**	**Page 4**
1.1.	**Use of Relative Retention Time or Relative Capacity Factor**	**Page 4**
1.2.	**The contribution of equatorial and axial substituents in the (–)-quinic acid moiety**	**Page 8**
1.3.	**Chlorogenic acids with one or more *cis*-hydroxycinnamoyl moieties**	**Page 12**
1.4.	**Quinic acid diastereo-isomers**	**Page 12**
1.5.	**Chlorogenic acids not containing (–)-quinic acid (L-quinic acid) IUPAC**	**Page 12**
2.	**Standards, surrogate standards, and calibrants**	**Page 16**
2.1.	**Determining relative retention times**	**Page 16**
2.2.	**Calibrants for quantitative studies**	**Page 17**
3.	**Chlorogenic Acids Identification and Fingerprinting by LC–MS**	**Page 20**
3.1.	**Use of ion-trap-MS^n hierarchical keys for regio-isomer identification**	**Page 20**
3.1.1.	***Automated fragmentation of most intense ion***	**Page 20**
3.1.2.	***Targeted fragmentations***	**Page 25**
3.1.3.	***Distinguishing chlorogenic acid subgroups with identical accurate masses***	**Page 30**
3.1.4.	***Discriminating between chlorogenic acids subgroups with accurate masses differing by less than 1 amu***	**Page 33**
3.1.5.	***Distinguishing Acyl-quinic Acids not containing (–)-Quinic Acid (L-Quinic Acid) IUPAC***	**Page 36**
3.1.6.	***Distinguishing quinic acid diastereo-isomers***	**Page 37**
3.1.7.	***Standardisation of Ion-trap-MS protocol***	**Page 37**
3.1.8.	***Non-ion-trap MS Instruments***	**Page 39**
4.	**Brief Notes on Quinic Acid Biosynthesis**	**Page 40**
5.	**References**	**Page 42**

Referees

The following chlorogenic acid researchers are thanked for taking the time to read and comment upon the content of this document before its uploading to Researchgate

Professor Gary Williamson	**University of Leeds, UK**
Dr Asimina Kerimi	**University of Leeds, UK**
Dr Dimitrina Zheleva-Dimitrova	**Medical University of Sofia, Bulgaria**
Dr Edwin Madala	**University of Johannesburg, South Africa**

Objectives

These notes are designed to draw attention to the complexity of the chlorogenic acids and confusion surrounding their naming, characterisation and identification. They have been placed on Researchgate to make them freely available, and have been reviewed by the undermentioned chlorogenic acid researchers in an attempt to ensure that no errors of fact have crept through, and that the information is presented clearly. Please use them in conjunction with Parts 1 and 2 covering nomenclature and NMR characterisation. Please notify me of any errors that are found and these notes will be updated as necessary.

Summary

Based on this examination of the literature it is recommended that the most efficient method of characterising acyl-quinic acids and structurally related compounds is by LC–ion trap-MS^n, using fragment-targeted MS^n as required to assist identification and seek minor components that might otherwise be overlooked. Using this approach many acyl-quinic acids can be characterised to regio-isomer level in a single run and without isolation, and without the need for accurate mass. However, accurate mass is helpful in certain situations. Provided that the operating conditions are duplicated, the hierarchical keys prepared by the research groups of Clifford and Kuhnert are rugged and reliable.

Many characterisations are possible with QTOF and triple quadrupole MS instruments, but great care is required to ensure an appropriate collision energy is used to ensure that regio-isomers produce distinctive fragmentations permitting their full assignment. However, these will not necessarily duplicate the fragmentation profiles achieved with ion trap-MS and the hierarchical keys may be invalidated.

Whatever MS is used, it is recommended that the LC–MS system is calibrated with a surrogate standard, such as an extract of a green coffee bean, so that the operating parameters can be adjusted to yield the same pattern of fragmentations as reported by the Clifford and Kuhnert research groups. If this can be achieved, identification of even minor chlorogenic acids is greatly simplified. It is important to ensure that sufficient scans, not less than ten and preferably 20, are

obtained to ensure a reliable fragment profile is obtained, and for minor peaks this might require a sample concentration step.

If the presence of *cis* isomers is suspected, UV-irradiation and / or sodium ion-adduct LC–MS are recommended as recently developed by the Madala research group. If alkyl esters are suspected these can be easily prepared using a surrogate standard in the appropriate alcohol with an acid catalyst. This is a facile reaction and care must be exercised to avoid unwanted production of methyl esters when extracting in methanol.

In situations where full characterisation remains impossible, a reproducible LC–MS fingerprint should still be achievable, and this can be valuable to others who subsequently encounter the same compound.

If MS equipment is not available some characterisation is possible by reference to the relative retention times (relative capacity factors) and even when MS is available these data should be taken into consideration when assigning structures, ensuring that any assignment by MS is consistent with, or at least not greatly inconsistent with, the chromatographic behaviour.

For quantitative studies it is recommended that a good quality commercial 5-caffeoylquinic acid IUPAC be used as the calibrant, its molar absorption value reported, and data expressed as 5-CQA equivalents. This is much cheaper than purchasing small amounts of numerous individual chlorogenic acids and preparing multiple calibration curves, and more importantly, avoids the problem of impure standards generating false calibration curves.

1. Characterisation by Reversed-phase Liquid Chromatography

HPLC, especially reversed phase HPLC, has been used extensively to separate and isolate individual chlorogenic acids. More recently it has been routinely coupled with mass spectrometry, but significant information can be obtained even without MS. This additional information complements MS data and can assist identification.

The separation achieved depends on the column packing and the solvent program, but it is clear that on reversed phase column packings the sequence of elution of acyl quinic acids can generally be explained by the number of free equatorial and free axial hydroxyls in the quinic acid moiety, at least for the extensively studied derivatives of (–)-quinic acid (L-quinic acid) IUPAC.

Often studies report the analysis of a wide range of analytes in a single chromatogram, chlorogenic acids, several types of flavonoids, non-phenols such as terpenes, etc. While there is a need for such analyses and there is a time-saving advantage in an 'all in one' approach, it is not necessarily the best way to assign the structures of any one of the groups of analytes under investigation. It is recommended that the chlorogenic acids, which almost always occur as a complex mixture, be a specific focus so that reliable identification can be achieved as quickly as possible. This will usually be achieved with a simple linear shallow gradient over a relatively long time. Once the chlorogenic acids have been identified, or at least fingerprinted, the gradient system can be revised to speed up the analysis and accommodate other classes of analyte if desired.

1.1. Use of Relative Retention Time or Relative Capacity Factor

The retention time for a compound relative to another is simply the ratio of their retention times expressed as a decimal or a percentage. A better approach is to use the capacity factors rather than the retention times. The capacity factor uses the difference between the retention time and the time to the solvent front leaving the column (time corresponding to the void volume of the column) and is more accurate, especially when the time to the solvent front is large relative to the retention time. However, when examining published data and chromatograms this information might not be available. Both will give the same general picture.

Mono-acyl-quinic acids

When a simple linear gradient is used throughout an analysis, while it may not necessarily achieve the greatest total resolution of a complex mixture of chlorogenic acids and other phenolic and non-phenolic constituents, it does enable the relative capacity factor or relative retention time, to be

used as a structure diagnostic tool.*(1, 2)* This was once a standard approach but is now often overlooked. Such relationships are illustrated below for a column packing where the 4-acyl-quinic acid elutes before the 5-acyl-quinic acid, and obviously it will be very different for a system where 4-caffeoylquinic acid elutes after 5-caffeoylquinic acid.

Table 1. Relative retention times (C_{18}) as a function of regio-isomerism (IUPAC)			
	CQA	*p*CoQA	FQA
3-acyl	0.68	0.68	0.72
4-acyl	0.94	0.91	0.92
5-acyl	1.00	1.00	1.00

The data in Table 1 show that as a first approximation, a 3-acyl-quinic acid and a 4-acyl-quinic acid have a retention time some 30% shorter and some 8% shorter, respectively, than the corresponding 5-acyl-quinic acid.*(2)* There are relatively few data for 1-acyl-quinic acids, but 1-CQA had a retention time only 50% that of 5-CQA on a phenyl-hexyl column packing.*(3)*

Table 2. Relative retention times (C_{18}) as a function of the acyl residue			
	Caffeoyl	*p*-Coumaroyl	Feruloyl
3-acyl IUPAC	1.00	1.25	1.38
4-acyl IUPAC	1.00	1.17	1.27
5-acyl IUPAC	1.00	1.22	1.30

The data in Table 2 show that independent of the position of acylation, a *p*-coumaroylquinic acid elutes some 20% later and a feruloylquinic acid some 30% later than a caffeoylquinic acid.*(2)* A more extensive data set is presented in Table 3 for a diphenyl column packing.

Similarly, as the hydrophobicity of the acyl residue increases, so does the retention of the corresponding acyl-quinic acid. Accordingly, 3,4-dimethoxy-cinnamoylquinic acids elute after feruloylquinic acids, and are followed by sinapoylquinic acids, and trimethoxycinnamoylquinic acids. In contrast, galloylquinic acids elute before caffeoylquinic acids (and lack the ~320 nm λ_{max}). It is clear from the foregoing that an additional hydroxyl reduces the retention time, and an additional –OCH_3 increases the retention time, and these effects are seen also in the di-acyl-quinic acids.

Table 3. Relative Retention Times for mono-acyl-quinic acids on a diphenyl column packing														
	RT min	Relative Retention Time				RT min	Relative Retention Time				RT min	Relative Retention Time		
		trans	*cis*	epimer			*trans*	*cis*	epimer			*trans*	*cis*	epimer
1-CQA	12.0	0.65			1-pCoQA					1-FQA				
Cis-3-CQA	13.2		0.55		*Cis*-3-pCoQA	18.5		0.64		*Cis*-3-FQA	20.8		0.65	
3-CQA	13.7	0.74			3-pCoQA	17.2	0.69			3-FQA	21.3	0.76		
3-C-*muco*-QA	14.4			0.77						3-F-*muco*-QA	23.5			0.84
5-CQA	18.6	1.00			5-pCoQA	24.8	1.00			5-FQA	27.9	1.00		
epimer	19.5			1.05										
epimer	20.5			1.10										
Cis-4-CQA	21.3		0.89		*Cis*-4-pCoQA	25.8		0.90		*Cis*-4-FQA	30.0		0.94	
4-CQA	22.6	1.22			4-pCoQA	28.8	1.16			4-FQA	31.6	1.13		
Cis-5-CQA	23.8		1.00		*Cis*-5-pCoQA	28.5		1.00		*Cis*-5-FQA	32.0		1.00	
epimer	26.2			1.41										
epimer	28.2			1.52										

Data recalculated from Jaiswal *et al*. *(4, 5)*

Di-acyl-quinic acids

For diacyl-quinic acids without C1 substituents on a phenyl-hexyl column the retention times, relative to the dicaffeoylquinic acids as a group, were caffeoyl-feruloylquinic acids 1.19–1.20, diferuloylquinic acids 1.38–1.41, caffeoyl-dimethoxycinnamoylquinic acids 1.38–1.43, and feruloyl-dimethoxycinnamoylquinic acids 1.60–1.66, an increase of approximately 20% for each additional methyl group. Exactly the same 20% increase in retention per added methyl is seen when comparing *p*-coumaroyl-feruloylquinic acids and *p*-coumaroyl-dimethoxycinnamoylquinic acids with *p*-coumaroyl-caffeoylquinic acids on both C_{18} and phenyl-hexyl column packings.*(6)*

On a diphenyl column packing caffeoyl-sinapoylquinic acids eluted 30 to 40% later than the diCQA, demonstrating that adding an $–OCH_3$, as distinct from methylating an existing hydroxyl, also retards the molecule by some 20%. The 3-hydroxy-3-methyl-glutaroyl-caffeoylquinic acids eluted in the period 29 to 44 minutes compared with 33 to 38 minutes for the diCQA indicating a more complex interaction, in part reflecting the hydrophilic character of the aliphatic substituent, but almost certainly also its ability to hydrogen bond and increase the hydrophobicity in some isomers.

The di-*p*-coumaroylquinic acids elute approximately 40% later than the dicaffeoylquinic acids suggesting that the 'loss' of one hydroxyl group has much the same effect as methylating the hydroxyl and keeping the same number of substituents. Inevitably, this results in the diFQA, di-*p*-CoQA, CDQA and CSiQA coeluting, making discrimination in the UV unrealistic if even two sets are present in the same extract.

A similar comparison using a C_{18} column packing of the retention of the 3,5-diacyl-quinic acid and the 4,5-diacyl-quinic acid relative to the 3,4-diacyl-quinic acid (1.00), are 1.04 ± 0.02 and 1.12 ± 0.02 for nine groups (diCQA, CFQA, diFQA, CDQA, FDQA, di-*p*-CoQA, *p*CoCQA, *p*CoFQA and *p*CoDQA).*(6)*

There are comparatively few published data for the 1-substituted diacyl-quinic acids. Note, however, that the retention of 1,3-dicaffeoylquinic acid is only ~60% that of 3,4-dicaffeoylquinic acid on C_{18} and phenyl-hexyl column packings, but the 1,4-di- and 1,5-dicaffeoylquinic acids elute in the same time span as the 3,4-di-, 3,5-di- and 4,5-dicaffeoylquinic acids. Zhao *et al* reported a 1,3-CFQA (tentatively 1C,3FQA) eluting comparatively early from a reversed phase column packing with a retention ~70% that of a 3,4-CFQA (tentatively 3C,4FQA) essentially identical to the retention of 1,3-diCQA relative to 3,4-diCQA (67%),*(7)* albeit this isomer apparently eluted slightly later than 3,5-diCQA. It therefore seems reasonable to assume that the 1,3-diacyl members of the other diacyl-quinic acid subgroups will also elute earlier, and the structural feature responsible for this behaviour is discussed below.

