Abstract

1. The current study demonstrated that there is still new information to be obtained on the chemical and biological transformation of the widely studied flavonoid quercetin.
2. In rat hepatocytes thirty five metabolites of quercetin were observed by using high resolution mass spectrometry. The metabolites included glucuronides, sulphates, mixed sulphate/glucuronide metabolites and methylated versions of these metabolites.
3. Several metabolites were formed from chemical degradation products of quercetin which were found to form in Krebs-Henseleit (KH) buffer, degradants of quercetin were also formed in the buffer under the conditions used for hepatocyte incubations.
4. The degradants and metabolites of quercetin were characterised by using high resolution MS^2. It was observed that the glutathione (GSH) conjugates of quercetin formed in large amounts in ammonium bicarbonate solution although the pattern of conjugates formed was different from that observed in hepatocytes suggesting some degree on enzymatic control on GSH conjugate formation in the hepatocyte incubations.
5. GSH conjugates were not formed when GSH was included in incubations of quercetin in KH buffer alone and only small amounts of quercetin degradation occurred. Instead GSH was extensively converted into GSSG thus presumably reducing the levels of oxygen in the incubation thus preventing quercetin degradation.

Keywords Quercetin, hepatocytes, degradation, metabolism, glutathione adducts.

Introduction

There is strong evidence to suggest that dietary phenolic compounds can confer health benefits including protection against cardiovascular disease, neurodegenative disease and cancer (Del Rio *et al*, 2013). Phenolic compounds are comprised of a wide range of structural types including flavones, flavonols, isoflavones, flavanones, anthocyanins, chalcones and phenolic acids. One the most studied phenolic compounds is the flavonol quercetin. Quercetin is abundant in the diet where it occurs both as the aglycone and in conjugated form with, most commonly, glucose or the disaccharide rutinose, quercetin aglycone only occurs at low levels in foodstuffs. Quercetin itself can be directly

absorbed from the intestine but the more abundant glycosides have to be converted to the algycone via the action of a hydrolytic enzyme such as lactase phlorizidin hydrolase which occurs in the brush border membrane cells of the small intestine (Del Rio *et al*, 2013). Thus feeding of fried onions to subjects results in rapid appearance of quercetin sulphate and glucuronide metabolites in the blood stream following cleavage of the glucoside conjugates of quercetin which are abundant in onions (Mullen *et al*, 2006). The other abundant conjugate of quercetin in the diet is quercetin rutinoside which is not absorbed in the small intestine but passes into the large intestine where it is converted into the aglycone via the action of colonic microbiota. When tomato juice, which contains large amounts of quercetin 3-O rutinoside was given to humans the appearance of quercetin metabolites in plasma was much delayed in comparison with the absorption from onions and the levels absorbed were much lower (Jagananth et al, 2006). Part of the reason for the lower absorption of quercetin from the large intestine is due to the fact that the microbiota produce a range of degradation products from quercetin formed via cleavage of the C ring producing a range of phenolic acids (Serra *et al*, 2012). Mullen *et al* observed a range of quercetin metabolites in human plasma and urine following consumption of onions using LC-MS^2. Two sulfates, three glucuronides, two glucuronide sulphates, three diglucuronides, two methylquercetin diglucuronides plus a number of glucoside conjugates were observed (Mullen *et al*, 2004). A recent study identified fifteen metabolites of quercetin in human plasma following consumption of apple sauce with added apple peel or onion (Lee *et al*, 2012) using high resolution LC-MS/MS. The metabolites included one sulphate, three glucuronides, one diglucuronide sulphate, four diglucuronides, two methyl glucuronides, three methyldiglucuronides and a glutathione adduct. The levels of four major quercetin conjugates were determined in human plasma after three months of supplementation with quercetin (Cialdella-Kam *et al*, 2012). The most abundant conjugates detected were isorhamnetin-3-glucuronide, quercetin 3-glucuronide, quercetin 3-sulfate and quercetin diglucuronide. In addition this study examined the effects of quercetin supplementation on global metabolite profiles of the subjects in the study. The metabolites of a number of polyphenols including quercetin were analysed in humans following consumption of cranberry syrup (Iswaldi *et al*, 2013). Quercetin aglcyone and methylated and glucuronide metabolites of quercetin were detected.

There have also been numerous *in vitro* studies of quercetin metabolism. Incubation of quercetin with rat and human hepatocytes led to identification of 14 metabolites of quercetin including methylated, glucuronidated and sulphated metabolites (van der Woude *et al* , 2004). In this study the identity of the metabolites and thus

positions of methylation, glucuronidation and sulfation were confirmed by using NMR for full structure elucidation. Quercetin was incubated with murine hepatocytes (Hong and Mitchell, 2006) and eighteen metabolites of quercetin were identified in the incubations including methylated, glucuronidated and sulphated metabolites but in addition a range of glutathione adducts were observed. These included the glutathione adducts of methyl quercetin and quercetin quinone and glucuronides of glutathione quercetin and methylquercetin. In addition, the observations in hepatocytes led to the observation of the presence of several mercapturic acid conjugates of phenolic acids derived from quercetin in human urine following onion consumption.

An aspect of quercetin behaviour which has not been clearly integrated with its biological behaviour is the relative ease with which it undergoes chemical degradation. It was observed that eighteen degradation products of quercetin were formed upon its electrochemical degradation (Zhou *et al*, 2007). The products included phenolic acids formed via ring scission, oxidised forms of quercetin and dimers of quercetin. It was proposed that the degradation occurred via a carbocation which led to the scission of ring C. Another study used peroxidase from onions to oxidise quercetin and found similar degradation products (Osman and Makris, 2010). In this case it was proposed that the ring scission of the C ring took place via formation of a free radical at position 2 in ring C followed by addition of oxygen to form an oxygen bridge across ring C. The degradation of quercetin in aqueous solutions at 75 °C and 85°C in aqueous solution at pH 5.9 and pH 7.4 was studied using continuous infusion into a high resolution mass spectrometer (Barnes *et al*, 2013). Again degradation took place under these relatively mild pH conditions to a range of ring scission products and dimers.

Thus it would seem that although quercetin has been intensively studied it still remains an active subject of study and each new study seems to reveal further aspects of its chemistry and biochemistry. In the current study the metabolism of quercetin was carried out in rat hepatocytes using high resolution mass spectrometry to elucidate the metabolites formed. A metabolomic approach was taken in order to see if the range of metabolites and degradation products could be extended beyond what is currently known.

Experimental

Chemicals and Reagents

Quercetin (99%) and dimethylsulphoxide (DMSO) were purchased from Sigma Aldrich, Dorset UK. HPLC grade acetonitrile and Analar formic acid were obtained from Fisher Scientific (Loughborough, UK). Water was obtained from a Milli-Q water-purification system (Millipore, Watford UK).

