Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1007 (2003) 21–29
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Detection and identification of ^{14}C-labelled flavonol metabolites by high-performance liquid chromatography–radiocounting and tandem mass spectrometry

William Mullen[a], Richard C. Hartley[b], Alan Crozier[a,*]

[a]*Plant Products and Human Nutrition Group, Graham Kerr Building, Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, UK*
[b]*Department of Chemistry, University of Glasgow, Glasgow G12 8QQ, UK*

Received 3 March 2003; received in revised form 6 May 2003; accepted 7 May 2003

Abstract

One hour after the ingestion of feed containing [2-^{14}C]quercetin-4′-*O*-β-D-glucoside, an extract from the gastrointestinal tracts of three rats was purified by partitioning and solid-phase extraction techniques. The resultant ethyl acetate and aqueous fractions were then analysed by reversed-phase HPLC with on-line radioactivity detection and ion trap mass spectrometry capable of performing data dependent MS–MS analysis. The presence of the ^{14}C-labelled metabolites was determined with the radioactivity monitor and the mass spectra produced were used to identify 16 of the 17 metabolites detected. These included methylation of the aglycone and the formation of mono and diglucuronides and sulfate conjugates. In the light of these findings, the use of mass spectrometry, operating in various modes, to identify flavonols and their metabolites in body tissues and fluids is discussed.

Keywords: Radiocounting detection; Detection, LC; Flavonols; Quercetin glucoside; Glucosides

1. Introduction

Flavonols, which in plants act as UV protectants [1] and are involved in pollen tube growth in the stigma [2,3], also function as dietary antioxidants [4,5]. Epidemiological studies have highlighted a positive correlation between high flavonol intake and a reduction in coronary heart disease [6,7].

Flavonols exist almost exclusively as sugar conjugates in plant-derived foods and beverages, and high-performance liquid chromatography (HPLC) is usually the method of analysis in quantitative studies. Unfortunately, very few indeed of the many hundreds of characterised flavonol conjugates are available from commercial sources for use as reference compounds. To overcome this problem, extracts are subjected to acid hydrolysis and the amounts of flavonol aglycones released, typically myricetin, quercetin, kaempferol and isorhamnetin, are quantified by reversed-phase HPLC with detection at 365 nm. This analytical approach, pioneered by Hertog and co-workers in the early 1990s, has been used to measure flavonol levels in a wide variety of fruits, vegetables and beverages [8–15]. Improved sen-

*Corresponding author. Tel.: +44-141-330-4613; fax: +44-141-330-5971.
E-mail address: a.crozier@bio.gla.ac.uk (A. Crozier).

0021-9673/03/$ – see front matter
doi:10.1016/S0021-9673(03)00882-3

sitivity and selectivity that is necessary for the analysis of flavonols in plasma, urine and body tissues can be achieved through the use of either electrochemical detection [16] or post-column chelation with methanolic aluminium nitrate to form highly fluorescent flavonol derivatives [17]. Irrespective of the detection system used, HPLC of hydrolysed extracts provides no information on the identity of the parent conjugate(s) and, therefore, provides quasiquantitative estimates of flavonol levels. Some investigators have used enzymes, such as glucosidases, glucuronidases and sulfatases, to release flavonol aglycones [16,18]. This suffers similar problems to acid hydrolysis and is compounded as the substrate specificities of the enzymes utilised are far from absolute and ensuring complete hydrolysis is not straightforward.

The HPLC and post-column derivatization of flavonols with methanolic aluminium nitrate referred to above provides sensitive and selective detection of flavonols with a free 3-hydroxyl group [17]. Using this method, Aziz et al. [19] detected quercetin-4′-glucoside and isorhamnetin-4′-glucoside in plasma and urine collected from human volunteers after the consumption of onions which contain high levels of both conjugates [20]. This observation, however, is not in keeping with proposals, based on in vitro studies, that during passage through the gut wall flavonol glucosides are subjected to the action of hydrolases and glucuronidases and as a consequence flavonol glucuronides rather than glucosides appear in the bloodstream [21]. It has been proposed by Sesink et al. [22] that the peak identified by Aziz et al. [19] as quercetin-4′-glucoside was a quercetin glucuronide although a subsequent publication by this group reveal that none of their available quercetin glucuronide conjugates co-chromatograph with quercetin-4′-glucoside [23]. Whether the peak in question is a quercetin glucoside or a glucuronide is very much a matter of conjecture. However, there is one clear way to severely limit disputes about analytical accuracy and prevent protracted discussions about whether an HPLC peak has been misidentified, that is to use a detector that provides structural information on the compounds under investigation. This can be achieved through the use of tandem mass spectrometry (MS–MS). MS–MS is an extremely powerful and flexible tool for the analysis of trace levels of natural products in impure extracts as interpretation of fragmentation patterns, coupled in some instances with knowledge of HPLC retention properties, can facilitate the partial identification of components where reference compounds are not available [24,25].