Yang *et al*. investigating the metabolism of 1,5-diCQA in rats reported four putative CFQA isomers eluting an average of ~40% later and five putative diFQA isomers eluting an average of ~80% later than the substrate but did not fully assign the structures.*(8)*

If complex gradients are used, the general order of elution might be maintained, but the simple arithmetic nature of the effects will be lost. It is desirable that anyone wishing to utilise such relationships calibrates their own system, and this is essential if a significantly different column packing is employed. Although not expressed in the same format, a similar collection of data are presented by Lin and Harnly, and these illustrate well the effect of varying the

stationary phase.*(9)* These data are in no way inconsistent with the picture presented here, but do demonstrate clearly that these relationships are not rigid and reinforce the need for careful calibration.

Although such relationships are useful in situations where no MS data are available to assist identification, they are of greater value when checking assignments made on other criteria, such as MS fragmentation, especially, but not exclusively, fragmentations obtained on non-ion trap instruments.

1.2. The contribution of equatorial and axial substituents in the (–)-quinic acid moiety

This section to some extent duplicates the previous section, but examines the data from a different perspective. (–)-Quinic acid (L-quinic acid) IUPAC, as found in the majority of naturally-occurring chlorogenic acids, has two equatorial and two axial hydroxyl groups in its preferred carboxy-equatorial conformer. For a given series of mono-acyl derivatives, e.g. the *trans*-caffeoylquinic acids, 1-CQA and 3-CQA which have two free equatorial and one free axial hydroxyls, elute well in advance of 4-CQA and 5-CQA, which have two free axial hydroxyls and only one free equatorial — for example, see Clifford *et al*., 2005.*(3)*

Mono-acyl-quinic acids

The data for 1-CQA are comparatively limited, but it appears mostly to be the first to elute. In contrast, 4-CQA sometimes precedes 5-CQA,*(1, 2, 9)* sometimes co-elutes *(10)* and sometimes elutes last,*(9, 11-14)* clearly dependent on the nature of the column packing.

Note, however, it has been reported that under certain conditions 3-feruloylquinic acid elutes before 1-feruloylquinic acid and 3-isoferuloylquinic acid elutes before 1-isoferuloylquinic acid, but well in advance of the 5-acyl and 4-acyl analogues.*(15)* Also Zhang *et al*. have reported retention times for all eight caffeoylquinic acids (i.e. both geometric isomers for each of the four regio-isomers) and they report the order of elution as *cis*-3-CQA, *trans*-3-CQA, *cis*-4-CQA, *trans*-4-CQA, *trans*-5-CQA as *cis*-5-CQA, *cis*-1-CQA and *trans*-1-CQA on an ACQUITY UPLC BEH C18 column (2.1 × 100 mm i.d., 1.7 µm with a more complex solvent system and gradient than those used in the previous studies. (A = acetonitrile/methanol 3:1 v/v and B = water containing 0.1% formic acid v/v and a gradient 0–2 min, 2–12%A; 2–5 min, 12–17%A; 5–10min, 17–21%A).*(16)*

Di-acyl-quinic acids

A similar pattern is observed with the *trans*-dicaffeoylquinic acids: 1,3-diCQA elutes first (two free equatorial hydroxyls) and 4,5-diCQA elutes last (two free axial hydroxyls) with 1,4-diCQA, 1,5-diCQA, 3,4-diCQA and 3,5-diCQA (one of each) eluting between them, but closer to 4,5-diCQA, as a fairly tight group. The sequence of this difficult foursome is noticeably dependent on the operating conditions,*(3, 9)* and any change in column packing requires recalibration with authentic standards or well-characterised surrogate standards.

Tri-acyl-quinic acids

Similarly, one is probably safe in anticipating that 1,3,4-triCQA and 1,3,5-triCQA (one free equatorial hydroxyl) elute before 1,4,5-triCQA and 3,4,5-triCQA (one free axial hydroxyl) but there are few reliable data on which to predict the order within the two subgroups.*(9, 17, 18)* Such data as are available have 1,4,5-triCQA eluting before 3,4,5-triCQA,*(9)* and 1,3,5-triCQA before 1,3,4-tricaffeoylquinic acid.*(19)*

Tetra-acyl-quinic acids

Tetra-acyl-quinic acids have no free hydroxyl group on the quinic acid moiety. There are two distinct subgroups:

(i) those containing one or more aliphatic residues with a balance of hydroxycinnamoyl residues, and

(ii) those containing only hydroxycinnamoyl residues.

1,3,4,5-Tetra-caffeoylquinic acid is known,*(20-23)* but 1,3,4,5-tetra-galloylquinic acid is the best characterised of the second group, eluting a little later than 3,4,5-tri-galloylquinic acid.*(24)* The behaviour of those in group one is complicated by the potential for the distal carboxyl of one or more of the aliphatic residues to hydrogen bond and no simple relationships can be defined at present.*(25, 26)*

Depsides

The best known depsides are galloylquinic acid derivatives where one or more galloyl residues esterify one or more gallic acid residues attached directly to the quinic acid moiety. The retention time increases for each additional depsidic galloyl residue, but pentagalloylquinic acids with a 1,3,4,5-tetragalloyl core elute before pentagalloylquinic acids with a 3,4,5-trigalloyl core, i.e. two depsidic galloyl residues increase the retention to a greater extent than one depsidic galloyl residue plus another attached directly to C1 of the quinic acid.*(24)*

Depsidic shikimic acid derivatives (di-, tetra-, penta- and hexa-galloylshikimic acids) also are known,*(27, 28)* and may have been overlooked.

In so far as data are available, it appears that these guidelines presented in the various categories of chlorogenic acids discussed above are applicable to chlorogenic acids containing *trans*-hydroxycinnamic acids other than caffeic acid,*(6, 14, 24, 29)* but not to their alkyl esters, e.g. methyl caffeoylquinates, or quinides. There are no relevant data for hydroxybenzoic acids other than gallic acid.

Methyl chlorogenates

The methyl caffeoylquinates elute from a diphenyl column packing in the sequence methyl 3-CQ, methyl 1-CQ, methyl 4-CQ and methyl-5-CQ, rather than 1-CQA, 3-CQA, 5-CQA and 4-CQA. This revised order was also observed

for the methyl FQ and methyl *p*CoQ. In contrast the methyl diCQ, methyl diFQ, methyl CFQ, methyl *p*CoCQ and methyl *p*CoFQ elute in the same order as the free acids, but note that the only one member of each pair of the mixed diacyl methyl quinates was available for investigation. *(30)*

Mixed di-acyl-quinic acids

When the quinic acid moiety bears two different hydroxycinnamoyl substituents, for example one caffeoyl and one feruloyl moiety, the data available suggest that the 3,4-diacyl pair precede the 3,5-diacyl pair, and that the 4,5-diacyl pair elute last. There are few data for the 1-acyl analogues but re-examination (further discussed below) of data from Han *et al*.*(31)* suggests that 1,5-CFQA elute between the 3,4-CFQA and the 3,5-CFQA. The members of each pair have the same number of free axial and equatorial hydroxyl groups in the quinic acid moiety, and the same number of aromatic hydroxyls and OCH_3 groups in the acyl moieties, and some other feature must explain their resolution.

Although there are relatively few data from which to generalise it appears that if the more hydrophilic acyl residue is attached equatorially it will elute before the other member of the pair,*(6, 9)* i.e. 3F,4CQA elutes before 3C,4FQA, and 3F,5CQA before 3C,5FQA.

This relationship has also been observed by Clifford *et* al. for the 3,4- and 3,5-*p*CoCQA,*(6)* and is supported by data from Lin and Harnly, except that in their study the two 3,4-*p*CoCQA could not be resolved.*(9)*

It is well established that *p*-CoQA elute before the FQA, i.e. that *p*-coumaric acid is more hydrophilic than ferulic acid, and on this basis the relationship holds also for the 3,4- and 3,5-*p*CoFQA.*(13)*

However, for the 4,5-diacyl pair of mixed isomers both acyl groups are attached to equatorial hydroxyls. Such observations as are available indicate that the first of the pair to elute has the more hydrophilic substituent at C5, e.g. 4F,5CQA elutes before 4C,5FQA, 4*p*Co,5CQA before 5*p*Co,4CQA, and 4F,5*p*CoQA before 4*p*Co,5FQA.*(6, 9, 13)* As discussed in Part 2 of these notes the diaxial ^3J coupling constants reported by Pauli *et al*. suggest some flattening of the cyclohexane chair with the $H4_{ax}$–$H5_{ax}$ dihedral angle as judged from the Karplus relationship nearer 140° rather than 180°. Tolonen *et al* using ^{1}H-NMR data and the Altona–Hasnoot equation calculated the $H4_{ax}$–$H5_{ax}$ and $H5_{ax}$–$H6_{ax}$ angles in the range 148–163° for five mono- and di-acyl-quinic acids.*(32)* Possibly such distortion of the ideal chair conformation renders the acyl moiety at C5 more equatorial than that at C4, and thus effectively extends the relationship, first reported by Lin and Harnly, also to the 4,5-diacyl mixed isomers.

However, a very different pattern, with many more fragments, is presented for the CFQA in a study by Han *et al*.*(31)* They did not cite the studies by Clifford *et al*. or use their hierarchical keys,*(3, 14)* and used an ionisation voltage of 4.5 kV rather than 3.5 kV, and did not define the collision energy employed. These factors no doubt account for the different behaviour with more fragments and difficulty of assigning the individual isomers. Several of their assignments are doubtful because of significant discrepancies in the reported sequence of elution, for example 3-

CQA after 5-CQA, 3F,4CQA immediately before 4F,5CQA with 3C,4FQA last of this group of eight CFQA. Operating conditions must be standardised if the hierarchical keys from the Clifford and Kuhnert research groups are to be used.

The (3-hydroxy-3-methyl-glutaroyl)quinic acids elute very early, in the period 4 to 6 minutes from the diphenyl column packing relative to ≈19 min for 5-CQA. It is therefore not surprising that a mixed diacyl-quinic acid containing one or more aliphatic dicarboxylic acid moieties also elutes comparatively early, e.g. the (3-hydroxy-3-methyl-glutaroyl)-caffeoylquinic acids overlap with the elution of the dicaffeoylquinic acids not acylated at C1, the sequence of elution for the six regio-isomeric (3-hydroxy-3-methyl-glutaroyl)-caffeoylquinic acids is very different to that seen for the mixed diacyl-quinic acids discussed above, for example see Clifford *et al*,*(33)* probably reflecting the ability or otherwise to form internal hydrogen bonds, and further generalisations cannot be made.

Chlorogenic acid glycosides

Glycosides of chlorogenic acids, particularly caffeoylquinic acid glycosides and dicaffeoylquinic acid glycosides are increasingly reported. The hydrophilic sugar moiety reduces the retention time considerably, for example 5-CQA-4'-glycoside and 5-CQA-3'-glycoside, both elute before 5-CQA,*(34)* with retention times relative to 5-CQA of 0.72 and 0.95, respectively. Dicaffeoylquinic glycosides elute from a phenyl hexyl column packing earlier than 1,3-dicaffeoylquinic acid, the most hydrophilic of the six dicaffeoylquinic acids.*(17)* Because of the marked impact of glycosylation position on the retention time, the sequence of elution for the eight possible caffeoylquinic acid glycosides, and 24 dicaffeoylquinic acid glycosides, is complex and not fully established.*(17)*

Ma *et al* using a C_{18} column packing reported very hydrophilic caffeoylquinic acid diglycosides (retention time 0.27 to 0.82 relative to 5-CQA), caffeoylquinic acid glycosides (0.60 to 1.60) and dicaffeoylquinic acid glycosides (1.54 to 2.14).*(35)*

Reports of putative dicaffeoylquinic acids eluting before 5-caffeoylquinic acid IUPAC are very likely to be caffeoylquinic acid glycosides, and the following reports are likely to be incorrect assignments.*(36-38)* Similarly, a report of a putative caffeoyl-diferuloylquinic acid which eluted in advance of the diferuloylquinic acids is more likely to be a diferuloylquinic acid glycoside.*(39)*

Baeza *et al*. have reported in *Coffea arabica* novel caffeoyl-2,7-anhydro-3-deoxy-2-octulopyranosic acids which can be distinguished from the coexisting dimethoxycinnamoylquinic acids by accurate mass (m/z = 381.0820 and *m/z* = 381.1186, respectively).*(40)* Baeza *et al*. were not able to provide comprehensive fragmentation data but it is unlikely that the novel glycosides would produce a substantial *m/z* 207 that is characteristic at MS^2 for the dimethoxycinnamoylquinic acids.*(29)* Data are available for the fragmentation of the *p*-coumaroyl, *p*-hydroxybenzoyl and benzoyl analogues reported in *Erigeron*.*(41)*

1.3. Chlorogenic acids with one or more *cis*-hydroxycinnamoyl moieties

There is increasing recognition of and interest in acyl-quinic acids which contain at least one *cis*-hydroxycinnamic acid moiety. It has been demonstrated by several groups that *cis*-3-CQA and *cis*-4-CQA elute just before their *trans* counterparts, but *cis*-5-CQA elutes appreciably later than *trans*-5-CQA.*(42)* The late eluting *cis*-5-CQA has often been incorrectly identified as *trans*-1-CQA, probably because it is seen as the 'fourth' CQA isomer and *cis*–*trans* isomerism is not considered as a possibility. For example, see the following references where 1-CQA apparently elutes after 5-CQA.*(38, 43-46)*

These relationships hold for the analogous *p*-CoQA and FQA on a phenyl-hexyl column packing, *(42)* and on a diphenyl column packing for all but *cis*-3-pCoQA which has been reported to elute after its *trans* counterpart.*(5, 47)*

The later elution of the *cis*-5-acyl-quinic acids has been attributed to the formation of one or two hydrogen bonds that render the molecule more hydrophobic.*(42)* The behaviour of *cis*-1-CQA is not known. Some relative retention times are presented in Table 3.