Preparation of hepatocytes

Hepatocytes were isolated from adult male Sprague-Dawley (SD) rat (≈ 200 g of body weight) livers by collagenase digestion, a two-step perfusion process as described by (Moldeus *et al.* 1978) Subsequently, the hepatocyte suspension was washed with 50ml Krebs Henseleit buffer, pH 7.4, containing 12.5 mM HEPES (Liu *et al*., 2002). In order to determine cell viability a trypan blue exclusion test was carried out, and hepatocyte preparations used were at least 78 % viable.

Incubation With Hepatocytes and KH Buffer Blank

A stock solution of quercetin was prepared by dissolving 100 µmoles of quercetin in 1 ml of DMSO. The quercetin solution was incubated at 100 µM with 2×10^6 isolated hepatocytes per ml at 37°C under an atmosphere of 95% O_2, 5% CO_2 in rotating 50 ml round bottomed flasks. Blank incubations were carried out without cells. A control incubation was concomitantly run with hepatocytes but without drug. Four aliquots (0.5 ml) were taken from the incubation solutions at 0, 30 and 120 minutes and the reaction terminated by addition of 1ml of acetonitrile. The samples were stored at -80°C. Prior to analysis samples were thawed at room temperature, sonicated and centrifuged at 5000 rpm for 5 min to remove protein and collect the supernatants for analysis of conjugates. In order to test the degradation of quercetin in blank solution under same above conditions (95% O_2, 5% CO_2) incubation was carried out in a rotating 50 ml round bottomed flask in KH buffer alone. In addition 10 µl of quercetin solution (30.2 mg / 1ml of DMSO) was incubated in 10 ml of KH buffer to which had been added 10 µl of GSH solution (60 mg or 120 mg / 1ml in 0.2% w/v ammonium bicarbonate).

Preparation of GSH Conjugate of Quercetin

A 10 µl of quercetin solution (30.2 mg / 1ml of DMSO) was added to GSH solution (1mg /1ml in 0.2% ammonium bicarbonate). The sample was left at room temperature for 24 hours before analysis. For analysis the sample was diluted 10:1 with 0.1% formic acid and then analysed by LC-MS.

LC-MS Analysis

Preliminary identification of the chemical structure of the quercetin metabolites was performed by using a Dionex HPLC instrument connected to an Exactive Orbitrap (Thermo Fisher Scientific, UK). The HPLC was fitted with an ACE 5 C18-AR column (5 µm, 150mm x 4.6mm, HiChrom, Reading UK). The mobile phases consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient system was 10% B, (0 min); 80% B, (30-32 min); 10% B, (32-40 min). The flow rate of the mobile phase was 0.3 ml/min. Mass spectra were recorded using electrospray

ionization (ESI) in negative and positive modes. Samples were run in negative mode using a needle voltage of - 4.0 kV and source temperature 320°C. Optimum nebulization was achieved using nitrogen at sheath gas flow rate 50 and auxiliary gas flow rate 17 (units not specified by the manufacturer). Scanning of total ion chromatograms (TIC) range was between 75-1000 *m/z*. For confirmation of the structure of the metabolites MS^2 was carried out by using an LTQ Orbitrap (Thermo Fisher Scientific, UK) with the same chromatographic conditions as were used with the Exactive. Finally, the data were collected and processed using Xcalibur software Ver. 2.0, Thermo- Fisher Corporation, UK, SIEVE software Ver. 1.2.1, Thermo- Fisher Corporation, UK and Metworks Software Ver. 1.3, Thermo- Fisher Corporation, UK.***Results and Discussion***

There was no significant loss of viability of hepatocytes incubated in KH buffer alone or with KH buffer + quercetin. Quercetin was metabolised very rapidly by hepatocytes and on an area % basis only 1.8% remained at 120 min (table 1). Upon inspection of the data the range of metabolites obtained for quercetin appeared to be more complex than described previously and included ring scission products and their metabolites which have been described as products of microbial degradation (Serra *et al*, 2012). It was observed that most of these "metabolites" also occurred in the blank incubations of quercetin in KH buffer alone. Thus it was thought perhaps that the KH incubation buffer might somehow be contaminated with microbes. However, a fresh batch of sterile buffer was prepared, tested for sterility, and the same range of degradants was observed. These degradants, which are rapidly formed *in vitro* at physiological pH under an atmosphere containing 95% oxygen, contribute to the overall metabolism of quercetin since they form in the incubation medium and are then metabolised by the hepatocytes. In order to simplify consideration of the hepatic metabolites of quercetin the degradants formed are considered first. The observed degradants in KH buffer are summarised in table 2 and figure 1 shows the proposed structures for the major chemical degradants of quercetin. Most of these degradants form almost immediately when quercetin is dissolved in the incubation buffer and only *ca* 25% of the quercetin remains after a few minutes. The major component is degradant D1 which presumably exists in equilibrium with quercetin in aqueous solution. It is likely that D1 forms via attack of oxygen on quercetin which results in D2 where an oxygen bridge is formed as proposed previously (Osoman and Makris 2010). It is possible that attack by hydroxyl plays are part in the degradation but a recent publication demonstrated that in the absence of oxygen quercetin does not degrade even in alkaline solution (Ramešová *et al*, 2012). D2 can be observed in the degraded mixture but at much lower levels than D1. D1 has been described as being a chalcone but a chalcone structure is not consistent with elimination of CO_2 which is observed in