In this report we demonstrate the use of HPLC–MS–MS in the identification of 16 intestinal metabolites of [2-^{14}C]quercetin-4′-glucoside from rats. Samples were analysed by reversed-phase HPLC with an on-line radioactivity monitor in series with a tandem mass spectrometer operating in the full scan mode. Full details of the biological, rather than analytical aspects of this study, in which radiolabelled components in plasma, liver, kidneys and other organs were investigated are reported elsewhere [26].

2. Experimental

2.1. Synthesis of [2-^{14}C]-4′-O-β-D-glucoside

[2-^{14}C]Quercetin-4′-*O*-β-D-glucoside (specific activity 3.75 mCi mmol^{-1}) was synthesized in four steps from barium [^{14}C]carbonate by the method we have previously reported for the synthesis of [2-^{13}C]quercetin-4′-*O*-β-D-glucoside [27] except that the intermediate ester was not purified by filtration through alumina. The compound was pure by ^{1}H nuclear magnetic resonance (NMR) spectroscopy and only one radioactive species, that co-chromatographed with quercetin-4′-glucoside, was detected by HPLC–radiocounting (RC).

2.2. Animals and sample preparation

After an overnight fast, three male rats of the Rowett Hooded Lister strain (mean mass 430±4 g) were each offered 1 g of stock rat feed (CRM, Special Diet Services, Witham, UK) containing 3 mg of [2-^{14}C]quercetin-4′-glucoside to give a dose of $58.6 \cdot 10^{6}$ dpm. Rats consumed 99.3% of the ration within 2 min. After 60 min, animals were terminally anaesthetised with isofluorane and their gastrointestinal tract, comprising the stomach, small and large intestines and their contents, removed. The intestines were frozen in liquid nitrogen and lyophilized after

which they were ground to a powder using a mortar and pestle and stored at −80 °C prior to extraction and fractionation.

2.3. Extraction and fractionation of samples

A 1.0-g aliquot of freeze–dried intestine was extracted by continuous shaking with 15 ml of 50% methanol in 0.1 *M* phosphate buffer (pH 7.0) containing 20 m*M* sodium diethydithiocarbamate. After 30 min the mixture was centrifuged at 2000 *g* for 20 min. The methanolic supernatant was decanted and the pellet re-extracted a further two times. The three methanolic supernatants were combined and the methanol removed in vacuo. The remaining aqueous phase was adjusted to pH 3.0 and partitioned three times with an equal volume of ethyl acetate. The ethyl acetate extracts were combined and reduced to dryness in vacuo prior to the measurement of radioactivity and HPLC–MS–MS analysis. A gentle stream of nitrogen was used to remove residual ethyl acetate from aqueous phase before it was loaded onto a 2 g C_{18} Sep-Pak cartridge (Waters, Milford, MA, USA) which was then washed with 15 ml of distilled water adjusted to pH 3.0 with 5% H_2SO_4. The cartridge was then eluted with 30 ml of methanol to remove polar radiolabelled metabolites. The methanolic extract was dried in vacuo and aliquots taken for radioactivity measurements and analysis by HPLC–MS–MS.

2.4. HPLC with diode array, radioactivity and MS–MS detection

Samples were analysed on a P4000 liquid chromatograph fitted with an AS 3000 autosampler and with the initial detection by a UV6000 diode array absorbance monitor scanning from 250 to 700 nm (Thermo Finnigan, San Jose, CA, USA). Separations were carried out using a 240×4.6 mm I.D., 4 μm Synergi Max-RP column (Phenomenex, Macclesfield, UK), maintained at 40 °C, and eluted with a 60 min gradient of 5–40% acetonitrile in 1% formic acid at a flow-rate of 1 ml min^{-1}. After passing through the flow cell of the absorbance monitor, the column eluate was directed to a radioactivity monitor (Reeve Analytical Model 9701, Lab Logic, Sheffield, UK) fitted with a 500 μl heterogeneous flow cell packed with cerium-activated lithium glass scintillant [28], after which it was split and 50% of the eluate directed to a Finnigan LCQ Duo mass spectrometer with an electrospray interface in the negative ion mode operating in full scan data dependent MS–MS mode from 100 to 2000 u. Mass spectrometer conditions: capillary temperature 270 °C, sheath gas flow 80 units, auxillary gas flow 15 units, source voltage 4.5 kV.