The diCQA are more complex, there being two mono-*cis*- and one di-*cis*-diCQA for each di-*trans* isomer, but the behaviour is similar to the mono-acyl, with a *cis*-5-substituent delaying elution. Note that the mono- and di-*cis*-1,4-, 3,4-, 1,5- and 3,5-dicaffeoylquinic acids all elute quite close to their di-*trans* counterparts, making this portion of a chromatogram even more crowded and very difficult to resolve. Makola *et al* have recently demonstrated enhanced resolution of *cis* isomers using alkali metal chelation,*(48)* and these developments are more fully discussed in the following LC–MS section.

1.4. Quinic acid diastereo-isomers

Recent studies from the Kuhnert research group have established that it is possible to resolve several free quinic acid diastereo-isomers using isocratic reversed phase chromatography. However, the five compounds differ very little in their retention time, ranging from 4.4 minutes to 3.9 minutes,*(49)* and in the absence of authentic synthetic standards discrimination requires the use of MS^n spectra as discussed below.

1.5. Chlorogenic acids not containing (–)-quinic acid (L-quinic acid) IUPAC

The evidence available for the natural occurrence in unprocessed plant material of chlorogenic acids with a quinic acid moiety other than (–)-quinic acid (L-quinic acid) IUPAC was discussed in Part 2. A striking feature of the majority of data claiming the presence of such chlorogenic acids, and an aspect that has received no comment or discussion, is the fact that the novel compound(s) were rarely observed in company with a full set of the more common compounds. For example, the putative 3-caffeoyl-*muco*-quinic acid reported by Haribal *et al*. was not accompanied by 3-CQA, 4-CQA and 5-CQA IUPAC. When one or two novel di-caffeoylquinic derivatives have been reported they have not been accompanied by 3,4-diCQA, 3,5-diCQA and 4,5-diCQA IUPAC, never mind the three isomeric 1-acyl relatives. This peculiarity does not *per se* establish that the claim of a novel compound is erroneous, but it does

immediately allow the possibility that one of the commoner chlorogenic acids has been wrongly identified. Similarly, there has never been any suggestion that more than one or two novel chlorogenic acids are present, again possibly raising questions about the reliability of the assignment, but more importantly highlighting the need for a specific method of analysis to seek what might be minor components eluting in a complex and crowded part of a chromatogram.

Acyl*-epi-*quinic acids

Be that as it may, it was concluded in Part 2 that there was convincing evidence for the natural occurrence of at least one *epi*-quinic acid derivative based on the significantly different chromatographic behaviour of (–)-quinic acid (L-quinic acid) IUPAC when compared with the product released by saponification of the novel chlorogenic acid. The compound isolated by Wang *et al*. appears to 4,5-dicaffeoyl-*epi*-quinic acid IUPAC. 3,4-DiCQA IUPAC was not detected, but 3,5-diCQA IUPAC and 4,5-diCQA IUPAC were present, eluting later than the novel compound. The early elution of the novel compound is unexpected for an isomer which in the carboxy-equatorial conformation would have only two free axial hydroxyl groups, and this behaviour suggests that it chromatographs as the carboxy-axial conformer, the conformation favoured by *epi*-quinic acid with two free equatorial hydroxyl groups — See Part 1, Table 5.

Taking the novel chlorogenic acid as the anchor, and calculating the relative retention times from visual inspection of an enlarged copy of the published chromatogram, gives relative retention times of 1.00, 1.03 and 1.12, identical to the relative retention times for the 3,4-di-acyl, 3,5-di-acyl and 4,5-di-acyl-(–)-quinic acids in nine chlorogenic acid subgroups as discussed above. While this must raise some concern about the reliability of the identification, the distinctive NMR data and demonstrated presence of a quinic acid moiety other than (–)-quinic acid must be given greater weight. It does, however, demonstrate how complex a mixture might be present in this portion of the chromatogram, and reinforce the need for a specific method to seek out such chlorogenic acids.

Incompletely characterised epimers

In contrast to the foregoing there are records of four incompletely characterised caffeoylquinic acids, albeit minor components, that are accompanied by a comprehensive set of the commoner caffeoylquinic acids, and are thus chromatographically distinct from the *trans* and *cis* isomers thereof.

Two of these with distinctive fragmentations have been observed in maté, eluting later than *cis*-5-CQA, and are thus appreciably more hydrophobic.*(47)* Two more have been observed in certain Asteraceae, eluting between *trans*-5-CQA and *cis*-4-CQA, also clearly distinguished by a distinctive MS^2 base peak,*(5)* as presented in Table 13.

Synthetic acyl*-muco-*quinic acids

Data from Jaiswal *et al*. for synthetic 3-caffeoyl- and 3-feruloyl-*muco*-quinic acid record their elution approximately 5% later than 3-CQA and approximately 10% later than 3-FQA, respectively, and well in advance of 5-CQA and 5-FQA, respectively. These two acyl-*muco*-quinic acids have two free equatorial and one free axial hydroxyls and an equatorially disposed hydroxycinnamoyl moiety in the favoured conformer, and might therefore have been expected to elute even earlier. It follows from the retention time that 3-caffeoyl-*muco*-quinic acid cannot be one of the four incompletely characterised caffeoylquinic epimers referred to above.

Acyl-shikimic acids

Conjugates of shikimic acid are increasingly reported and the caffeoyl-shikimic acids, feruloylshikimic acids, etc. are of particular interest because they are isobaric with, and coelute with, the caffeoylquinides, feruloylquinides, etc., from which they can be discriminated by their fragmentation.

The data in Table 4 were obtained with synthetic products. 5-acyl elutes before the 4-acyl and the 3-acyl elute last.

Table 4. Relative Retention Times for mono-acyl-shikimic acids on a diphenyl column packing

	Retention Time	Relative Retention Time		Retention Time	Relative Retention Time		Retention Time	Relative Retention Time
5-CSA	≈23	1.00	5-*p*CoSA	28.5	1.00	5-FSA	30.8	1.00
4-CSA	23.8	≈1.03	4-*p*CoSA	29.2	1.03	4-FSA	31.8	1.03
3-CSA	25.4	≈1.11	3-*p*CoSA	32.0	1.12	3-FSA	34.0	1.10

Data recalculated from Jaiswal *et al.(50, 51)*

Note: It has not been possible to locate a retention time for 5-CSA. The data in red are calculated using the relative retention times for the *p*-CoSA and FSA illustrating the usefulness of such data. The values in red should, however, be treated as estimates.

Synthetic acyl-1,5-γ-quinides

It is interesting to note that 4-caffeoyl-1,5-γ-quinide with two free equatorial hydroxyls elutes from a phenyl-hexyl column packing before 3-caffeoyl-1,5-γ-quinide with one free equatorial and one free axial hydroxyl, thus maintaining the relationship observed for acyl-quinic acids on this column packing.

However, the elution order of synthetic acyl-quinides from a diphenyl column is, 3-C-1,5-γ-quinide, 4-C-1,5-γ-quinide and 1-C-1,5-γ-quinide. The same order is observed for the corresponding feruloyl-quinides and *p*-coumaroyl-quinides,*(50)* and differs for that observed with the corresponding acyl-quinic acids and their methyl esters. These three sets of data clearly demonstrate the importance of the column packing and the quinic acid carboxyl in determining the retention.

Table 5. Relative Retention Times for mono-acyl-1,5-γ-quinides on a diphenyl column packing

	Retention Time	Relative Retention Time		Retention Time	Relative Retention Time		Retention Time	Relative Retention Time
3-C-1,5-γ-quinide	27.0	1.00	3-*p*Co-1,5-γ-quinide	32.0	1.00	3-F-1,5-γ-quinide	34.6	1.00
4-C-1,5-γ-quinide	30.5	1.13	4-*p*Co-1,5-γ-quinide	35.3	1.10	4-F-1,5-γ-quinide	37.5	1.08
1-C-1,5-γ-quinide	32.9	1.22	1-*p*Co-1,5-γ-quinide	38.2	1.19	1-F-1,5-γ-quinide	40.5	1.17

Data recalculated from Jaiswal *et al.(50, 51)*

Anomalous chromatographic data

The chromatographic behaviour of chlorogenic acids described in the foregoing, with minor variations dependent on the nature of the column packing and solvent gradient, can be found in hundreds of publications.

However, there are several data sets where the reported chromatographic behaviour is completely different. These have been obtained by a South Korean research group using a Shiseido (Chuoku) Capecll Pak C_{18} column with 0.05% phosphoric acid in water for solvent A and methanol for solvent B, and an isocratic step gradient, as follows: 40% B isocratic from 0–10 min, 50% B isocratic 10–20 min, 60% B isocratic 20–30 min and 40% B 30–35 min.*(52-59)*

The reported elution order is 3,4-diCQA eluting at 2.7 min (before the mono-acyl quinic acids, 3-*p*CoQA at 2.9 min, 5-CQA at 4.5 min, 3-CQA at 8.9 min, with 3,5-diCeQA at 9.6 min, 3,5-diCQA at 12.9 min and 4,5-diCQA at 17.1 min. Nugroho *et al*. describe their standards as having been supplied by Professor Kang Ro Lee (see Kwon *et al. (60)*) and in this original account there is no reference to 3,4-diCQA. In some publications 3,5-diC-*epi*-quinic acid is referred to as 3,5-diC-*muco*-quinic acid,*(55-57)* and what appears to be 3-*p*CoQA is referred to as '3-O-*p*-coumaroyl-caffeoylquinic acids',*(53)* possibly a typographical error, but further confusing the accounts.

The assignment of 3,5-diCeQA, or 3,5-diC-*muco*-QA, as discussed elsewhere, is considered doubtful. That constraint, coupled with the idiosyncratic analytical procedure make it difficult to relate their otherwise interesting data to those obtained from the bulk of the literature.

2. Standards, surrogate standards, and calibrants

In this section the term 'calibrant' is used in two contexts. The first is to provide relative retention times or relative capacity factors for which either expensive commercial standards or relatively cheap surrogate standards,*(35)* or mixture thereof, can be used. Surrogate standards would be well characterised preparations such as an in-house generated extract of a green coffee bean. The second context is as a standard for quantitative studies.

2.1. Determining relative retention times

Calibration of a chromatographic system so as to enable relative retention times or relative capacity factors to be used, requires substantial data sets covering most, if not all, members of several chlorogenic acid subgroups.

5-Caffeoylquinic acid IUPAC and 1,3-dicaffeoylquinic acid IUPAC are readily available and quite cheap, and can be used as the anchor for such calibrations. Many other chlorogenic acids are listed by chemical suppliers, but often with confusing and sometimes incorrect nomenclature as discussed in Part 1, and usually at a substantial price for milligram quantities that frequently have significant impurities. 1,5-DiCQA IUPAC may be worth purchasing if 1,4-diCQA is required because it is a better starting material than 1,3-diCQA from which to produce that isomer by acyl migration.

For many chlorogenic acids a cheaper and often much more convenient approach is to use surrogate standards, in combination with acyl migration in base,*(1, 2, 9)* partial acid hydrolysis,*(3, 9)* and UV irradiation *(42)* applied to a limited set of commercial standards (or in house isolates) to manufacture a fairly comprehensive set of standards.

Acyl migration of 5-caffeoylquinic acid in either ammonia or tetramethylammonium hydroxide will easily provide the 3-caffeoylquinic and 4-caffeoylquinic acids, and mild acid hydrolysis of 1,3-dicaffeoylquinic acid will additionally provide 1-caffeoylquinic acid.

An extract of a green arabica coffee bean will provide 3-CQA, 4-CQA and 5-CQA, 3-FQA, 4-FQA and 5-FQA, plus 3,4-diCQA, 3,5-diCQA and 4,5-diCQA. Almost always 5-CQA, 5-FQA and 3,5-diCQA are the dominant members of their respective subgroups. There is a risk that 3-FQA and 4-FQA might coelute with other chlorogenic acids and not be locatable unless MS is available. Although 5-*p*CoQA is usually detectable in an extract of a green arabica coffee bean the 3- and 4-*p*CoQA isomers may not be and a better source of these is fermented, i.e. alcoholic, apple juice, variously known as cider in the UK or hard cider in some countries. Note that rather unusually 4-*p*CoQA and 5-CQA dominate their respective subgroups and this has often led to 4-*p*CoQA being described incorrectly as 5-*p*CoQA.