the MS^2 spectrum of D1 (figure 2), where in order to readily devise a mechanism for CO_2 elimination two oxygen atoms have to be bonded to the same carbon atom . The spectrum of D1 is unusually complex for a MS^2 spectrum and many of the fragments are due to alternate elimination of oxygen and CO. We have tried to rationalise the fragmentation pattern observed in the spectrum in figure 3 and the complexity of the spectrum reinforces the impression that quercetin is an unusual molecule. D1 appears to act as the key intermediate in degradation which decomposes into mainly D3- D6 plus several minor degradants including some involving adduct formation such as the adducts formed to produce a dimer of quercetin and an adduct between quercetin and D1. Direct observation of quercetin degradation in an aqueous buffer by NMR is not an option because of its poor water solubility. Quercetin is soluble in NaOH but rapidly degrades so that it was not possible to isolate the key intermediate D1 by dissolving in NaOH. Table 3 shows the masses of the fragment ions in MS^2 spectra of those degradants which were sufficiently abundant to obtain and good quality MS^2 spectra. After 120 min of incubation in KH buffer the quercetin has almost completely decomposed. Quercetin is obviously highly reactive as reported before (Zhou *et al*, 2007) but so far no studies have reported the degradation of quercetin under physiological conditions and the resultant contribution of the degradants to the metabolic profile of quercetin. Table 1 shows the major metabolites formed following incubation of quercetin with rat hepatocytes for 30 min. and 120 min. The quercetin is largely metabolised by 30 min with the major metabolites being monoglucuronides, a diglucuronide, a methyl glucuronide and a mixed glucuronide sulphate. Small amounts of monosulfates and methyl monosulfates are also present. At 120 minutes the main metabolites are mixed glucuronide sulphates. The proposed metabolites are shown in figure 4. Most of these metabolites have been described before (van der Woude *et al* , 2004, Hong and Mitchell, 2006) although this the first study where they have been characterised by using high resolution mass spectrometry. In addition to quercetin metabolites, a small amount of the sulphate of D3 was formed. D3 is a major degradant of quercetin in KH buffer in the absence of hepatocytes but its levels are much lower in hepatocyte incubations. Thus, it would appear that the chemical degradation of quercetin in hepatocyte incubations is less extensive in comparison with its degradation in KH buffer alone which may be due to inhibition of degradation as a result of glucuronidation, sulphation, methylation and GSH conjugate formation. In addition to small amounts of D3 sulphate being formed, there were small amounts of putatively identified metabolites resulting from the methylation of D3 followed by glucuronidation, sulphation and sulphation in combination with glycine conjugation. Quercetin also forms three glutathione conjugates in small amounts (figure 5) which presumably result from addition of GSH at different positions within

the ring system; a GSH conjugate of D3 can also be observed in the hepatocyte incubations. It would seem likely that a favoured site for reaction of GSH would be at position 2 in the C ring of quercetin where it was proposed that addition of oxygen occurs (Osman and Makris, 2010). Also small amounts of GSH conjugates of the quercetin quinone can be observed as shown in figure 5. These GSH conjugates can be readily formed when quercetin is incubated in ammonium bicarbonate solution although the relative abundance of the conjugates is different from those formed in hepatocytes suggesting that in hepatocytes the reaction may be enzymatically controlled rather than a simple chemical reaction. In the case of the incubation in ammonium bicarbonate two peaks for the conjugate of equal intensity result and this would be consistent with addition at position 2 where two diastereomeric adducts would form since addition of GSH generates a chiral centre. Also larger amounts of a quinone GSH conjugate are formed in the ammonium bicarbonate incubation. The presence of GSH in the ammonium carbonate incubation does not inhibit the degradation of quercetin and the major degradants D3-D6 are all formed. In addition GSH adducts of D3-D6 can also be observed. When quercetin is incubated in KH buffer containing GSH under an atmosphere containing 95% oxygen, in contrast to the same experiment in ammonium bicarbonate, there was no reaction with GSH and very little degradation of quercetin occurred up to 180 minutes. During the course of the incubation, as observed by mass spectrometry, much of the GSH was converted to GSSG suggesting that the GSH was removing oxygen from the solution through itself becoming oxidised. This further supports the idea that the initiation of quercetin degradation occurs via the formation of the oxygen bridge intermediate D2. The MS^2 spectra for some quercetin metabolites and quercetin degradant metabolites are shown in table 4 and these are consistent with the assigned structures.

Conclusions

The levels of quercetin used in the incubations in rat hepatocytes were about ten times the likely exposure from dietary absorption when comparing for instance the total area under the curve over 24 h for quercetin metabolites (*ca* 3 µg compared to 30 µg in the current case) absorbed from onion powder (Lee *et al*, 2012). However, from the data in table 1 it can be seen that the level of quercetin at physiological pH rapidly declines to *ca* 25% of the original concentration thus the levels used in the incubation may not be that different from the levels of exposure in human dosing studies. Although quercetin has been extensively studied it seems that its chemistry and metabolism are far from being fully understood. Its high reactivity means that, as well as acting as a free radical scavenger, it would be likely to form adducts with reactive structures such as thiol groups within proteins. However, it would appear from

the current work that its reactivity is reduced by conjugation, for instance with glucuronic acid, and this might explain the lack of an effect on the viability of the hepatocytes. It was observed in a trial using quercetin as an anti-tumour agent that nephrotoxicity was a dose limiting factor and that phase II metabolism was important in reducing toxicity (Ferry *et al* 2006) The mechanism of quercetin degradation remains to be fully explained but it should be possible to make a more thorough study of this by using NMR particularly in ^{13}C NMR which would be able to map the changes in carbon substitution during degradation. Although quercetin has been proposed as an antioxidant compound it may have interesting pro-oxidant and oxygen sensing properties.

Acknowledgements

We would like to thank Scottish Life Sciences Alliance (SULSA) for funding for mass spectrometry equipment. KO acknowledges the Libyan Government for funding through a PhD scholarship.

References

Barnes JS, Foss Jr FW, Schug KA (2013) Thermally Accelerated Oxidative Degradation of Quercetin Using Continuous Flow Kinetic Electrospray-Ion Trap-Time of Flight Mass Spectrometry. Journal of the American Society for Mass Spectrometry 24: 1513-1522.

Beekmann K, Actis-Goretta L, van Bladeren PJ, Dionisi F, Destaillats F, Rietjens IMCM. A state-of-the-art overview of the effect of metabolic conjugation on the biological activity of flavonoids. Food & Function. 2012;3(10):1008-18.

Boots AW, Kubben N, Haenen G, Bast A. Oxidized quercetin reacts with thiols rather than with ascorbate: implication for quercetin supplementation. Biochemical and Biophysical Research Communications. 2003;308(3):560-5.

Bousova I, Skalova L. Inhibition and induction of glutathione S-transferases by flavonoids: possible pharmacological and toxicological consequences. Drug Metab Rev. 2012;44(4):267-86.

Cialdella-Kam L, Nieman DC, Sha W, Meaney MP, Knab AM, et al. (2012) Dose–response to 3 months of quercetin-containing supplements on metabolite and quercetin conjugate profile in adults. British Journal of Nutrition 1: 1-11.

Del Rio D, Rodriguez-Mateos A, Spencer JP, *et al* (2013) Dietary (poly) phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. Antioxidants Redox Sig. 18:1818-1892.

Ferry, D. R.; Smith, A.; Malkhandi, J.; Fyfe, D. W.; Anderson, D.; Baker, J.; Kerr, D., Phase I clinical trial of the flavonoid quercetin: pharmacokinetics and evidence for in vivo tyrosine kinase inhibition. *Clinical Cancer Research* **1996,** *2*, 659-668.

Frauzino Araujo KC, Costa EMdMB, Pazini F, Valadares MC, de Oliveira V. Bioconversion of quercetin and rutin and the cytotoxicity activities of the transformed products. Food and Chemical Toxicology. 2013; 51:93-6.