3. Results

An aliquot of the feed was extracted with methanol and analysed by HPLC–RC–MS–MS which demonstrated that the [2-^{14}C]quercetin-4′-glucoside was radiochemically pure. Full scan MS analysis showed a molecular ion ($[M\text{-}H]^-$) at m/z 463 and an ion at m/z 465 from the ^{14}C label was also present. Calculated abundance of the ^{14}C fragment was 11.4% from the specific activity of the label and this is in keeping with the relative abundance of the m/z 463 and 465 ions. MS–MS was carried out on the m/z 463 ion rather than the smaller m/z 465 fragment. This yielded a secondary mass spectrum (MS^2) with the major ion at m/z 301, which corresponds to the aglycone quercetin.

The sample of rat intestines and contents, obtained 60 min after dosing, contained 88.7% of the ingested radioactivity. When fractionated 92% of the recovered radioactivity was associated with the ethyl acetate extract and 8% with the aqueous fraction. Aliquots of both extracts, each containing $30{\cdot}10^3$ dpm of radioactivity were analysed by reversed-phase HPLC with on line radioactivity detection and data dependent full scan MS–MS. The radioactivity and $A_{365\ nm}$ chromatograms obtained are illustrated in Fig. 1. In total there are 18 significant radioactive peaks and it can be seen that not all the peaks in the $A_{365\ nm}$ traces are radiolabelled components. The mass spectral data, which are summarised in Table 1, facilitated the identification of 17 of the 18 radiolabelled peaks. The relative amounts of these compounds, based on the amount of radioactivity in each peak, are also shown in Table 1.

Peak 11 which accounts for 26.2% of the radioactivity in the intestine is the residual [2-^{14}C]quercetin-