A green robusta coffee bean can be used to locate many other minor chlorogenic acid subgroups not present in the arabica if MS detection is available. If green coffee beans are not available an instant coffee can be used for the CQA, FQA and diCQA, but MS detection is probably essential because of the more complex chromatogram. This is also a good source of caffeoyl- and feruloyl-quinides, and potentially 3-caffeoyl-*muco*-quinic acid and 3-feruloyl-*muco*-quinic acid albeit these are minor components formed during roasting.

2.2. Calibrants for quantitative studies

A calibrant for quantitative studies must be of high purity. A commercial standard described as ‘chromatographically pure’ may simply mean that at a chosen wavelength, probably 320 nm for chlorogenic acids, only one substantial peak will be seen in the chromatogram. An examination at 280 nm might reveal many other components. In addition there might be ash, water and / or organic solvents present, none of which would be detected by chromatography with UV detection. Any of these impurities will contribute to the mass weighed when preparing a calibration curve and thus result in a more dilute solution than expected.

Early studies on chlorogenic acids where a novel compound was first reported almost always involved relatively large scale preparations yielding grams of the material, sufficient to permit repeated recrystallisations, drying *in vacuo*, proximate analysis including ash content, etc. The final preparation would then be characterised by melting point, specific rotation, UV–vis spectrum and molar absorbance, possibly plus IR and NMR spectra if such procedures existed at that date. The collected molar absorbance values make interesting reading *(61, 62)* — see below. For a given acyl residue, mono-acyl regio-isomers differ by no more than approximately ±4% of mean, and by no more than ±6% for the dicaffeoylquinic acids.

Accordingly one would expect the slope of calibration curves (response factors) for any set of mono- or di-acyl-quinic acids to differ by no more than ±6% of mean. Data from Ruan *et al*. for 3-CQA, 4-CQA and 5-CQA IUPAC do indeed meet that expectation.*(63)* However, compare the other four sets of data which show substantial variations up to approximately three-fold,*(53, 64-66)* and data from Liu *et al*. and Che *et al*. (not shown) provide further examples.*(67, 68)* Clearly, compositional data obtained by such calibration curves will be unreliable, and might distort which isomer dominates.

It is simpler, and cheaper, to use a good quality 5-CQA IUPAC standard and express all data as 5-CQA equivalents, with a correction if desired for the diacyl-quinic acids. If the molar absorption value for the 5-CQA is provided others can then properly compare their quantitative data.

Accurate quantification requires also the avoidance of artefacts during sample preparation. The potential for acyl migration, partial hydrolysis, alkyl ester formation and addition of water across the cinnamic acid double bond, are well documented — for example see Clifford (1985),*(69)* Deshpande *et* al. (2014)*(70)* and Wianowski *et al*. (2015).*(71)*

Table 6. Molar absorbance values and response factors

	Clifford 1985; Kuhnert *et al.,* 2012			Matsui et al 2007		Nugroho et al 2009		Gouveia and Castilho 2012		Kim *et al.* 2012		Ruan *et al* 2012	
	E in MeOH	Relative response		Response factor	Relative response factor	Response factor	Relative response factor	Response factor	Relative response factor	Response factor	Relative response factor	Response factor	Relative response factor
		within group	against 5-CQA		within group		within group		within group		within group		within group
1-CQA	18200	0.93	0.93										
3-CQA	18200	0.93	0.93	27850	0.51	304.05	1.26					15709	0.99
4-CQA	18200	0.93	0.93	9315	0.17							16703	1.05
5-CQA	19500	1.00	1.00	54383	1.00	241.02	1.00	0.54	1.00	16.74	1.00	15875	1.00
1-FQA	18600	0.97	0.95										
3-FQA	19000	0.99	0.97										
4-FQA	19500	1.01	1.00										
5-FQA	19250	1.00	0.99										
1-pCoQA	20400	1.00	1.05										
3-pCoQA	20000	0.98	1.03										
4-pCoQA	20900	1.02	1.07										
5-pCoQA	20400	1.00	1.05										
1,3-diCQA	31600	0.95	1.62					1.44	1.19			13621	
1,4-diCQA	33900	1.02	1.74										
1,5-diCQA	31600	0.95	1.62					0.90	0.75				
3,4-diCQA	33900	1.02	1.74	6907	0.44	66.85	0.47	1.17	0.97	22.45	1.66		
3,5-diCQA	35500	1.07	1.82	7485	0.48	113.68	0.80	1.35	1.12	28.45	2.11		

3. Chlorogenic Acids Identification and Fingerprinting by LC–MS

For the purposes of this exercise the MS equipment can be divided into two types — ion-trap instruments, and other types. Some of these are so-called accurate mass, others accurate only to unit mass, and while access to an accurate mass instrument is useful, an ion-trap that can only achieve unit mass accuracy is more powerful than an accurate mass non-ion-trap instrument for determining chlorogenic acid regio-chemistry. LC–Ion-trap analysis is the gold standard for chlorogenic acids characterisation, permitting all chlorogenic acids in an extract to be fingerprinted and most fully identified in a single analysis without a need for isolation. Minor components can be accommodated easily, either by a larger injection volume or pre-concentration, and usually, overlapping peaks can be resolved in the fragmentation spectrum even if not chromatographically.

3.1. Use of ion-trap-MS^n hierarchical keys for regio-isomer identification

Ion-trap instruments are designed to ionise the molecules of interest, select ions, i.e. trap ions, of a particular *m/z* ratio, and if required cause them to fragment and record the *m/z* values of these daughter ions that form the MS^2 spectrum. If desired one of these daughter ions can be selectively trapped and fragmented to produce the MS^3 spectrum, and this can be repeated several more times provided the signals are sufficiently strong. For chlorogenic acids negative ion operation is to be recommended — structural information for chlorogenic acids is much more difficult, if not impossible to obtain, in positive ion, and it is generally false economy to try and fully characterise flavonoids and chlorogenic acids in one run.

Having selected negative ionisation, defined fragmentation conditions and other operating parameters, an ion trap can be operated in two distinct modes. The first is an automated system where the instrument attempts to ionise everything in a selected *m/z* range (e.g. 100–800 *m/z*), fragments everything to produce numerous MS^2 spectra, automatically selects the strongest ion in each MS^2 spectrum, and repeats the fragmentation step to produce MS^3 spectra, and so on. This can be a useful general screening procedure, and sometimes is all that is required to give a full identification.

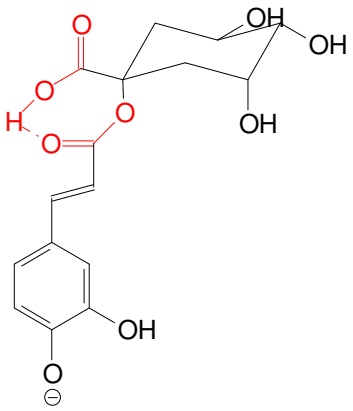
The second mode of operation is much more targeted, provides more detailed information that is often essential for identification of the regio-isomer, but is focussed on a particular chlorogenic acid family, or even a single chlorogenic acid. The following examples illustrate the relative merits of these two approaches.

3.1.1. Automated fragmentation of most intense ion

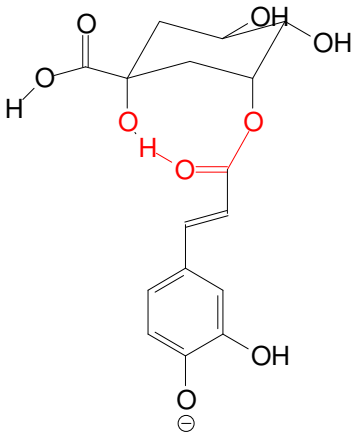
The following table summarises in a simplified format the fragmentation data most important for assigning regio-isomers — full spectra and precise operating conditions, are given in the paper describing the original hierarchical key.*(14)* The various hydrogen bonding networks that facilitate the distinctive fragmentations are illustrated below.

Table 7. Base peak and selected secondary fragments for CQA, FQA and *p*CoQA at MS^2 and MS^3					
	MS^1	MS^2	MS^3		
1-CQA	353	191	85		
3-CQA	353	191 + 179 (50%)	85 + 127 (95)		
4-CQA	353	173 + 179 (70%)	93		
5-CQA	353	191 + 179 (<5%)	85 + 127 (95)		
1-FQA	367	191	85		
3-FQA	367	193	149		
4-FQA	367	173 + 191 (90%)	111		
5-FQA	367	191	85 + 127 (95)		
1-*p*CoQA	337	191	85		
3-*p*CoQA	337	163	119		
4-*p*CoQA	337	173	93		
5-*p*CoQA	337	191	85 + 127 (95)		

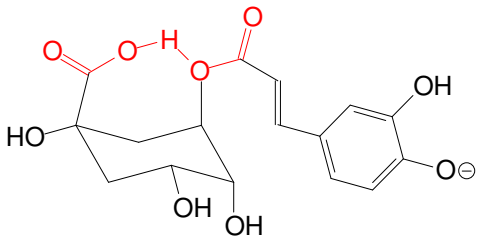
The H-bonding arrays responsible for the different fragmentation of the four caffeoylquinic acid regio-isomers are illustrated below.



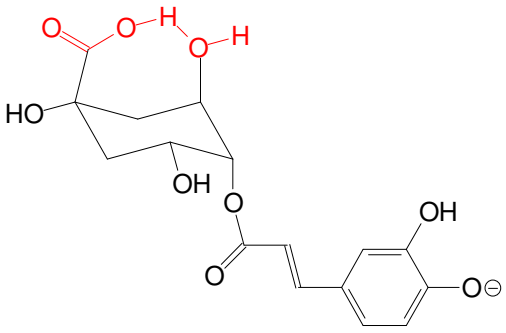
1-CQA



3-CQA



Inverted chair 5-CQA



Inverted chair 4-CQA

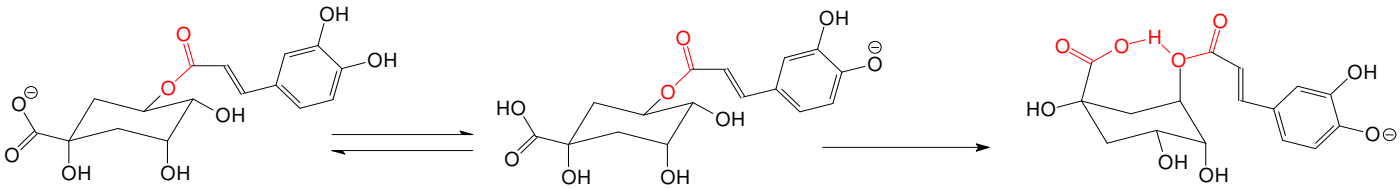
It is clear from these data that for each set of four regio-isomers it is not immediately possible to distinguish the 1-acyl from the 5-acyl. However, these can be easily distinguished by their relative retention times as discussed above, with the 1-acyl eluting well in advance of 5-acyl.

If there is any doubt, the use of a surrogate standard such as an extract of green coffee beans which does not contain 1-acyl-quinic acids will clarify the situation. Moreover, if this surrogate standard is used before and after UV-irradiation (1 hour) it will allow the *cis* isomers to be distinguished from the *trans* isomers and eliminate any risk of

cis-5-acyl-quinic acids being assigned as *trans*-1-acyl-quinic acids.*(42)* Another approach to characterise *cis* isomers is to use sodium-ion adducts,*(48)* as discussed below.

Note that investigations using a quadrupole ion-mobility time-of-flight mass spectrometer have established that *trans*-4-caffeoylquinic acid and both *cis*- and *trans*-5-caffeoylquinic acid produce ions with the charge located on the quinic acid carboxyl as well as on the phenolic hydroxyl. The four prototropic isomers derived from 5-CQA (two *cis* and two *trans*) are resolved in the ion mobilograms. Each pair of prototropic ions differ slightly, but distinctly, in their fragmentation, but this does not invalidate the ion trap hierarchical keys for regio-isomer identification.*(72)*

Kuhnert *et al*. suggest that the reason these prototropic isomers can be resolved arises from them having significantly different cross sectional area and the phenolate ion being stabilised by hydrogen bonding after inversion of the carboxy equatorial chair, as illustrated below for 5-CQA. For 1-CQA and 3-CQA the hydrogen bonds favour the carboxy equatorial conformer and this may explain why only one prototropisomer was detected when 3-CQA was examined.



Carboxylate and phenolate ions of 5-caffeoylquinic acid, and hydrogen bond-stabilised phenolate ion from Kuhnert *et al*.*(72)* The hydrogen bond-stabilised inverted chair conformer yields a more intense fragment at *m/z* 191 relative to the parent ion at *m/z* 353.

Dicaffeoylquinic acids

Table 8. Base peak and selected secondary fragments for diCQA at MS^2, MS^3 and MS^4				
	MS^1	**MS^2**	**MS^3**	**MS^4**
1,3-diCQA	515	353 + 335 (35%)	191 + 179 (70%)	85
1,4-diCQA	515	353 + 335 (25%) 317 (35%) 299 (50%) 255 (20%) 203 (55%)	173	93
1,5-diCQA	515	353 + 335 (7%) 203 (3%)	191 + 179 (7%)	85
3,4-diCQA	515	353 + 335 (16%) 299 (5%) 255 (5%) 203 (7%)	173 + 191 (50%)	93 + 111 (50%)
3,5-diCQA	515	353 + 203 (10%)	191 + 179 (50%)	85
4,5-diCQA	515	353 + 317 (7%) 299 (12%) 255 (8%) 203 (15%)	173 + 191 (30%)	93 + 111 (35%)

Data are taken from Clifford *et al*.*(3, 14)*

The following observations are clear:

(i) The six diCQA regio-isomers have distinct fragmentations.
(ii) Each regio-isomer loses a caffeic acid residue at MS^2 followed by another at MS^3.