Hong Y-J, Mitchell AE (2006) Identification of glutathione-related quercetin metabolites in humans. Chemical Research in Toxicology 19: 1525-1532.

Iswaldi I, Arraez-Roman D, Gomez-Caravaca AM, Contreras MDM, Uberos J, Segura-Carretero A, et al. Identification of polyphenols and their metabolites in human urine after cranberry-syrup consumption. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association. 2013;55:484-92.

Jaganath IB, Mullen W, Edwards CA, and Crozier A. The relative contribution of the small and large intestine to the absorption and metabolism of rutin in man. Free Radic Res 40: 1035–1046, 2006.

Kaushik D, O'Fallon K, Clarkson PM, Dunne CP, Conca KR, Michniak-Kohn B. Comparison of Quercetin Pharmacokinetics Following Oral Supplementation in Humans. Journal of Food Science. 2012;77(11):H231-H8.

Lee J, Ebeler SE, Zweigenbaum JA, Mitchell AE. UHPLC-(ESI)QTOF MS/MS Profiling of Quercetin Metabolites in Human Plasma Postconsumption of Applesauce Enriched with Apple Peel and Onion. J Agric Food Chem. 2012;60(34):8510-20.

Liu X-L, Li L-J, Chen Z . (2002). Isolation and primary culture of rat hepatocytes. Hep. Pancreatic Dis. Int. 1: 77-79.

Moldeus, P, Hogberg, J, Orrenius, S. (1978). Isolation and use of liver cells. Methods Enzymol. 52: 60-71.

Moalin M, van Strijdonck GPF, Bast A, Haenen G. Competition between Ascorbate and Glutathione for the Oxidized Form of Methylated Quercetin Metabolites and Analogues: Tamarixetin, 4 ' O-Methylquercetin, Has the Lowest Thiol Reactivity. J Agric Food Chem. 2012: 60(36):9292-7.

Mullen W, Edwards CA, and Crozier, A. Absorption, excretion and metabolic profiling of methyl-, glucuronyl-, glucosyl and sulpho-conjugates of quercetin in human plasma and urine after ingestion of onions. Br J Nutr 96:107–116, 2006.

Murota K, Shimizu S, Chujo H, Moon JH, Terao J (2000) Efficiency of absorption and metabolic conversion of quercetin and its glucosides in human intestinal cell line Caco-2. Archives of Biochemistry and Biophysics 384: 391-397.

Osman A, Makris DP (2010) Comparison of fisetin and quercetin oxidation with a cell-free extract of onion trimmings and peel, plant waste, containing peroxidase enzyme: a further insight into flavonol degradation mechanism. International Journal of Food Science & Technology 45: 2265-2271.

Ramešová S, Sokolová R, Degano I, Bulíčková J, Žabka J, Gál M. (2012) On the stability of the bioactive flavonoids quercetin and luteolin under oxygen-free conditions Anal Bioanal Chem 402:975–982

Serra A, Macià A, Romero M-P, Reguant J, Ortega N, et al. (2012) Metabolic pathways of the colonic metabolism of flavonoids (flavonols, flavones and flavanones) and phenolic acids. Food Chemistry 130: 383-393.

van der Woude H, Boersma MG, Vervoort J, Rietjens I (2004) Identification of 14 quercetin phase II mono- and mixed conjugates and their formation by rat and human phase II in vitro model systems. Chemical Research in Toxicology 17: 1520-1530.

Zhou A, Kikandi S, Sadik OA (2007) Electrochemical degradation of quercetin: Isolation and structural elucidation of the degradation products. Electrochemistry Communications 9: 2246-2255.

Table 1 Metabolites of quercetin and its degradants putatively identified according to accurate mass in incubations with rat hepatocytes.

Metabolite/Degradant	$[M-H]^-$	Elemental Composition	Rt min.	Area % 30 min	Area % 120 min
D3	153.01	$C_7H_5O_4$	11.2	2.85	2.02
Quercetin	301.03	$C_{15}H_9O_7$	21.5	0.58	1.80
Quercetin GSH Conjugate M1	606.10	$C_{25}H_{24}O_{13}N_3$ S	14.0	0.18	0.213
Quercetin GSH Conjugate M2	606.10	$C_{25}H_{24}O_{13}N_3$ S	14.5	6.93	3.95
Quercetin GSH Conjugate M3	606.10	$C_{25}H_{24}O_{13}N_3S$	15.45	NA	0.0580
Quercetin GSH Conjugate M4	604.08	$C_{25}H_{22}O_{13}N_3S$	15.7	0.475	0.0500
Quercetin GSH Conjugate M5	604.08	$C_{25}H_{22}O_{13}N_3S$	16.2	2.69	0.540
M6	458.08	$C_{17}H_{20}O_{10}N_3S$	6.7	0.60	1.083
M7	458.08	$C_{17}H_{20}O_{10}N_3S$	9.4	NA	0.167
M8	232.97	$C_7H_5O_7S$	6.1	1.067	0.587
M9	246.99	$C_8H_7O_7S$	10.0	NA	0.605
M10	246.99	$C_8H_7O_7S$	10.5	NA	0.280
M11	304.01	$C_{10}H_{10}O_8NS$	6.7	NA	1.84
M12	304.01	$C_{10}H_{10}O_8NS$	9.3	NA	0.470
Quercetin sulphate M13	380.99	$C_{15}H_9O_{10}S$	17.5	NA	0.138
Quercetin sulphate M14	380.99	$C_{15}H_9O_{10}S$	19.3	2.43	0.47
Quercetin glucuronide M15	477.06	$C_{21}H_{17}O_{13}$	15.3	12.8	4.54
Quercetin 3-glucuronide M16	477.06	$C_{21}H_{17}O_{13}$	17.5	10.20	1.86
M17	343.06	$C_{14}H_{15}O_{10}$	6.7	0.0277	0.346
Methylated quercetin M18	315.05	$C_{16}H_{11}O_7$	23.9	0.074	0.068
Methylated quercetin glucuronide M19	491.08	$C_{22}H_{19}O_{13}$	16.6	NA	5.60
Methylated quercetin glucuronide M20	491.08	$C_{22}H_{19}O_{13}$	17.4	5.75	0.81
Methylated quercetin glucuronide M21	491.08	$C_{22}H_{19}O_{13}$	17.7	3.22	0.79
Methylated quercetin glucuronide M22	491.08	$C_{22}H_{19}O_{13}$	18.1	2.48	0.56
quercetin monglucuronide sulphate M23	557.02	$C_{21}H_{17}O_{16}S$	13.9	16.55	4.40
quercetin monglucuronide sulphate M24	557.02	$C_{21}H_{17}O_{16}S$	14.5	3.56	18.4
quercetin monglucuronide sulphate M25	557.02	$C_{21}H_{17}O_{16}S$	15.0	2.17	11.4
Methylated quercetin monglucuronide sulphate M26	571.04	$C_{22}H_{19}O_{16}S$	13.8	0.707	4.40