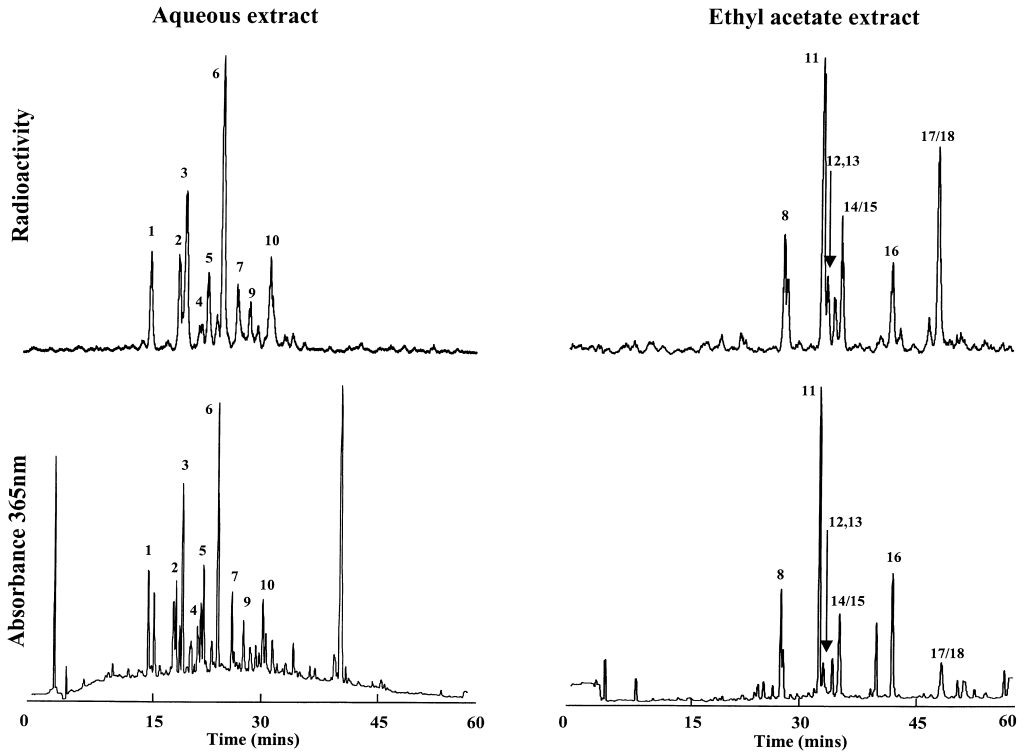

Fig. 1. Gradient reversed-phase HPLC of aqueous and ethyl acetate extracts from rat intestines 1 h after the ingestion of [2-^{14}C]quercetin-4′-glucoside. Samples, $30{\cdot}10^3$ dpm, separated on a 250×4.6 mm I.D., 4 μm Synergi Max-RP column at 40 °C and eluted at 1.0 ml min^{-1} with a 60 min gradient of 5–40% acetonitrile in water containing 1% formic acid using a diode array detector at 365 nm and a radioactivity monitor. Numbered peaks represent components subsequently analysed by MS–MS with an electrospray interface in the negative ion mode—see Table 1.

4′-glucoside characterised by a $[M\text{-}H]^-$ at m/z 463 which yields a MS^2 fragment at m/z 301 which corresponds with quercetin and the 162 u loss due to cleavage of the glucosyl moiety (Table 1). Table 1 also contains mass spectra information on the radiolabelled peaks all of which were identified with the exception of peak 2. The identified metabolites included the aglycone quercetin (peak 17, $[M\text{-}H]^-$ at m/z 301, with MS^2 fragment ions at m/z 179, 151) together with a combination of methylated, sulfated and glucuronide derivatives of quercetin. Peak 18 was quercetin sulfate with a $[M\text{-}H]^-$ at m/z 381, which produced an MS^2 at m/z 301 (loss of 80 u corresponds to loss of sulfur trioxide from a sulfate group). Peaks 8 and 15 were quercetin glucuronides with a $[M\text{-}H]^-$ at m/z 477, which fragments with loss of 176 u, corresponding to loss of a glucuronyl unit, yielding an MS^2 ion at m/z 301. Peak 8 co-chromatographed with a quercetin-3-glucuronide standard. Four quercetin diglucuronides, peaks 1, 4, 6 and 7, were detected. All were characterised by a $[M\text{-}H]^-$ at m/z 653 and $[M\text{-}H]^-$ −176 MS^2 fragments at m/z 477 and 301. Peak 9 was a quercetin glucuronide sulfate with a $[M\text{-}H]^-$ at m/z 557 and MS^2 ions at m/z 477 (loss of sulfur trioxide), 381 (loss of glucuronyl unit) and 301 (loss of sulfur trioxide and glucuronyl units).

A number of methylated quercetin derivatives were also characterised. Peak 17, for instance, was a methylquercetin sulfate, characterised by the same fragmentation as peak 18 but at m/z values 14 u higher. Peaks 12–14 were all methylquercetin glucuronides with a $[M\text{-}H]^-$ at m/z 491, which produced an MS^2 at m/z 315 corresponding to methylquercetin through cleavage of the glucuronyl unit. Peak 10 which had a $[M\text{-}H]^-$ at m/z 571 that yielded MS^2