Further interpretation requires a comparison of the diCQA MS^3 spectra with the CQA MS^2 spectra obtained under the same conditions (see Table 7) in order to determine the location of the second caffeoyl moiety. When an MS^3 spectrum matches the MS^2 spectrum of

(i) 3-CQA, the component is either 1,3-diCQA or 3,5-diCQA
(ii) 4-CQA, the component is 1,4-diCQA 3,4-diCQA or 4,5-diCQA
(iii) 1-CQA and 5-CQA the component is 1,5-diCQA

From the previous section it is well established that 1,3-diCQA is the first to elute and 4,5-diCQA is the last. Comparison of the data with those obtained for a surrogate standard such as a green coffee extract which does not contain 1-acyl-quinic acids will immediately permit the remaining regio-isomers to be assigned.

It is possible that mono- and di-*cis*-isomers will be present. Their fragmentation is identical to that for the di-*trans*-isomer and may not be chromatographically resolved, but they will be minor components and unlikely to be confused with the dominant di-*trans* isomer. UV irradiation can help locate them. Sodium-adduct LC–MS can help assign them as discussed below.

Note, that the 1,4-dicaffeoyl-, 3,4-dicaffeoyl- and 4,5-dicaffeoyl-quinic acids differ markedly in terms of the secondary peaks in their MS^2 spectra, particularly the intensity of the dehydrated ion at *m/z* 335. This observation is useful when assigning other diacyl-quinic acid subgroups. It is essential when using the targeted mode of operation to assign tri-acyl-quinic acids, and chlorogenic acids incorporating aliphatic acids or having any substituent at C1.

Caffeoyl-feruloylquinic acids

Table 9. Base peak and selected secondary fragments for CFQA at MS^2, MS^3 and MS^4

	MS^1	MS^2	MS^3	MS^4	
3F,4CQA	529	353 + 367 (90%) 335 (75%) 349 (50%)	173 + 179 (90%)	134	
3C,4FQA	529	367 + 335 (20%) 349 (5%)	173	93	
3F,5CQA	529	367 + 335 (9%)	193	134	
3C,5FQA	529	353 + 367 (50%)	191 + 179 (60%)	127	
4F,5CQA	529	367	173 + 193 (60%)	93	
4C,5FQA	529	353 + 367 (25%)	173 + 179 (80%)	93	

Data are taken from Clifford *et al*. *(14)*

An examination of the MS^2 data for the six CFQA clearly identifies whether the first fragmentation step removes a caffeoyl or a feruloyl moiety. The MS^3 fragmentation then defines the structure of the MS^2 ion and which hydroxycinnamic acid is present. Accordingly the first CFQA is easily assigned as 3F,4CQA because the MS^2 base peak is clearly $[4\text{-CQA}]^-$, the feruloyl moiety was at either C3 or C5, and 3,4-diacyl-quinic acids consistently elute before 4,5-diacyl-quinic acids as discussed in the preceding section. Notice also that in the MS^2 spectra the dehydrated ions at *m/z* 335 and 349 are prominent for the 3,4-CFQA but not detectable for the 4,5-CFQA. The other five CFQA can be assigned by similar arguments and none of the assignments so obtained are in any way equivocal or conflicting. There are insufficient data for the 1-acyl CFQA but it should be possible to recognise them and possibly to assign them by their fragmentation supported by the use of a green robusta coffee bean extract as a surrogate standard.

Tetra-acyl-quinic acids

Comparatively few tetra-acyl-quinic acids have been reported, and the majority of them incorporate aliphatic acid residues in which the distal carboxylate can participate in the fragmentation.*(25, 26)* Of those not containing an aliphatic acid residue only 1,3,4,5-tetra-galloylquinic acid has been studied in detail. This yields *m/z* 799 as expected at MS^1, but *m/z* 601 rather than *m/z* 647 at MS^2, followed by *m/z* 431, *m/z* 169 and *m/z* 124 as the MS^3, MS^4 and MS^5 base peaks, respectively.*(24)* It will be interesting to see if this distinctive behaviour applies to other similar tetra-acyl-quinic acids.

Cis-*Chlorogenic Acids*

Makola *et al* using QTOF-MS have demonstrated that *cis*-isomers of the hydroxycinnamoyl-quinic acids bind alkali metal ions such as Na^+, K^+ or Li^+, whereas the *trans*-isomers do not. By careful control of the fragmentation conditions they were able to distinguish the four geometric isomers of dicaffeoylquinic acids at MS^2 by comparing the relative intensity of ions at *m/z* 353 and 375 when sodium was complexed.*(48)* There would appear to be potential for targeted ion trap MS^3 also to perform this analysis by following the fragmentation of the sodium ion-adduct.

3.1.2. Targeted fragmentations

The following examples illustrate the usefulness, indeed in some cases the essentialness, of the targeted fragmentation mode of ion-trap MS analysis.

Diferuloylquinic acids

Table 10. Base peak and selected secondary fragments for diFQA at MS^2, MS^3 and MS^4

	MS^1	MS^2	MS^3	MS^4	
3,4-diFQA	543	349 + 367 (17%)	173	134	
3,5-diFQA	543	367 + 349 (35%)	193		
4,5-diFQA	543	367 + 349 (17%)	173	93	

The data obtained from the analysis of a green coffee extract,*(29)* which lacks 1-acyl-quinic acids, are summarised above. The second and third eluting diFQA isomers are assigned in exactly the same way as the corresponding diCQA, but the data for first eluting diFQA isomer are slightly different to those observed for 3,4-diCQA. The MS^2 base peak is a dehydrated ion at *m/z* 349 rather than *m/z* 367. On this occasion there is no difficulty in assigning this isomer — there are only three possibilities in a coffee extract, two of which are easily defined, and so the third is assignable by default: this fact plus the early elution and the MS^3 base peak at *m/z* 173, combine to make the assignment as 3,4-diFQA perfectly reasonable.

However, confirmation is easily achieved by switching to the second operating mode and making a targeted analysis rather than letting the instrument automatically select the strongest fragment ion for the subsequent fragmentation.

Accordingly instructing the instrument to trap *m/z* 543 and then trap fragment *m/z* 367 (rather than automatically the base peak), and then perform MS^3 yields *m/z* 173 as the base peak fragment of *m/z* 367. This observation makes a perfect match with the behaviour previously described, and the hierarchical key.

The ability to target *m/z* (543 + 367), although not absolutely essential on this occasion, is a feature provided only by ion-trap instruments. There are situations where this facility is essential to the full structure determination.

Note that the tendency to produce a dehydrated base peak, and in some cases intense dehydrated secondary ions, increases markedly as the number of $–OCH_3$ substituents in the acyl residues increases — see Clifford *et al*. (2006) for a summary of the available data.*(29)*

Tri-(hydroxycinnamoyl)-quinic acids

The tri-acyl-quinic acids in species that (i) do not acylate C1 of quinic acid, and (ii) do not incorporate aliphatic acids, are quite easily assigned, because the C5, C3 and C4 substituents will be lost in that order, and can be identified by the mass losses leading to the MS^2, MS^3 and MS^4 base peaks, respectively, which correspond to the MS^1, MS^2 and MS^3 base peaks of the equivalent diacyl-quinic acid obtained under the same conditions. For example see the assignment of seven such chlorogenic acids in green coffee beans.*(73)*

There are comparatively few ion trap-MS data for 1-substitiuted tri-acyl-quinic acids. Two tricaffeoylquinic acids were reported in an extract of *Chrysanthemum morifolium*,*(17)* and three in an extract of *Erigeron breviscapus*,*(19, 74)* all of which have been characterised by ion trap-MS. In each case the last eluting from a phenyl-hexyl or a C_{18} column packing was characterised as 3,4,5-triCQA which has only one free axial hydroxyl on the quinic acid moiety. The one or two earlier-eluting triCQA always yielded a distinctive MS^2 base peak at *m/z* 497 with a secondary fragment ion at *m/z* 515. By targeting *m/z* (677+ 515) these were assigned as 1,3,5-triCQA and 1,3,4-triCQA in order of elution, consistent with both having one free equatorial hydroxyl in the quinic acid moiety.*(17, 19, 74)*

3,4,5-Trigalloylquinic acid and two more hydrophilic regio-isomers behave very similar to the corresponding caffeoylquinic acids described above.*(24)*

Karaköse *et al*. reported an *m/z* 515 base peak at MS^2 for a tricaffeoylquinic acid eluting after 3,4,5-triCQA which on the basis of its MS^3 fragmentation was assigned as 1,3,5-triCQA *(18)* despite having a relatively strong *m/z* 173 (90%). This late elution from a diphenyl column packing is unexpected for a tricaffeoylquinic acid with one free equatorial hydroxyl, and it may be an incorrect assignment for 1,4,5-triCQA. Further investigation is required but there is clearly a printing or typographical error in the last four rows of data presented in Table 1 of this paper.

(3-Hydroxy-3-methyl-glutaroyl)-caffeoylquinic acids

An investigation of an extract of *Gardenia jasminoides* detected several chlorogenic acids containing 3-hydroxy-3-methyl-glutaric acid.*(33)* This species does not produce 1-acyl-quinic acids.

There were three hydrophilic compounds that produced MS^1 base peaks at *m/z* 335, all of which produced an MS^2 base peak at *m/z* 191. Accordingly these were assigned as novel non-aromatic derivatives of quinic acid, but it was not possible to assign the regio-isomers by fragmentation behaviour in contrast to the previously studied hydroxycinnamoylquinic acids. The fumaroyl-quinic and succinoyl-quinic acids, prepared synthetically, behave analogously, suggesting that fragmentation is driven by the distal aliphatic carboxyl. The regio-isomers were tentatively assigned by their order of elution.

The same study located six isomers at *m/z* 497, tentatively assigned as (3-hydroxy-3-methyl-glutaroyl)-caffeoylquinic acids. Five isomers produced an MS^2 base peak at *m/z* 353, indicating loss of the aliphatic moiety, and the sixth yielded *m/z* 335. This could have been produced by loss of the aliphatic moiety, or loss of the caffeoyl moiety accompanied by dehydration. The location of the caffeoyl moiety was identified by a targeted MS^2 fragmentation for *m/z* (497 + 353) and as expected two isomers were defined as having a caffeoyl at C3, two at C4 and two at C5, but it was clear that the sequence of elution was no longer the 3,4-diacyl-, 3,5-diacyl and 4,5-diacyl previously associated with the hydroxycinnamoylquinic acids.

Full characterisation required the location of the aliphatic residue to be determined. The analogous targeted MS^2 fragmentation *m/z* (497 + 335) is not helpful because the *m/z* 335 is either a 'dehydrated' caffeoylquinic acid ion or a 'non-dehydrated' (3-hydroxy-3-methyl-glutaroyl)-quinic acid ion. To overcome this limitation targeted MS^3 fragmentations were required to define the MS^2 base peak. Using (497 + 335 + 191) clearly demonstrated that all five contained an intact quinic acid moiety, and therefore the MS^2 base peak could not have been the 'dehydrated' caffeoylquinic acid ion.

An accurate mass instrument would be able to distinguish the two ions at *m/z* 335, but only the ion-trap, with or without accurate mass, could easily assign all six at regio-isomeric level.

Tri-(hydroxycinnamoyl)-quinic acids substituted at C1 — methoxyoxalyl-dicaffeoylquinic acids

Analysis of an extract of *Cichorium intybus*, which is known to produce 1-acyl-quinic acids, detected two peaks at *m/z* 601 suggesting that these were methoxyoxalyl-dicaffeoylquinic acids. In the automated base peak fragmentation mode both yielded *m/z* 395, *m/z* 233 and *m/z* 173 at MS^2, MS^3, and MS^4, respectively, clearly placing the methoxyoxalyl moiety at C4 in both. Note that because the oxalic acid moiety is present as its methyl ester, there is no distal carboxylate to interfere in the fragmentation as is seen with, for example, 3-hydroxy-3-methyl-glutaric acid. No further assignment was possible because both had virtually identical fragmentation even when minor secondary peaks were considered. A fragment with *m/z* 515, corresponding to $[diCQA]^-$, was the strongest secondary fragment at MS^2. On the basis of these data these two compounds were assigned as 4-methoxyoxalyl-dicaffeoylquinic acids, but it was not immediately obvious whether or not these were regio-isomers or geometric isomers.

In order to determine the structure of the $[diCQA]^-$ ions, a targeted analysis targeting *m/z* (601 + 515) was used in order to generate the structure diagnostic MS^3 and MS^4 spectra of these tri-acyl-quinic acids, which respectively correspond to the MS^2 and MS^3 spectra of diCQA obtained under the same conditions. This targeted approach

clearly defined one regio-isomer as 4-methoxyoxalyl-1,5-dicaffeoylquinic acid and the other as 4-methoxyoxalyl-3,5-dicaffeoylquinic acid.*(4)* This could not have been achieved without targeting a specific MS^2 fragment ion and obtaining its MS^3 spectrum to use in the hierarchical key as previously described.