Methylated quercetin monglucuronide sulphate M27	571.04	$C_{22}H_{19}O_{16}S$	14.2	0.867	5.72
Methylated quercetin monglucuronide sulphate M28	571.04	$C_{22}H_{19}O_{16}S$	14.9	0.692	5.92
Methylated quercetin monglucuronide sulphate M29	571.04	$C_{22}H_{19}O_{16}S$	15.6	0.203	2.30
Quercetin-3,7-diglucuronide M30	653.1	$C_{27}H_{25}O_{19}$	13.5	13.25	14.15
Quercetin-3,7-diglucuronide M31	653.1	$C_{27}H_{25}O_{19}$	14.3	1.53	1.63
Quercetin-3,7-diglucuronide M32	653.1	$C_{27}H_{25}O_{19}$	14.7	2.13	1.89
Methylated quercetin sulphate M33	395.008	$C_{16}H_{11}O_{10}S$	14.2	NA	0.045
Methylated quercetin sulphate M34	395.008	$C_{16}H_{11}O_{10}S$	19.05	0.053	0.243
Methylated quercetin sulphate M35	395.008	$C_{16}H_{11}O_{10}S$	19.5	0.055	0.11

Table 2 Formation of quercetin degradants in KH buffer at 0, 30 and 120 minutes. Putative identification of degradants according to accurate mass with < 2 ppm deviation from the proposed composition.

Degradant	$[M-H]^-$	Elemental Composition	Rt min.	Area % 0 min	Area % 30 min	Area % 120 min
$C_7H_5O_4$ D3	153.0195	$C_7H_5O_4$	11.9	9.7	15.3	37
$C_7H_5O_5$ D4	169.0145	$C_7H_5O_5$	11.2	2.5	4.1	9.1
$C_8H_5O_5$ D5	181.0146	$C_8H_5O_5$	6.8	0.62	1.15	3.3
$C_8H_5O_6$ D6	197.0095	$C_8H_5O_6$	10.2	6.6	7.9	13.5
C8H7O6	199.0252	$C_8H_7O_6$	6.3	0.68	1.2	1.2
quercetin -CO	273.041	$C_{14}H_9O_6$	15.8	0.16	0.13	0.09
quercetin quinone D8	299.0203	$C_{15}H_7O_7$	22.7	3.5	3.0	2.7
quercetin	301.0358	$C_{15}H_9O_7$	22.2	25.8	27.4	6.20
chalcone quinone D7	315.0155	$C_{15}H_7O_8$	18.3	1.40	0.92	0.50
Chalcone D1	317.0308	$C_{15}H_9O_8$	15.8	44.5	33.9	20.3
oxidised chalcone oxygen bridge	331.0104	$C_{15}H_7O_9$	14.7	0.75	0.77	1.6
quercetin oxygen bridge D2	333.0261	$C_{15}H_9O_9$	19.8	1.0	0.71	0.43
Adduct 169/273	425.0525	$C_{21}H_{13}O_{10}$	18.4	0.05	0.22	1.3
197 + quercetin adduct	497.0372	$C_{23}H_{13}O_{13}$	16.6	0.68	0.99	1.3
quercetin dimer	601.0635	$C_{30}H_{17}O_{14}$	23.9	0.16	0.52	0.41
quercetin + 317	617.0581	$C_{30}H_{17}O_{15}$	19.8	0.82	0.75	0.51

Table 3 MS^2 (35eV) data for the major degradants formed from quercetin in KH buffer. *Observed as a degradant following treatment with 1 M NaOH

Degradant	$[M-H]^-$	MS/MS fragments
$C_7H_5O_4$ D3	153.0195	109.0298 ($C_6H_5O_2$ - CO_2, 100%)
$C_7H_5O_5$ D4	169.0145	151.004 ($C_7H_3O_4$ - H_2O,100%) 125.0246 ($C_6H_5O_3$ - CO_2, 1.24%) 107.0139 ($C_6H_3O_2$ - H_2O and - CO_2, 0.54%)
$C_8H_5O_5$ D5	181.0146	109.0295 ($C_6H_5O_2$ - $COCO_2$)? →153.02 (- CO_2, 38.79%)
$C_8H_5O_6$ D6	197.0095	153.0196 ($C_7H_5O_4$ - CO_2, 100%), 125.02 ($C_6H_5O_2$ - $COCO_2$, 1.90%)
quercetin –CO $C_{14}H_9O_6$	273.041	258.0176 ($C_{13}H_6O_6$ - CH_3, 48.19%), 245.0465 ($C_{13}H_9O_5$ - CO, 100%), 231.03076 ($C_{12}H_7O_5$ - $COCH_2$, 65.40%) 229.0515 ($C_{13}H_9O_4$ - CO_2, 84.49%), 217.0510 ($C_{12}H_9O_4$, - $COCO_2$, 11.34%)
chalcone quinone D7 $C_{15}H_7O_8$	315.0155	287.0207 ($C_{14}H_7O_7$ - CO, 100%), 243.0308 ($C_{13}H_7O_5$ - $COCO_2$, 11.99%) 151.0043 ($C_7H_3O_4$ - $C_8H_4O_4$, 8.52%), 271.06 ($C_{14}H_7O_6$ - CO_2, 13.26%)
*Chalcone D9 $C_{15}H_9O_8$	317.0308	299.0194($C_{15}H_7O_7$ - H_2O, 100 %), 271.0247 (- H_2O-CO), 255.0298 ($C_{14}H_7O_5$ - CO_2 -H_2O, 10.6%),231.0298 ($C_{12}H_7O_5$ - $C_3H_2O_3$, 9.4%), 194.9934 (($C_8H_3O_6$ 19.5%),166.9985 (($C_7H_3O_5$, 9.4%)
chalcone D1 $C_{15}H_9O_8$	317.0308	299.0182 ($C_{15}H_7O_7$ - H_2O, 53.19%) 273.0410 ($C_{14}H_9O_6$ - CO_2, 12.04%) 255.0309 ($C_{14}H_7O_5$ - CO_2 -H_2O, 7.86%) 231.0308 ($C_{12}H_7O_5$ - $C_3H_2O_3$, 1.32%) 206.9943 ($C_9H_3O_6$ - $C_6H_6O_2$, 36.04%) 190.9991 ($C_9H_3O_5$ - $C_6H_6O_3$, 100%) 178.9994 ($C_8H_3O_5$) 163.0044 ($C_8H_3O_4$ -$C_7H_6O_4$, 18.94%) 153.0200($C_7H_5O_4$) 135.0092 ($C_7H_3O_3$)
quercetin oxygen bridge D2 $C_{15}H_9O_9$	333.0261	289.0363 ($C_{14}H_9O_7$ - CO_2, 29.46%) 181.0149 ($C_7H_5O_5$ - $C_8H_4O_4$, 100%) 169.0151 ($C_7H_5O_4$ - $C_8H_4O_5$, 90.47%) 109.0282 ($C_6H_5O_2$ - $C_9H_4O_7$, 7.72%)
adduct 169/273 $C_{21}H_{13}O_{10}$	425.0525	407.0419 ($C_{21}H_{11}O_9$ - H_2O, 9.24%) 299.0207 ($C_{15}H_7O_{7,}$ - $C_6H_6O_3$ 38.66%) 273.0412 ($C_{14}H_9O_6$, - $C_7H_4O_7$ 25.81%)
197 + quercetin $C_{23}H_{13}O_{13}$	497.0372	345.0262 ($C_{16}H_9O_9$ - $C_7H_4O_4$, 100%) 301.0363 [$C_{15}H_9O_7$ (-196 $C_8H_4O_6$, 61.79%)]
quercetin dimer $C_{30}H_{17}O_{14}$	601.0635	449.0553 ($C_{23}H_{13}O_{10}$ - $C_7H_4O_4$, 100%) 431.0420 ($C_{23}H_{11}O_{10}$ - $C_7H_6O_4$, 1.96%)
quercetin + 317 $C_{30}H_{17}O_{15}$	617.0581	465.0481 ($C_{23}H_{13}O_{10}$ - $C_7H_4O_5$, 100%)