Table 1
Identification of metabolites of [2-^{14}C]quercetin-4′-glucoside

Peak	t_R (min)	% Total	Compound	[M-H]$^-$ (m/z)	MS^2 fragment ions (m/z)
1	16.7	0.8	Quercetin diglucuronide	653	477 ([M-H]$^-$-GlcUA), 301 [Q]([M-H]$^-$-GlcUA-GlcUA)
2	20.6	0.8	Unknown		
3	21.6	1.3	Methylquercetin diglucuronide	667	491 ([M-H]$^-$-GlcUA), 315 [MQ]([M-H]$^-$-GlcUA-GlcUA)
4	23.7	0.2	Quercetin diglucuronide	653	477 ([M-H]$^-$-GlcUA), 301 [Q]([M-H]$^-$-GlcUA-GlcUA)
5	24.3	0.6	Methylquercetin diglucuronide	667	491 ([M-H]$^-$-GlcUA), 315 [MQ]([M-H]$^-$-GlcUA-GlcUA
6	26.4	2.5	Quercetin diglucuronide	653	477 ([M-H]$^-$-GlcUA), 301 [Q]([M-H]$^-$-GlcUA-GlcUA)
7	28.3	0.6	Quercetin diglucuronide	653	477 ([M-H]$^-$-GlcUA), 301 [Q]([M-H]$^-$-GlcUA-GlcUA)
8	29.6	10.6	Quercetin-3-glucuronide	477	301 [Q]([M-H]$^-$-GlcUA)
9	30.3	6.9	Quercetin glucuronide sulfate	557	477 ([M-H]$^-$-SO_3), 381 ([M-H]$^-$-GlcUA), 301 [Q]([M-H]$^-$-SO_3-GlcUA)
10	32.6	0.7	Methylquercetin glucuronide sulfate	571	491 ([M-H]$^-$-SO_3), 315 [MQ]([M-H]$^-$-SO_3-GlcUA)
11	34.5	26.2	Quercetin-4′-glucoside	463	301 [Q]([M-H]$^-$-Glc)
12	35.3	6.5	Methylquercetin glucuronide	491	315 [MQ]([M-H]$^-$-GlcUA)
13	36.5	2.5	Methylquercetin glucuronide	491	315 [MQ]([M-H]$^-$-GlcUA)
14	37.4	2.4	Methylquercetin glucuronide	491	315 [MQ]([M-H]$^-$-GlcUA)
15	37.4	12.2	Quercetin glucuronide	477	301 [Q]([M-H]$^-$-GlcUA)
16	44.6	7.2	Quercetin	301	179, 151
17	51.1	8.9	Methylquercetin sulfate	395	315 [MQ](M-H]$^-$-SO_3)
18	51.2	8.9	Quercetin sulfate	381	301 [Q]([M-H]$^-$-SO_3)

HPLC–RC retention times, negative ion MS–MS fragmentation patterns and identities of metabolites detected in rat tissues 60 min after oral ingestion of [2-^{14}C]quercetin-4′-glucoside. Peak numbers refer to peaks in Fig. 1.
Q—Quercetin, MQ—methylquercetin, GlcUA—glucuronyl unit; Glc—glucosyl unit; [M-H]$^-$—molecular ion. Concentrations of co-eluting peaks were calculated using the signal intensity from the mass spectrometer, it was assumed that all metabolites gave an equal response.

fragment ions at m/z 491 (loss of sulfur trioxide) and 315 (a loss of 176 u corresponding to glucuronyl unit) was a methylquercetin glucuronide sulfate. Finally peaks 3 and 5 were methylquercetin diglucuronides with a [M-H]$^-$ at m/z 667 and MS^2 ions at m/z 491 ([M-H]$^-$ −176, loss of a glucuronyl unit) and 315 ([M-H]$^-$ −352, cleavage of two glucuronyl moieties) (Table 1).

4. Discussion

This is the first study in which the extensive and varied metabolism of quercetin-4′-glucoside, or indeed any other flavonol, has been revealed by full scan MS–MS. The sample analysed showed 73.8% of the original compound has been deglycosylated and further metabolised by varying combinations of methylation, glucuronidation and/or sulfation. Full scan MS–MS proved suitable for the identification of all of these metabolites. Further information about the position of the various substituent groups could be achieved by the use of NMR. However, NMR requires not only high sample purity but also the presence of substantially more analyte than the low-ng quantities required for MS–MS.

HPLC linked to tandem mass spectrometry is becoming more widely available and provides the most powerful tool currently available for the identification of flavonol conjugates in biological samples. However, a mass spectrometer can be used in many ways depending on the circumstances of the analysis and the samples under study. At one extreme, to increase the sensitivity and selectivity of the analysis, the mass spectrometer can be set up to detect only one ion, selected ion monitoring (SIM) mode. Alternatively when limited availability of sample is not a problem, full scan analysis can provide detailed structural information at the expense of sensitivity. Selected reaction monitoring (SRM) is another mode of analysis that offers high selectivity and sensitivity. However, SRM can only be carried out with triple quadrupole or ion trap mass spectrometers. With an ion trap mass spectrometer, like the instrument used in the current study, the parent ion of the compound of interest is ‘‘trapped’’ and all other ions excluded. The ‘‘trapped’’ ion is then fragmented and a specific fragment ion monitored. In the case of a quercetin

monoglucuronide, the mass spectrometer would be set up, in negative ion mode, to detect the m/z 301 quercetin ion resulting from the fragmentation of the $[M–H]^-$ at m/z 477. Wittig et al. [29] used this method to identify five quercetin monoglucuronide metabolites in human plasma after ingestion of an 800 g onion supplement. The limitation with SRM is that it can only be used to detect what you already know or suspect to be present in a sample. It is not suitable for the determination of novel metabolites.