This approach can be used with similar compounds in which aliphatic or hydroxycinnamic acid moieties are present and the use of the same strategy to assign a greater range of methoxyoxalyl and / or fumaroyl-quinic acids is reported fully by Jaiswal *et al*. in a separate publication.*(26)*

Note that methoxyoxalic and malonic acid are isobaric, and both occur in acyl-quinic acids of which the botanical distribution is poorly defined and might overlap. If a targeted MS analysis is not possible it may be necessary to hydrolyse the acyl-quinic acids and characterise the released aliphatic acids by LC–MS to be certain which is present.*(26)*

Chlorogenic acid glycosides

As discussed in a previous section these sugar derivatives elute considerably earlier than their non-glycosylated equivalents, and, more importantly, before the non-glycosylated chlorogenic acid of similar molecular mass. Glycosidic derivatives of caffeoylquinic acids, feruloylquinic acids, *p*-coumaroylquinic acids, dicaffeoylquinic acids and caffeoylferuloylquinic acids have been reported. The caffeoylquinic glycosides are presently the most reported, and these are discussed further.

Another distinctive feature of caffeoylquinic acid glycosides is the production at MS^2 of a fragment ion at *m/z* 341, *m/z* 323, or both, accompanied by an ion corresponding to caffeoylquinic acid, i.e. *m/z* 353.*(17, 34)* The ion at *m/z* 353 is produced by loss of the sugar residue, whereas the ion at *m/z* 341 arises from the removal of the quinic acid moiety. Either of these can be the base peak, and may be accompanied by a 'dehydrated' ion at *m/z* 323, which also may be the base peak.*(17)*

These two ions (*m/z* 341 and 323) are not produced by hydroxycinnamoyl-quinic acids. In order to define the acyl-quinic acid substitution a targeted MS^3 fragmentation *m/z* (515 + 353) is required. A 3'-glycoside can be identified by the resonance stabilised fragment ion at *m/z* 323 when it occurs as base peak. For example, Jaiswal *et al*. reported four isomeric 5-caffeoylquinic acid glycosides in *Lonicera henryi*, two producing the *m/z* 323 as MS^2 base peak, and two the *m/z* 353 base peak. Using UV-irradiation and the targeted MS^3 protocol these were assigned as the *cis* and *trans* isomers of 5-caffeoylquinic acid-3'-glycoside and 4'-glycoside, respectively. *(34, 75)*

Caffeoylquinic acid glycosides (*m/z* = 515.1402) can be distinguished from dicaffeoylquinic acids (*m/z* = 515.1189) by accurate mass, and in the absence of accurate mass data can be distinguished also by their short retention time and usually by the MS^2 fragment ion at *m/z* 341. Note, however, that there are reports of putative dicaffeoylquinic acids eluting before 5-caffeoylquinic acid IUPAC that yield MS^2 fragment ions at *m/z* 353 and 191, but apparently not at *m/z* 341 — for example see Herraiz *et al*. (2016)*(36)* and Abdennacer *et al*. (2015).*(37)* — which might well be caffeoylquinic acid glycosides. There is also a report of a putative depsidic (*p*-coumaroyl-caffeoyl)quinic acid with analogous behaviour,*(76)* and further investigation is required.

Of potential importance is the use in these studies of either a triple-quadrupole-MS,*(37)* or a Brucker ion-trap-MS and operating conditions *(36)(59)* different from those used by the Kuhnert research group, and it is possible that this explains the different fragmentation of compounds that on the basis of their retention times appear to be acyl-quinic acid glycosides.

In principle, a full characterisation of dicaffeoylquinic acid glycosides (*m/z* = 677.1718) can be achieved by a targeted MS^4 analysis at *m/z* (677 + 515 + 353),*(17, 75)* However, there are two quite different possibilities for the identity of the fragment ion at *m/z* 515 — [dicaffeoylquinic acid]$^-$ (accurate mass *m/z* = 515.1189), and [caffeoylquinic acid-glycoside]$^-$ (accurate mass *m/z* = 515.1402). Only the [caffeoylquinic acid glycoside]$^-$ can produce *m/z* 341, but either could give rise to *m/z* 353, and it is therefore not possible certainly to determine the origin of *m/z* 353 unless accurate mass is available.

Based on the known behaviour of caffeoylquinic acid glycosides discussed above, it is reasonable to assume that both ions will be produced by at least some isomers. If both are produced, both are trapped (regardless of whether or not accurate mass is available) and subsequently fragmented, and as a consequence it is not possible to determine which caffeic acid residue was lost first.

An added complication is the occurrence of caffeoylquinic acid diglycosides (biosides) (*m/z* = 677.1929), one of which has also been reported in *Lonicera henryi*. The compound in question yielded *m/z* 515 at MS^2 indicating the loss of one sugar moiety and *m/z* 173 at MS^3, indicating that the caffeic acid moiety was at C4.*(75)* This compound also produced a significant MS^2 secondary ion at *m/z* 497, an ion seen as the MS^2 base peak in two compounds reported in *Chrysanthemum*,*(17)* and originally thought to be dicaffeoylquinic acid glycosides, but possibly wrongly assigned. A report of an apparent caffeoylquinic acid diglycoside eluting at 63 min seems much more likely to be a tricaffeoylquinic acid.*(77)*

Further studies on a wider range of these glycosides are required to gain a better insight into their behaviour. Reducing the collision energy for the first fragmentation might help by producing only one of the two fragmentations that yield the same nominal mass.

Mammalian sulphate conjugates of acyl-quinic acids

Caffeoylquinic acid sulphates and feruloylquinic acid sulphates have been observed as human metabolites of chlorogenic acids. The sulphate conjugates elute well in advance of the free acids.*(78, 79)* The sulphate moiety is readily lost at MS^1 and subsequent MS^2 fragmentation can define the regio-chemistry as previously described for the chlorogenic acids.

3.1.3. Distinguishing chlorogenic acid subgroups with identical accurate masses

When two different classes of chlorogenic acids have identical accurate masses, they can be distinguished only by some other parameter, such as relative retention time, or mass fragmentation. Mass fragmentation may achieve this even without accurate mass.

Discriminating between hydroxycinnamoyl-quinides and hydroxycinnamoyl-shikimic acids

It is possible not only to distinguish these two subgroups, but also to determine their regio-chemistry by the use of MS^2 and MS^3 spectra.*(51)* The table below illustrates this capability for caffeoyl-quinides and caffeoyl-shikimates (*m/z* = 335.0772). Data for the *p*-coumaroyl and feruloyl analogues are also available. The report of a feruloylquinide in water melon *(80)* is probably an incorrect assignment of a feruloylshikimic acid.*(81)* Similarly, the report of a caffeoylquinide in *Erigeron breviscapus* is also suspect.*(82)*

Sulphate conjugates of caffeoylquinides have been observed as mammalian metabolites of chlorogenic acids. The sulphate moiety is readily lost at MS^1 yielding *m/z* 335 as MS^2 base peak and regio-chemistry can be assigned at MS^3.*(78, 79)* Acyl-quinides and acyl-shikimic acids have more rigid structures than acyl-quinic acids, and this is reflected in the less variable nature of the fragmentations among regio-isomers.

Table 11. Distinguishing Caffeoylquinides and Caffeoyl-shikimic Acids

	MS^1	MS^2	MS^3		
1-CQL	335	161 + 135 (90%)	133		
3-CQL	335	161 + 135 (10%)	133		
4-CQL	335	161 + 173 (70%)	133		
3-CSA	335	179 + 161 (80%) 135 (50%)	135		
4-CSA See Note: a	335	179 + 161 (80%) 135 (20%)	135		
5-CSA	335	179 + 173 (11%) 135 (20%)	135		

Note: a The data presented in *(51)* and *(18)* are not consistent

Discriminating between methyl esters and methyl ethers

Methyl caffeoylquinates, feruloylquinic acids and isoferuloylquinic acids (*m/z* = 367.1035), methyl dicaffeoylquinates, caffeoyl-feruloylquinic acids and caffeoyl-isoferuloylquinic acids (*m/z* = 529.1351), have identical accurate masses. Data below summarise the key features required for discrimination. The full spectra can be found in the original publications, along with data for other methyl chlorogenates.*(14, 15, 30)*

With care, the methyl CQ can be assigned to regio-isomers at MS^2. The fragmentation is completely different to that associated with the feruloylquinic and isoferuloylquinic acids. The isoferuloylquinic acids have not so far been encountered in plants, being known only as mammalian metabolites of caffeoylquinic acids. Note that the fragment ion at *m/z* 173 is no longer exclusively associated with the 4-acyl isomer.

The fragmentation observed for the three methyl diCQ is completely different from that of the CFQA, but not what might have been expected based upon the behaviour of the diCQA relative to the CQA. The MS^3 fragmentation for methyl 3,4-diCQ resembles methyl 3-CQ rather than methyl 4-CQ, and suggests that the C4 caffeoyl moiety is removed more easily than the C3.

Similarly, the MS^3 fragmentations for methyl 3,5-diCQ and methyl 4,5-diCQ resemble methyl 5-CQ rather than methyl 3-CQ and methyl 4-CQ, suggesting that the C5 caffeoyl moiety is the most difficult to remove. This is the reverse of the behaviour seen for the diCQA.

Note that there are literature reports of FQA and CFQA that apparently produce fragments at *m/z* 179 or *m/z* 161, but not at *m/z* 193 or *m/z* 367, and it is possible that these should have been assigned as methyl CQ and methyl diCQ.*(83-86)*

Table 12. Distinguishing Methyl caffeoylquinates, Feruloylquinic Acids and Isoferuloylquinic Acids

	MS^1	MS^2	MS^3	MS^4
Methyl-1-CQ	367	179 + 135 (50%) 297 (20%)	135	
Methyl-3-CQ	367	161 + 135 (25%) 297 (5%)	133	
Methyl-4-CQ	367	161 + 135 (30%) 355 (5%)	133	
Methyl-5-CQ	367	179 + 135 (40%)	135	
1-FQA	367	191	85 + 127 (95) Note a	
3-FQA	367	193	149	
4-FQA	367	173 + 191 (90%)	111	
5-FQA	367	191	85 + 127 (95) Note a	
1-iFQA	367	193	134	
3-iFQA	367	173	143	
4-iFQA	367	173	93	
5-iFQA	367	173	93	
Methyl-3,4-diCQ	529	367	161 + 135 (16%)	133
Methyl-3,5-diCQ	529	367	179 + 135 (60%)	135
Methyl-4,5-diCQ	529	367	179 + 135 (70%)	135
3F,4CQA	529	353 + 367 (90%) 335 (75%)	173 + 179 (90%)	
3C,4FQA	529	367 + 335 (20%)	173	
3F,5CQA	529	367 + 335 (9%)	193	
3C,5FQA	529	353 + 367 (50%)	191 + 179 (60%)	
4F,5CQA	529	367	173 + 193 (60%)	
4C,5FQA	529	353 + 367 (25%)	173 + 179 (80%)	

Note a: Sometimes *m/z* 127 is the base peak with a slightly weaker signal at *m/z* 85

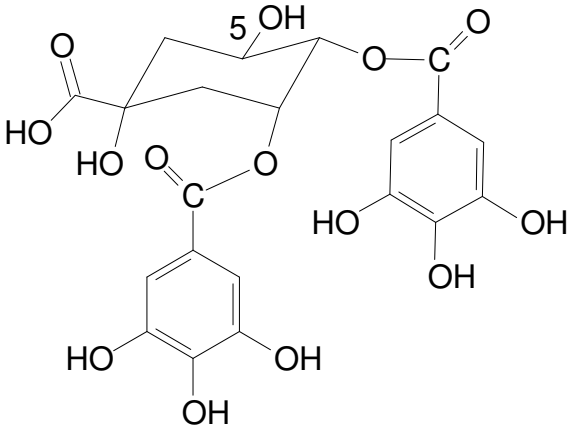
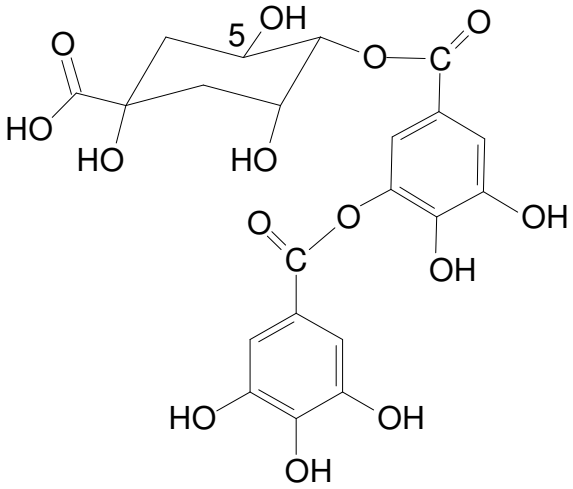
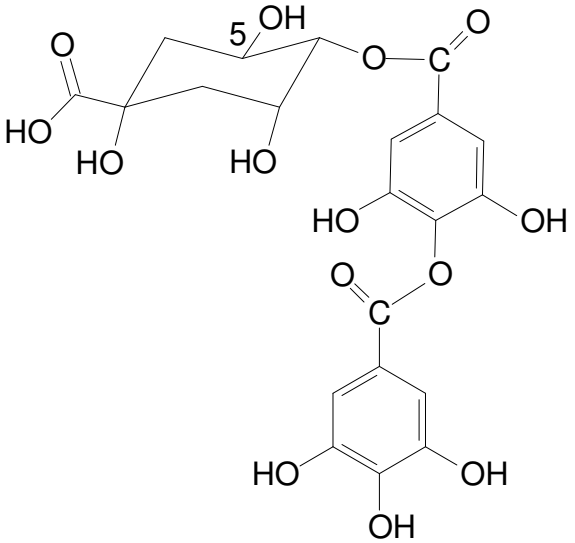
Distinguishing between sugar esters and glycosides

Although falling strictly outside the definition of chlorogenic acids it seems useful to mention sugar esters and glycosides of hydroxycinnamic acids because they usually occur alongside the chlorogenic acids. These two classes are easily distinguished by fragmentation, glycosides losing the sugar as a single unit, whereas when sugar esters are analysed the sugar moiety fragments. For example caffeoyl-3-β-D-glucoside and caffeoyl-4-β-D-glucoside both show *m/z* 341 and *m/z* 179 at MS^1 and MS^2, respectively. In contrast the various caffeoyl-glucose esters produce *m/z* 341 at MS^1 and a series of fragments varying by 30 amu at MS^2, e.g. *m/z* 311, 281, 251, and 221, plus 233 and 203, sometimes accompanied by *m/z* 179.*(17)* Jaiswal *et al* subsequently proved that all possible caffeoyl-glucose regio-isomers can be distinguished by their fragmentation.*(87, 88)*

Distinguishing between depsidic acyl residues and core acyl residues

An acyl residue may be attached directly to the quinic acid or shikimic acid moiety as a core acyl residue or be attached indirectly as a depsidic acyl residue, i.e. present as an acyl reside attached to a core acyl residue, as illustrated below.