Table 4 MS^2 (35eV) data for the major metabolites formed from quercetin in hepatocyte incubations. Gluc = glucuronic acid –OH, G= glutathione –SH, GSH= glutathione.

Metabolite	$[M-H]^-$	Rt min.	Fragment ions
Quercetin GSH Conjugate M1	606.10	14.5	333.01 (-G, 38.2%), 299.02 (-GSH, 100%)
Quercetin GSH Conjugate M2	606.10	15.1	333.01 (-G, 100%), 299.02 (-GSH, 2.6%)
Quercetin GSH Conjugate M3	606.10	16.2	333.01 (-GH, 50%)
Quercetin GSH Conjugate M4	604.08	16.9	330.99 (-G, 100%), 298 (-GSH, 0.98%)
Quercetin GSH Conjugate M5	604.08	21.9	301.03 (-GSH, 100%)
M8	232.97	7.0	153.02 ($-SO_3$, 100%), 109.029 ($-CO_2$, $-SO_3$ 39.5%)
M10	246.99	10.0	203.002 ($-CO_2$, 21.2%), 167.03 ($-SO_3$, 63.5%)
M11	304.01	7.4	260.02 ($-CO_2$, 1.2%), 224.05 ($-SO_3$, 100%)
M17	343.06	7.4	167.03 (-Gluc, 67.2%)
Quercetin sulphate M14	380.99	19.9	301.03 ($-SO_3$, 100%)
Quercetin glucuronide M15	477.06	16.2	301.03 (- Gluc, 100%)
Quercetin glucuronide M16	477.06	18.4	301.03 (- Gluc, 100%)
Quercetin methyl glucuronide M19	491.08	17.6	315.05 (-Gluc, 100%)
quercetin monglucuronide sulphate M22	557.02	15.0	477.06 ($-SO_3$, 100%), 380.99 (-Gluc, 12.6%), 301.04 (-Gluc and SO_3, 19.70%).
quercetin monglucuronide sulphate M24	557.02	15.6	477.06 ($-SO_3$, 100%), 380.99 (-Gluc, 28.6%), 301.04 (-Gluc and SO_3, 4.66%).
Methylated quercetin monglucuronide sulphate M23	571.04	14.3	491.08 ($-SO_3$, 100%), 395.01(-Gluc, 2.2%), 315.05($-SO_3$ and -Gluc, 6.91%)
Methylated quercetin monglucuronide sulphate M26	571.04	14.7	491.08 ($-SO_3$, 100%), 395.01 (-Gluc, 2.6%), 315.05 ($-SO_3$ and -Gluc, 23.7%)
Methylated quercetin monglucuronide sulphate M27	571.04	16.3	491.08 ($-SO_3$, 100%), 395.01 (-Gluc, 6.64%), 315.05 ($-SO_3$ and - Gluc, 6.97%)
Quercetin-3,7-diglucuronide M30	653.1	14.1	477.06 (-Gluc, 100%), 301.03 (-2Gluc, 8.3%)
Quercetin-3,7-diglucuronide M31	653.1	14.3	477.06 (-Gluc, 100%), 301.03 (-2Gluc, 7.76%)
Methylated quercetin sulphate M34	395.005	19.6	315.05 ($-SO_3$, 100%)
Methylated quercetin sulphate M35	395.005	20.1	315.05 ($-SO_3$, 100%)

Figure 1 Proposed structures for major degradants formed from quercetin in KH buffer.