Using a triple quadrupole mass spectrometer set up in the positive ionisation mode, Day and Williamson [23] employed LC–MS, as opposed to MS–MS, to identify metabolites of quercetin in human plasma after the consumption of a more manageable 200 g of onions. However, this study did not produce full scan mass spectra but used the SIM data to support identifications based on chromatographic retention times and enzyme hydrolysis data. In the SIM mode the mass spectrometer was set up to monitor four ions per analysis. Although a total of 12 putative quercetin glucuronide peaks (P1–P12) were suspected to be present on the basis of HPLC–$A_{365\ nm}$ traces and enzyme hydrolyses, the presence of only three was confirmed by SIM. From our findings one of the compounds in the group P1–P3 may be a methylquercetin diglucuronide. However, the molecular ion of this compound was not monitored so, if present, it went undetected. In addition as methylquercetin glucoside has the same molecular ion as quercetin glucuronide, the distinct possibility of a misidentification by SIM exists. Choice of the ionisation mode is critical in any mass spectrometric analysis. With our ion trap mass spectrometer the limit of detection for flavonols is at least an order of magnitude better in negative ion mode than positive ionisation and absorbance peaks as small as 3 mAU routinely provide full scan MS–MS data.

Analysis by SIM is much less selective than SRM and more likely to produce false positive identifications. Conversely, the secondary fragmentation that operates with both SRM and full scan MS–MS enables identifications to be made that would be missed or be more doubtful when analysed by SIM. The extracted ion chromatograms in Fig. 2, which are taken from the 20–50 min HPLC full scan MS analysis of the ethyl acetate extract in Fig. 1, is the equivalent of what is produced in a SIM analysis. It

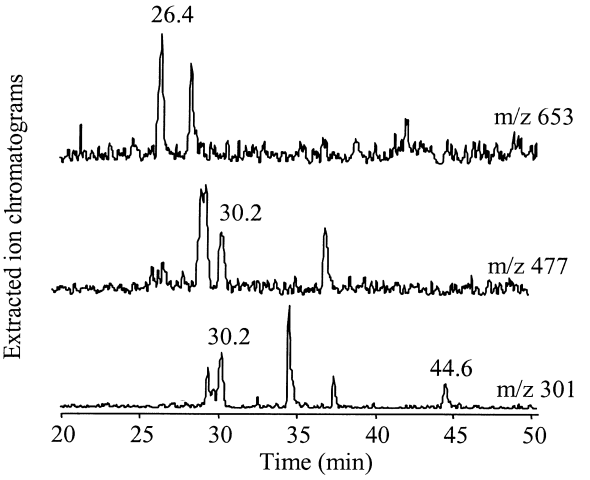

Fig. 2. Gradient reversed-phase HPLC of a $30{\cdot}10^3$ dpm ethyl acetate extract of rat intestine as described in Fig. 1 but with detection by tandem mass spectrometry with an electrospray interface operating in the negative ion mode. Traces show extracted ion chromatograms of the $[M-H]^-$ of quercetin diglucuronide (m/z 653), quercetin glucuronide (m/z 477) and quercetin (m/z 301).

can be used to show some of the limitations of SIM analysis when compared to MS–MS. The m/z 653 trace, the $[M-H]^-$ of quercetin diglucuronide, shows only a weak peak at t_R 26.4 min and no fragment peaks are present at either m/z 477, the monoglucuronide or m/z 301, the aglycone (Fig. 2). This would be poor evidence for the presence of a quercetin diglucuronide. However, the full scan MS–MS spectrum of this ion showed that it is indeed a quercetin diglucuronide (Table 1, peak 6). At the other extreme, the ion traces from m/z 477 and 301 indicate the presence of a quercetin-based glucuronide at t_R 30.2 min (Fig. 2). However, full scan MS–MS of the m/z 301 fragment revealed that this was not the case as it a yielded an MS^2 spectrum with major ions at m/z 283 and 265 which is quite distinct from the MS^2 spectrum of quercetin which has prominent ions at m/z 179 and 151. In contrast, MS–MS of the small m/z 301 peak at t_R 44.6 min (Fig. 2) yielded an MS^2 spectrum with major fragment ions at m/z 179 and 151, confirming the presence of free quercetin.