Table *. Core and depsidic acyl residues

		
3,4-Digalloylquinic Acid	**4-(Digalloyl)quinic Acid (*meta* depside)**	**4-(Digalloyl)quinic Acid (*para*-depside)**

Only gallic acid depsides are well documented, where the potential for additional internal hydrogen bonds is clear, and these have the potential to affect the retention time and mass fragmentation in ways not seen in, for example, the caffeoylquinic acids. Clifford *et al*. observed that, surprisingly, the aryl ester was more easily cleaved than the alicyclic ester, and there does not seem to be any simple and reliable way to distinguish between depsidic acyl residues and core acyl residues by the fragmentation pattern obtained using the conditions employed for the hydroxycinnamoylquinic acids.*(24)*

The recommended approach is to employ methanolysis to remove the depsidic acyl groups and leave only core acyl groups. Comparison of the mass profile before and after methanolysis easily distinguishes those compounds that are methanolysis-stable from those with depsidic acyl residues. If the changes induced by methanolysis are followed over time, the length of the depsidic chains can be deduced from the presence or otherwise of methyl gallate, methyl digallate, methyl trigallate, etc.

Gouveia *et al*. have reported in an extract of *Andryala* a very hydrophilic acyl-quinic acid with MS^1 and MS^2 base peaks at *m/z* 499 and 191, respectively, and with several secondary MS^2 fragments including *m/*z 173 (94%) and *m/*z 353 (13%), and which elutes before 5-caffeoylquinic acid IUPAC. It was identified tentatively as the depside 5-(*p*-coumaroyl-caffeoyl)quinic acid,*(76)* and if correct, is the first example of a hydroxycinnamoylquinic acid depside.

The early elution is unexpected and suggests the possibility of a *p*-coumaroylquinic glycoside, although the absence of a [*p*-coumaroyl-glycoside]$^-$ fragment as commonly seen in caffeoylquinic glycosides tends to argue otherwise. It should be noted, however, that two very early eluting putative dicaffeoylquinic acids have been observed with analogous behaviour,*(36, 37)* and further investigation is required. Of potential importance is the use of either a triple-quadrupole-MS,*(37)* or a Brucker ion-trap-MS and operating conditions *(36)(59* different from those used by the Kuhnert research group, and it is possible that this explains the different fragmentation of compounds that on the basis of their retention times appear to be acyl-quinic acid glycosides.

3.1.4. Discriminating between chlorogenic acids subgroups with accurate masses differing by less than 1 amu

These subgroups can be distinguished by accurate mass spectrometry, but not reliably by instruments accurate to only unit mass unless fragmentation data are available.

Discriminating between acyl-quinic acids, acyl-isocitric acids and quinic acid glycosides

Although comparatively little studied there are reports of hydroxycinnamic acid conjugates of a range of tricarboxylic acids, e.g. isocitric, hydroxy-citric, and their methyl esters. In the present context such esters of isocitric acid are of interest because they could be confused with the more extensively studied esters of quinic acid, possibly being mistaken for esters of one of the less common quinic acid isomers, e.g. caffeoyl-isocitric acid (*m/z* = 353.0509) and caffeoylquinic acid (*m/z* = 353.0873). Note that with only a single hydroxyl available in isocitric acid there can only be one mono-acyl regio-isomer in contrast to the quinic acid derivatives. There can be *cis* and *trans* isomers, and

there may be 2*R* and 2*S* acyl-isocitric acids that would not resolve on typical reverse-phase column packings. It is unusual, however, to find only a single acyl-quinic acid regio-isomer, and the presence in an extract of only one (or two, i.e. *cis* and *trans*, with identical mass spectra) is suggestive of an acyl-isocitric acid rather than an acyl-quinic acid. More recently, three quinic acid glycosides have also been reported (*m/z* 353.1084) in Kudingcha,*(68)* and these also might cause confusion but their very early elution, well before 1-CQA, should assist in their recognition despite also producing *m/z* 111 at MS^2..

The botanical distribution of these esters and glycosides is probably not fully established, but it is almost certain that they overlap with the acyl-quinic acids. Caffeoyl-isocitric acid was first reported in *Amaranthus cruentus*, characterised by NMR and synthesis,*(89)* with the first ion trap-MS data provided by Steingass *et al*.*(90)* for citric acid and isocitric acid, plus *p*-coumaroyl-isocitric acid and feruloyl-isocitric acid in pineapple, as summarised below. Similar data have been provided by Parveen *et al*. for the base peaks *(91)* but they did not report the relative intensity of the secondary ions.

Stintzing *et al*. have reported ion trap fragmentation data for six compounds in *Amaranthus spinosus* assigned as acyl-quinic acids,*(92)* but re-examination strongly suggests that these are three pairs of *cis* and *trans* acyl-isocitric acids with each pair having virtually identical fragmentation. The MS^2 ion at *m/z* 111 is an important feature of the acyl-isocitric acids, see below. Such an ion can be found in mono- and di-acyl-quinic acids but only at MS^3 and MS^4, respectively, but whether this is a reliable criterion in non-ion trap-MS instruments is unclear.

Table 13. Distinguishing Acyl-quinic acids and Acyl-isocitric Acids

	MS^1	MS^2	MS^3	Ref
Isocitric acid	191	173 + 155 (50%) 111 (60%)		*(90)*
Citric acid	191	111 + 173 (10%)		*(90)*
p-Coumaroyl-isocitric acid	337	173 + 111 (35%) 155 (5%)	111 +155 (15%) 85 (5%)	*(90)*
Feruloyl-isocitric acid	367	173 + 111 (35%) 155 (10%)	111 + 155 (15%) 85 (5%)	*(90)*
Caffeoyl-isocitric	353	173 + 191	111 + 155	*(91)*
Caffeoyl-isocitric acid	353	173 + 191 (62%) 155 (4%) 111 (15%)	111 + 155 (23%) 129 (6%)	*(92)*
p-Coumaroyl-isocitric acid	337	173 + 155 (4%) 111 (18%)	111 + 155 (25%)	*(92)*
Feruloyl-isocitric acid	367	173 + 155 (10%) 111 (31%)	111 + 155 (31%)	*(92)*

There can be little doubt that the compounds in Table 12 are structurally related, and the fragmentations match isocitric acid better than citric acid, and are a poor match for (–)-quinic acid (L-quinic acid) IUPAC, but in the absence of accurate mass data a less common quinic acid isomer remains a possibility. Such data as are available for authenticated quinic acid epimers are presented below in Table 13 and are quite distinct from those in Table 12.

Discriminating between caffeoylquinic acids and scopoletin

Scopolin, the glycoside of scopoletin, has an accurate mass of 354.0951 and yields an ion at *m/z* 353.0873 which is indistinguishable from the parent ion produced by caffeoylquinic acids. There are comparatively few publications reporting LC–ion trap-MS data for both scopoletin and caffeoylquinic acids, but it is clear that the chromatographic

behaviour of scopoletin varies significantly with the experimental conditions. Scopoletin might elute before *(93)* or after *(94)* 5-CQA IUPAC, and between 5-CQA and 4-CQA IUPAC when 4-CQA elutes after 5-CQA.*(95)* Although it has not been possible to locate LC–ion trap-MS fragmentation data for scopoletin under the conditions used to discriminate between the caffeoylquinic acid regio-isomers it is clear that scopoletin can also yield fragment ions at *m/z* 353, 191, 179, 173 and 161 and that there is a real risk of confusion.*(94)* Discrimination might be more easily achieved in positive ion mode because it has been reported that an ion at *m/z* 193 is diagnostic for the scopolin fragment following deglycosylation.*(93, 94)*

3.1.5. Distinguishing Acyl-quinic Acids not containing (–)-Quinic Acid (L-Quinic Acid) IUPAC

Table 14. Chlorogenic Acids not containing (–)-quinic Acid (L-Quinic Acid) IUPAC					
	MS^1	**MS^2**	**MS^3**	**MS^4**	**Reference**
3-caffeoyl-*muco*-*quinic acid*	353	191	127 + 173 (70%) 85 (50%) 109 (25%) 145 (20%)	109	*(50)*
3-feruloyl-*muco*-quinic acid	367	191	127 + 173 (30%) 85 (25%) 109 (15%) 145 (15%)	109	*(50)*
Caffeoylquinic acid epimer from maté	353	191 + 179 (50%) 173 (99%) 135 (10%) See note 1 below	155 + 127 (28%) 171 (46%)		*(47)*
Caffeoylquinic acid epimer from maté	353	179 + 191 (51%) 161 (10%) 135 (20%)	135		*(47)*
Caffeoylquinic acid epimer from *Rudbeckia hirta*	353	191 + 295 (13%)	173 + 127 (15%) 93 (39%)		*(5)*
Caffeoylquinic acid epimer from *Helianthus tuberosus* and *Carlina acaulis*	353	191 + 295 (15%)	173 + 127 (36%) 85 (46%)		*(5)*
Note 1: the MS^2 ions at *m/z* 191 and 173 are of almost identical intensity and which appears as base peak will vary from analysis to analysis. It is convention in such cases to assign the larger fragment as the base peak.					

There is no doubt that these caffeoyl- and feruloyl-quinic acids are distinct from those known to contain (–)-quinic acid IUPAC, and distinct from the acyl-isocitric acids discussed in the preceding section. Only the first two in Table 13, prepared synthetically, but found as products of roasting in roasted coffee beans, have been fully characterised. There are more for which MS data are not available — see Part 2 of these notes.

3.1.6. Distinguishing quinic acid diastereo-isomers

A recent publication from the Kuhnert research group has established that several free quinic acid diastereo-isomers can be discriminated using MS^n and a carefully standardised fragmentation protocol — a Bruker Daltonics HCT Ultra ion trap-MS operating at 0.75 volts collision energy.*(49)* The results are summarised below.

Table 15. MS^n data for quinic acid diastereo-isomers

	MS^1	MS^2	MS^3	Notes
(–)-Quinic acid	190.7	126.8	108.9	
muco-Quinic acid	190.7	126.8	108.8	Resolved by retention time
(±)-*epi*-Quinic acid	190.7	126.8, 172.7 (95%)	111.0 154.8	Fragment at *m/z* 154.8 is derived from targeting *m/z* 172.7
cis-Quinic acid	190.7	93.1		
scyllo-Quinic acid	190.7	172.6, 126.8 (97%)	93.0	

MS^3 was required to distinguish between *scyllo*-quinic acid (MS^3 base peak *m/z* 93) and (±)-*epi*-quinic acid (MS^3 base peak *m/z* 111). In contrast (–)-quinic acid and *muco*-quinic acid behaved identically at MS^2 and MS^3 but could be distinguished by their retention time, *muco*-quinic acid eluting earlier than (–)-quinic acid in isocratic reversed phase chromatography.*(49)*

3.1.7 Standardisation of Ion-trap-MS protocol

The data from the groups of Clifford *et al*. and Kuhnert *et al*. have been obtained using a ThermoFinnigan LCQ deca+ and a Bruker Daltronics HCT Ultra, respectively, with a standardised protocol obtained using commercial 5-caffeoylquinic acid IUPAC as the model. The precise operating conditions are given in the cited papers, and it has been demonstrated that the fragmentations obtained by the two systems are identical, and consistent from day to day. However, note that the conditions required for discriminating between the free quinic acid diastereo-isomers are different from those used for the acyl-quinic acids.*(49)* Although in principle it should be possible to characterise the acylated diastereo-isomers other than (–)-quinic acid using Deshpande *et al's* method by targeting the *m/z* 191 fragment, it is possible that further modification might be required.