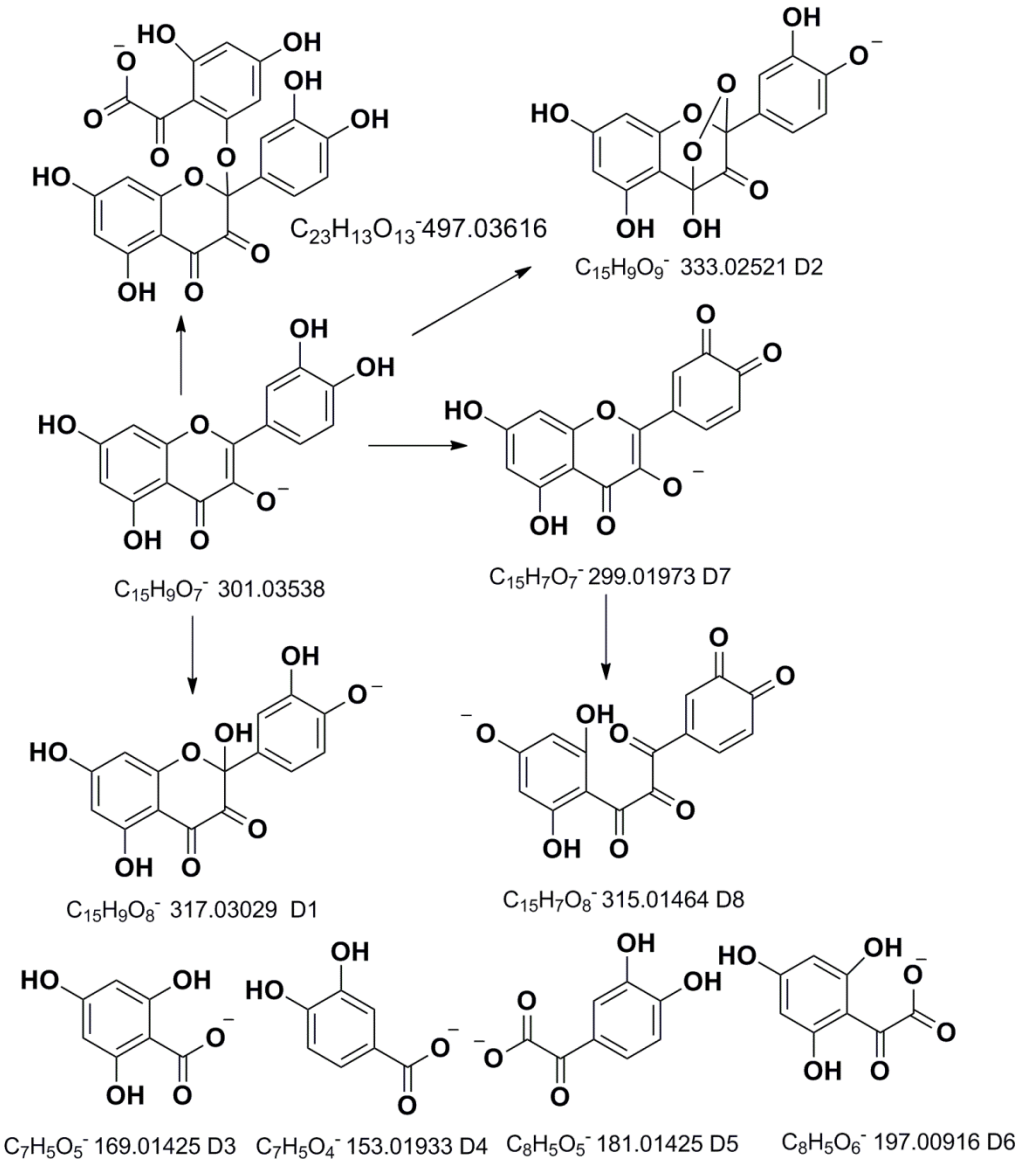

Figure 2 MS^2 spectrum (35eV) of the key intermediate D1 of quercetin degradation.

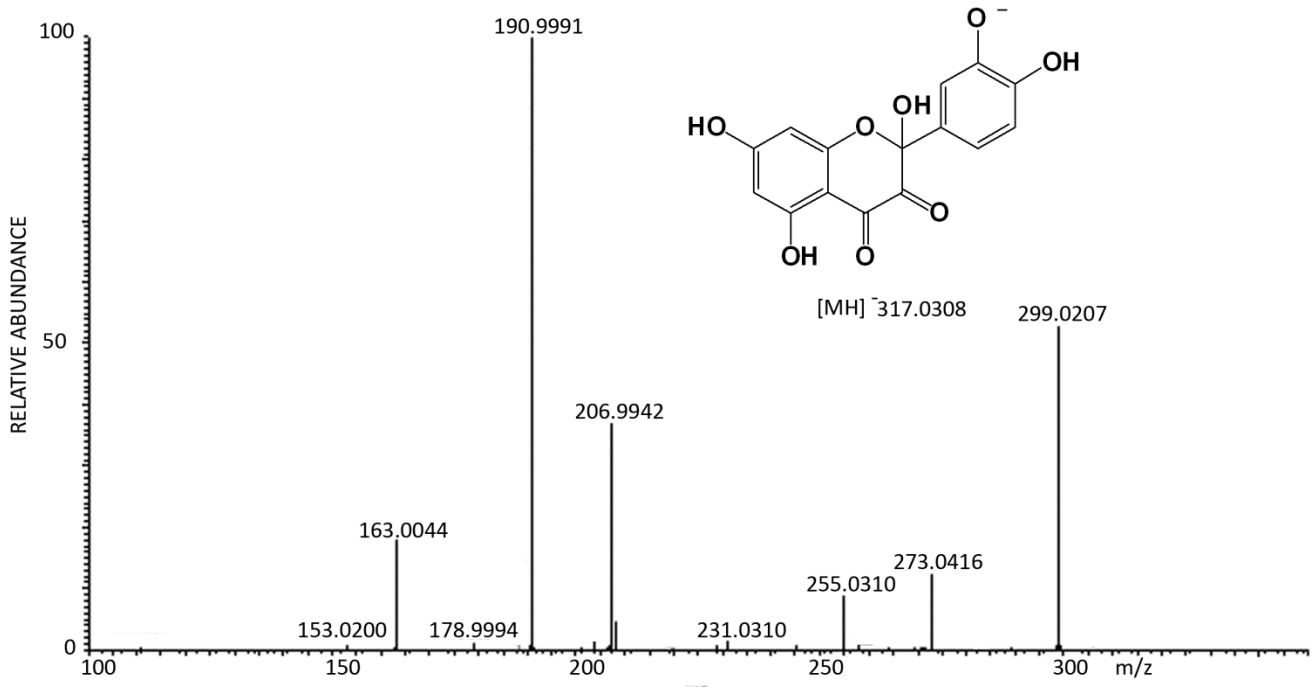

Figure 3 Proposed MS^2 (35eV) fragmentation pathways of the key intermediate D1 in quercetin degradation.