The mass spectrometric method used in this study is known as data dependent MS–MS. The ion trap is set up to select the most significant ion in each full

scan, isolate it from all the other ions, and fragment it yielding a secondary mass spectrum The secondary spectrum, therefore, contains only ions originating from the ''trapped'' parent ion. A further refinement to this analytical technique is the ability to program the detector to only look at the same ion a limited number of times (e.g., three) over a set time window, (e.g., 30 s) depending on the peak width. This enables full scan MS–MS of individual components in merged peaks to be obtained, as seen in peaks 14 and 15 and also minor peaks in the presence of larger ones, as in peak 13 on the shoulder of peak 12 (Fig. 1). A further advantage is that the system can be programmed not to carry out MS–MS on background ions so that ''bleed'' from the column or the mobile phase can also be rejected from the analysis.

Our study illustrates the power and simplicity of MS–MS analysis for the partial identification of trace levels of natural products. This point is well illustrated with the analysis of peak 7 where an uninformative full scan mass spectrum (Fig. 3A) yielded a clean secondary spectrum (Fig. 3B). The primary mass spectrum, equivalent to that obtained by single stage MS, contained numerous ions and has no diagnostic value. However, fragmentation of the m/z 653 ion produced a daughter spectrum comprised of ions at m/z 477 ($[M\text{-}H]^-$ −176, loss of a glucuronyl unit) and m/z 301(quercetin, $[M\text{-}H]^-$ −176–176, loss of two glucuronyl units). All the fragments in a secondary spectrum must come from the parent ion and as a consequence the mass spectrum illustrated in Fig. 3B represents convincing