It is very important to note that a change in the protocol, especially the collision energy, can markedly alter the fragmentation, to the extent that the hierarchical keys for the chlorogenic acids can no longer be used for identification. For example, see the data provided by Han *et al*.*(31)* who used a very similar ThermoFinnigan ion-trap-MS but increased the ion spray voltage from 3.5 kV as used by the group of Clifford *et al*. to 4.5 kV. These

effects are particularly obvious for the di-acyl-quinic acids, with the production of numerous fragments not seen by Clifford *et al*. or Kuhnert *et al*. making it impossible to assign structures using the hierarchical keys generated at 3.5 kV.

Similarly, although the Gouveia research group use a Bruker ion-trap-MS very similar to that used by the Kuhnert research group, there are some subtle differences observed in the fragmentations obtained, particularly in the fragmentation of the *m/z* 191 [quinic acid]$^-$ fragment ion, and this might explain the different behaviour of an early eluting compound tentatively assigned as 5-(*p*-coumaroyl-caffeoyl)quinic acid but which might be a *p*-coumaroyl-quinic acid glycoside,*(76)* as discussed above. Xie *et al*. using a Bruker MicroTOF Q MS reported identical fragmentation of three diCQA when using a protocol designed simultaneously to determine flavonoids,*(96)* clearly demonstrating the advantage of optimising independently for each class of compound.

Zhang *et al*. using an MSD Trap XCT Plus mass spectrometer and previously characterised chlorogenic acid standards have developed an LC–MS^n procedure that generates fragmentation-diagnostic ions (DFI) that allows the categorisation of chlorogenic acids into several subgroups.*(97)* Compared with the hierarchical keys developed by the Clifford and Kuhnert research groups there appear to be some limitations in this DFI method. The DFI method will no doubt perform perfectly for routine analyses of Flos Lonicerae (for which it was designed) it lacks the predictive power essential when confronted with a previously unidentified chlorogenic acid.

This disadvantage is best illustrated by considering the fragmentation of, for example, 3,5-diCQA in both methods. In the original Clifford *et al*. hierarchical key method 3,5-diCQA yields [3-CQA]$^-$ at MS^2 and that fragment ion at MS^3 behaves exactly the same as 3-CQA itself at MS^2. As explained above, this consistent behaviour can then be utilised in identifying all other diacyl-quinic acids so far investigated, and many tri-acyl- and some tetra-acyl-quinic acids. This facility is not available in the DFI method where both 3-CQA and 5-CQA yield *m/z* 191 and *m/z* 173 at MS^2 and MS^3, whereas 3,5-diCQA yields *m/z* 191 and 127, respectively.

Similarly, although in both methods 4-CQA is the only CQA regio-isomer to yield *m/z* 173 at MS^2, in the DFI method 3,4-diCQA and 4,5-diCQA yield *m/z* 173 at MS^3, but in contrast 1,4-diCQA yields *m/z* 191. In addition, only one of the six putative CFQA reported by Zhang *et al.* shows an *m/z* 367→193 MS^3 transition which is a key diagnostic of the original hierarchical key, making discrimination of caffeoyl-feruloylquinic acids and methyl dicaffeoylquinates more difficult. There is also a possibility that the Zhang *et al*. have confused *cis*-5-CQA eluting appreciably later than *trans*-5-CQA with 1-CQA,*(97)* but this might arise from the use of a different column packing and a complex, non-linear gradient,*(16)* designed to give maximal chromatographic resolution in a single run.

Whatever the exact explanation for the different behaviour, it is clear that with any change in equipment or operating parameters careful recalibration is necessary.

It is also important to recognise that whatever equipment is used there may be some scan-to-scan variation in the intensity of individual fragments, especially when two or more fragments are formed to a similar extent. This effect is likely to be most prominent when signals are weak, i.e. for compounds present at very low concentrations, and for higher order spectra. Because the profile of fragments is crucial in assigning regio-isomers, and in discriminating

between different classes of chlorogenic acids, it is essential to ensure that sufficient scans are obtained at every stage to ensure that a good average spectrum is obtained. At least 10 scans should be taken, preferably at least 20. If this is not possible it is desirable to prepare a more concentrated extract or use a larger injection if the column can accommodate it.

3.1.8. Non-ion-trap MS Instruments

With careful control of the collision energy it is possible to obtain the same MS^2 fragmentation data on QTOF-MS instruments, but great care is required if using a triple quadrupole instrument because of the much increased fragmentation energy employed.

Ramirez-Ambrosi *et al*. used LC–QTOF-MS for chlorogenic acids characterisation but commented that it was not possible to assign them to regio-isomer level because the fragmentation was always the same.*(98)* A similar problem was encountered by Qi *et al*. even at moderate fragmentor voltage, and surprisingly these authors assigned fragments that clearly are associated with the caffeoyl moiety (*m/z* 179, 161 and 135) to the quinic acid moiety.*(99)* In contrast Madala *et al*. have developed a QTOF-MS method that will produce MS^2 and MS^3 spectra that match closely those obtained in the ion trap.*(100, 101)*

The effect of selected fragmentation energy on the MS^2 spectrum when a triple-quadrupole MS is used is very well illustrated by the study of Matsui *et al*.*(64)* Triple quadrupole-MS has been used successfully to fingerprint regio-isomers but structure assignment required the use of two different collision energies in positive ion mode (15 and 30 eV) and three different collision energies in negative ion mode (20, 40 and 60 eV) in order to assign regio-isomers. The MS^2 fragments were not identical to those obtained in an ion trap. Effectively, a new hierarchical key was developed,*(102)* and the lack of higher order spectra when using a triple quadrupole instrument limits the capability. Lin and Harnly who also used triple quadrupole MS had to resort to previously isolated and NMR characterised standards to achieve assignment at regio-isomer level,*(9, 103)* a much more time-consuming process, and as discussed in Part 2 of these notes, assignment by NMR has its own difficulties and limitations. More recently Willems *et al* (2016) have developed a triple quadrupole MS method that can discriminate 3-CQA, 4-CQA and 5-CQA in negative ion mode.*(104)* The behaviour of 1-caffeoylquinic acid was not reported, and it is uncertain whether this method could be applied to feruloylquinic acids, *p*-coumaroylquinic acids, etc. It seems unlikely that it could be applied to dicaffeoylquinic acids, caffeoyl-feruloylquinic acids and the more complex chlorogenic acids.

4. Brief Notes on Quinic Acid Biosynthesis

The pathway to (–)-quinic acid (L-quinic acid) IUPAC is well established, but the route(s) to other quinic acids seems not to have been investigated. As discussed in these three sets of notes there is good evidence for the occurrence in unprocessed plant material of acyl derivatives also of *epi*-quinic acid of known configuration,*(105)* plus 2-hydroxyquinic acid,*(106)* 4-deoxy-quinic acid *(107)* and 1,2,4,5-tetrahydroxy-6-methyl-1,6-dicarboxylic acid *(108)* for which the configurations are not fully determined. However, as discussed in Part 2 of these notes, the configurations at C3, C4 and C5 may well correspond to those of (–)-quinic acid and / or (–)-*epi*-quinic acid thus suggesting some common elements in their biosynthesis.

As illustrated below, the starting point for the biosynthesis of (–)-quinic acid is the conversion of phosphoenol-pyruvate and D-erythrose-4-phosphate to 3-deoxy-D-arabino-heptulosonate-7-phosphate by a synthase enzyme. This intermediate is converted to 3-dehydroshikimate by a dehydroquinate synthase (dehydroquinate dehydratase) and (–)-quinate and (–)-shikimate are then produced by quinate and shikimate dehydrogenase, respectively.

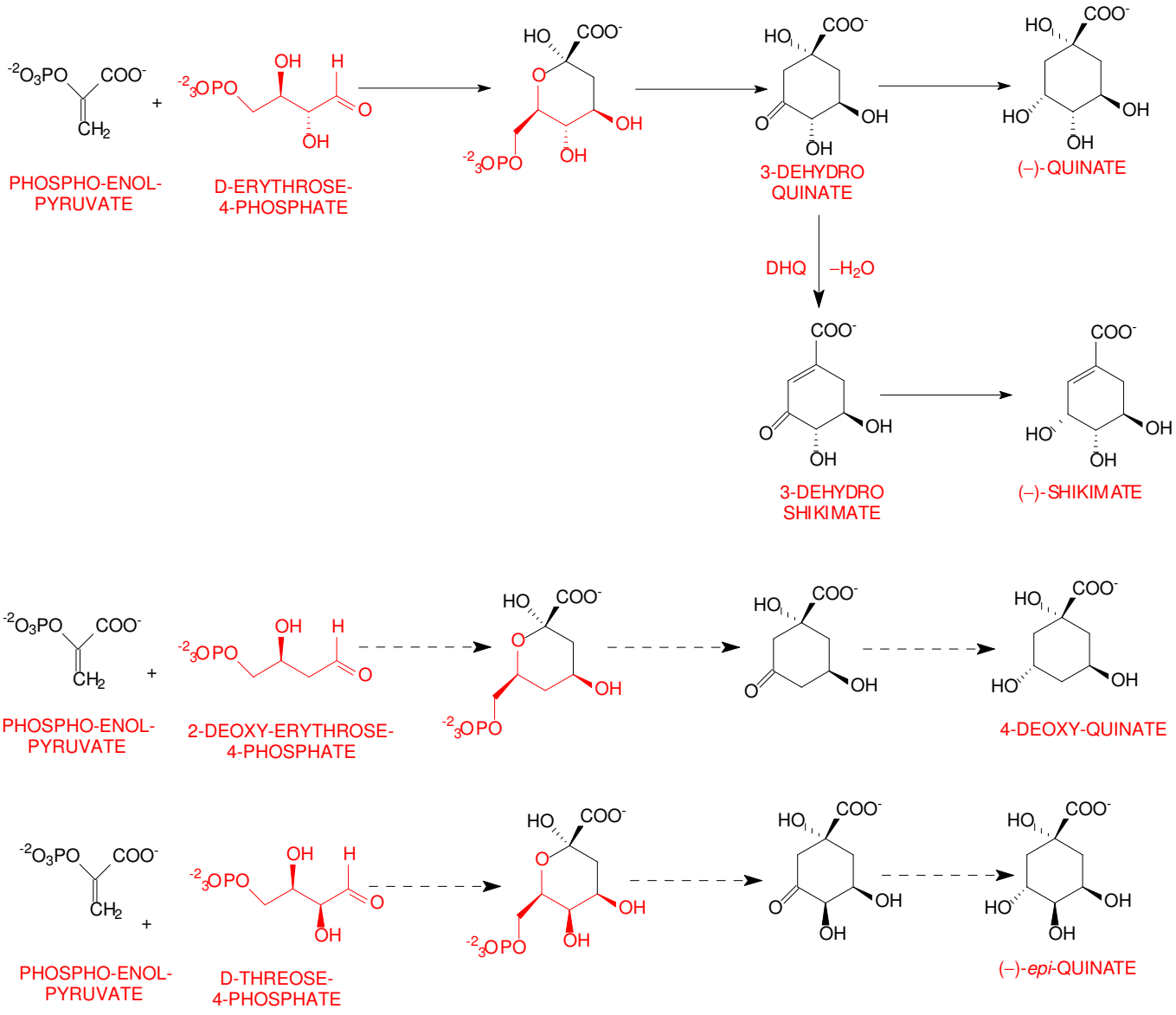
If D-erythrose-4-phosphate is replaced by D-threose-4-phosphate one might expect the formation of (–)-*epi*-quinic acid by the same pathway. D-threose-4-phosphate is a known substrate for the relevant synthase enzyme in *E. coli.(109)* It has been suggested that the widespread ribulose-5-phosphate 3-epimerase (EC 5.1.3.1) may have erythrose-4-phosphate isomerase activity and might, thus, be able to produce this precursor from D-erythrose-4-phosphate. The potential to produce (–)-*epi*-quinic acid by such a minor and plausible alteration to a well-established pathway tends to suggest that acyl derivatives thereof may well have been overlooked.

It is interesting to note that 2-deoxy-erythrose-4-phosphate also participates in the shikimate pathway in *E. coli (110, 111)* and is plausibly the precursor for the synthesis of 4-deoxy-quinic acid, and if this is indeed the source of the acyl derivative in *Arachis* the configuration of 4-deoxyquinic acid moiety would correspond at C3 and C5 with that of both (–)-quinic acid and (–)-*epi*-quinic acid. It has been shown that the C4-OH is required for the activity of dehydroquinase Type I, but it is not required for the activity of dehydroquinase Type II,*(112)* thus making such a pathway eminently plausible.

Although the biological significance is not entirely clear, it has been observed that chemically synthesised *muco*-quinic acid 3-phosphate is an inhibitor of DHQ.*(113)*

The insertion at C2 of a hydroxyl at some convenient point in the pathway to (–)-quinic acid would yield 2-hydroxyquinic acid, and the same insertion at some convenient point in the hypothetical pathway to 4-deoxy-quinic acid would provide a substantial part of the skeleton of 1,2,4,5-tetrahydroxy-6-methyl-1,6-dicarboxylic acid. The origin of the additional methyl and carboxyl at C6 remains obscure.

Biosynthesis of (–)-quinic acid and (–)-shikimic acid and hypothetical routes to 4-deoxy-quinic acid and (–)-*epi*-quinic acid



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