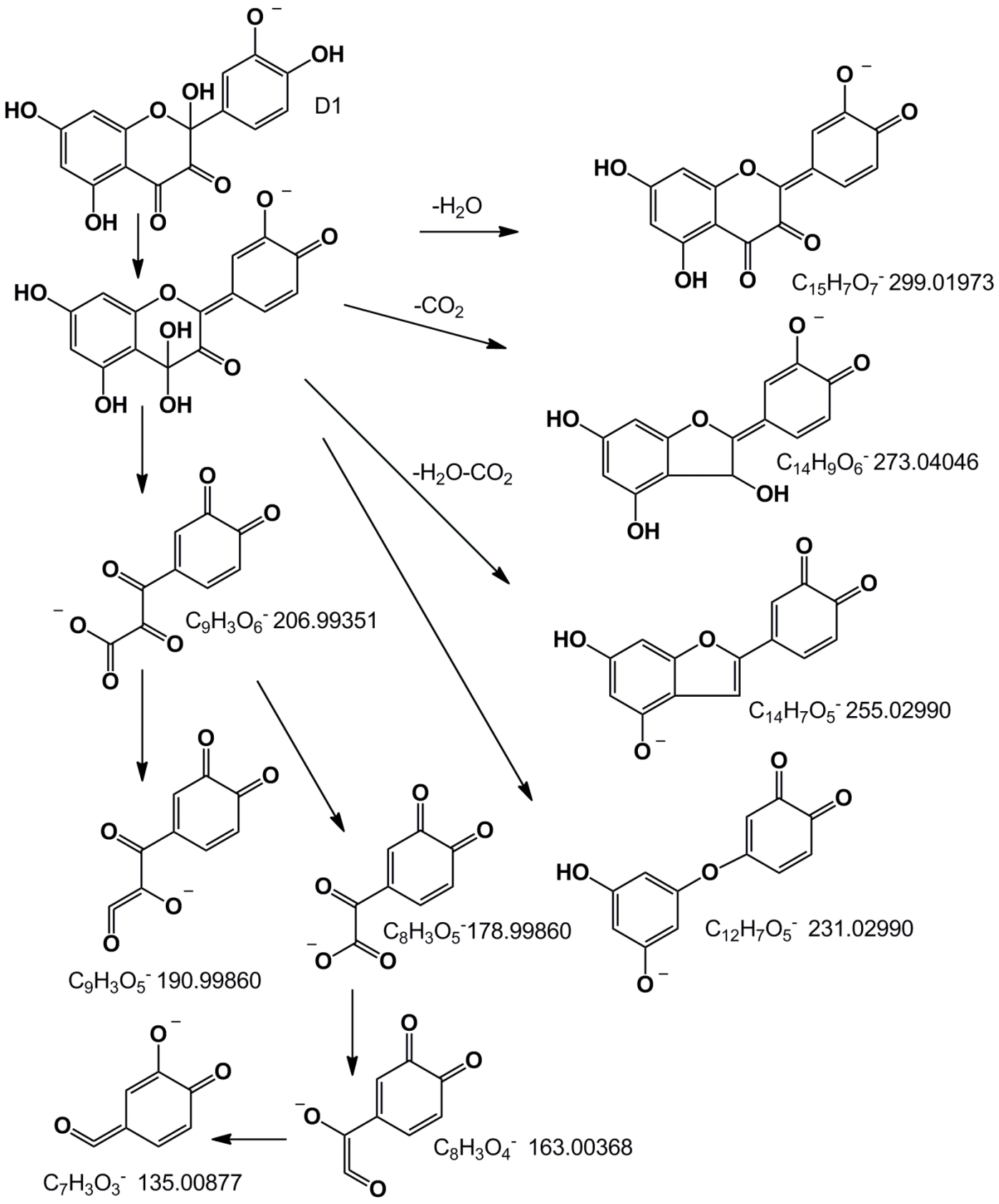

Figure 4 Proposed structures of the metabolites of quercetin formed in incubations with rat hepatocytes. Gluc = glucuronic acid.

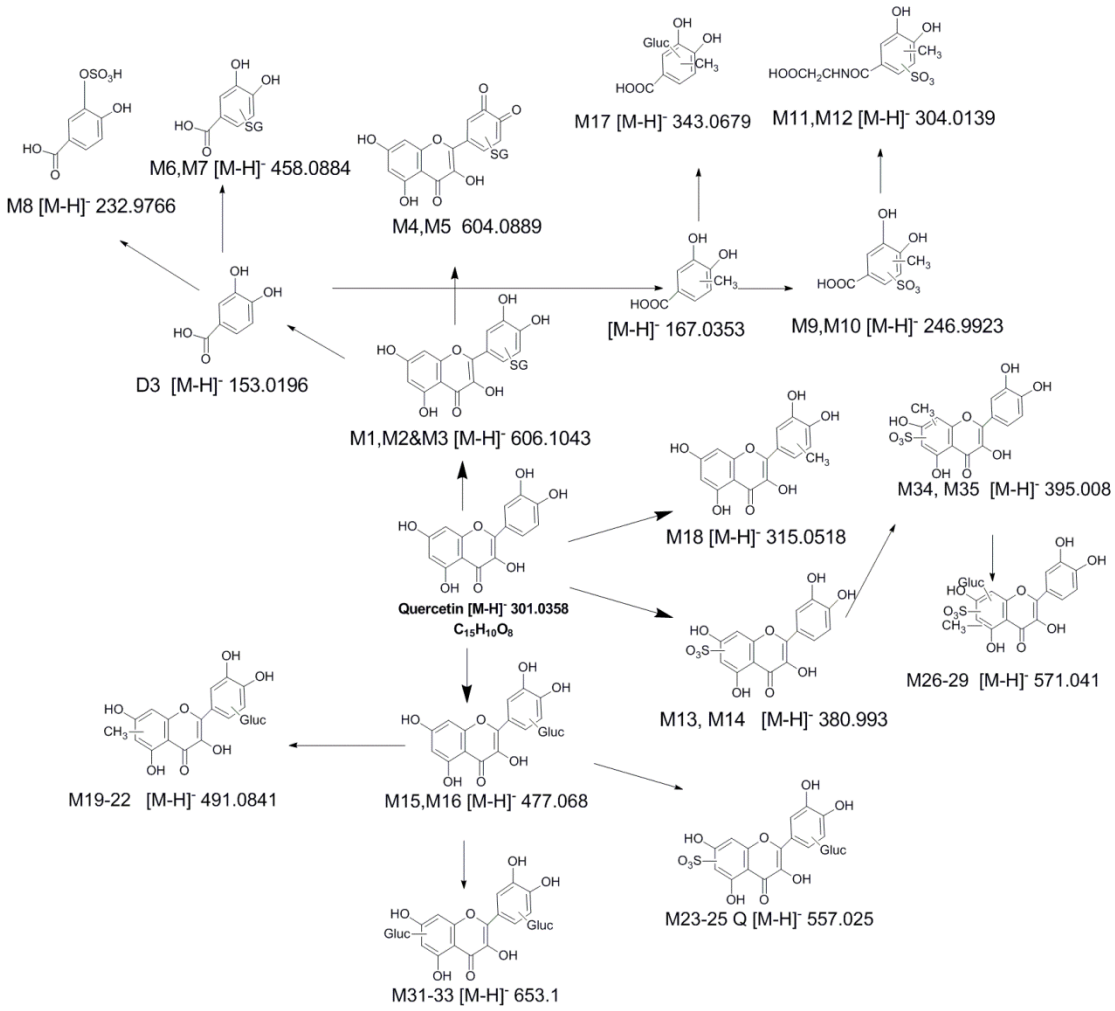

Figure 5 Extracted ion traces showing formation of GSH conjugates of quercetin in hepatocytes and ammonium bicarbonate solution.