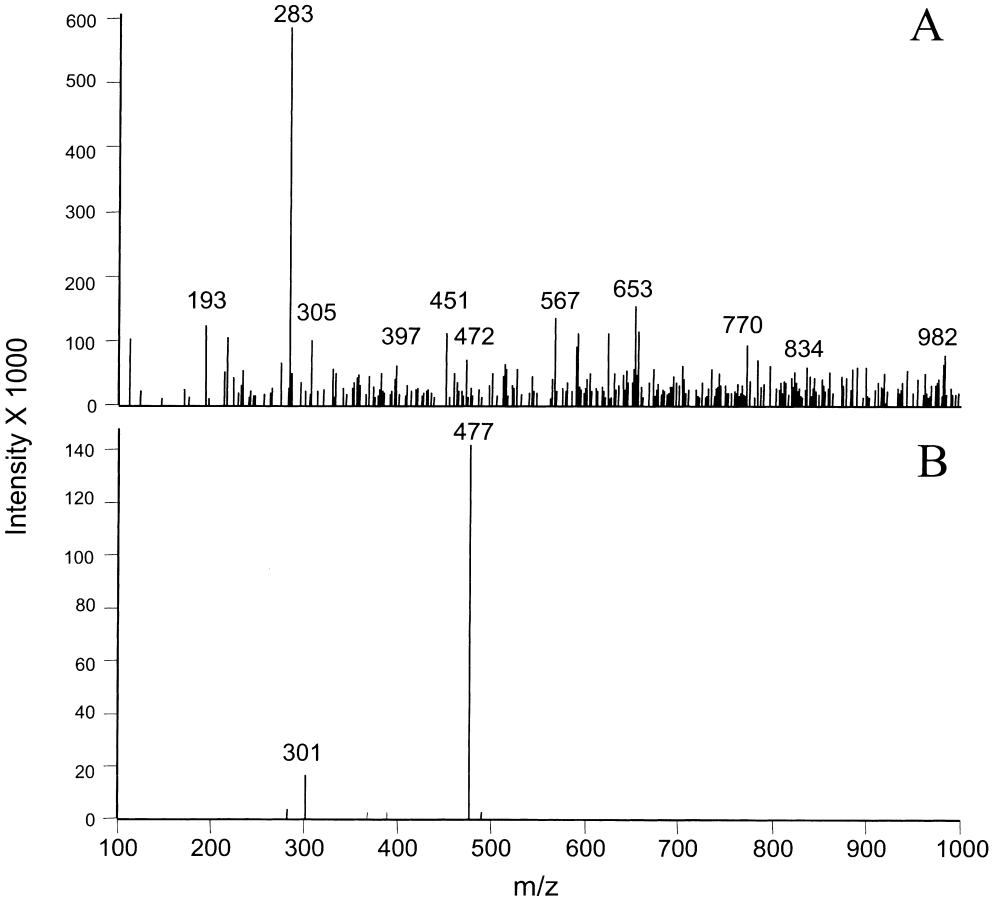

Fig. 3. (A) The parent negative ion mass spectrum obtained in the LC–MS–MS analysis of peak 7 (see Fig. 1). (B) The daughter negative ion mass spectrum obtained by fragmentation of m/z 653 in the parent mass spectrum.

evidence for the presence of a quercetin diglucuronide.

The value of HPLC with full scan MS–MS for the structural elucidation of metabolites has also been demonstrated in a recent report on the identification of 17 isoflavone conjugates in the urine of female rats fed on a soya-rich diet [30]. The daughter mass spectra obtained are in keeping with those obtained in the present study with the initial fragmentation resulting in cleavage of the bond linking the aglycone and the conjugating sugar as outlined by Giusti et al. [31]. However, this is not the case in the claimed HPLC–MS–MS identifications of an epicatechin glucuronide and 3′-*O*-methyl epicatechin glucuronide in rat brains after oral ingestion of high doses of (−)-epicatechin [32]. In this report by Abd El Mohsen et al. [32], the illustrated MS^2 spectra, in marked contrast to the spectrum in Fig. 3B, contained 20–30 unexplained ions, some at 10–20% relative abundance and one exceeding 50%. To ignore the presence of these ions and to base identifications on the presence of three or four known ions, some of very low relative intensity, is a highly subjective interpretation of mass spectral data. The unexplained ions in the MS^2 spectra of Abd El Mohsen et al. [32] may be due to the presence of sizable amounts of co-eluting compound(s) with the same molecular mass. This would mean that the quantitative estimates were inaccurate. An alternative explanation is that the mass spectrometer was either not set up correctly or was malfunctioning, plausible as both the MS^2 spectra contained several ions at higher m/z values than the cited fragmented parent ions. Whatever the reasons, it is clear that this work is not a good example of the use of HPLC–MS–MS for the identification of flavonoid metabolites. In contrast, the studies of Wittig et al. [29] and Day et al. [23] and the data presented in this paper all demonstrate the value of various modes of mass spectrometric detection for the analysis of flavonols and their metabolites.

Acknowledgements

The authors wish to thank Stuart T. Caldwell (Department of Chemistry, University of Glasgow, Glasgow, UK) for the synthesis of [^{14}C]quercetin-4′-glucoside and Dr. Garry Duthie, Rowett Research Institute, Aberdeen, UK for carrying out the [^{14}C]quercetin-4′-glucoside feed to rats. We are also grateful to Alison Sutcliffe and Brigitte Graf for technical assistance with the extraction and purification. The sample of quercetin-3-glucuronide was generously supplied by Professor Garry Williamson, Food Research Institute, Norwich, UK. The LC–MS–MS system used in this study was purchased with a BBSRC grant to A.C. and J.R. Coggins.

References

[1] R.E. Koes, F. Quattrocchio, J.N.M. Mol, BioEssays 16 (1994) 123.
[2] Y. Mo, C. Nagel, L.P. Taylor, Proc. Natl. Acad. Sci. USA 89 (1992) 7213.
[3] T. Vogt, P. Pollak, N. Tarlyn, L.P. Taylor, Plant Cell 6 (1994) 11.
[4] F. Shadhihi, P.K. Wanadundara, Crit. Rev. Food Sci. Nutr. 32 (1992) 67.
[5] J.A. Vinson, Y.A. Dabbagh, M.M. Sherry, J. Jang, J. Agric. Food Chem. 43 (1995) 5754.
[6] M.G.L. Hertog, E.J.M. Feskens, P.C.H. Hollman, M.B. Katan, D. Kromhout, Lancet 342 (1993) 1007.
[7] P. Knegt, R. Jarvinen, A. Reuanen, Br. Med. J. 312 (1996) 478.
[8] M.G.L. Hertog, P.C.H. Hollman, D.P. Venema, J. Agric. Food Chem. 40 (1992) 1591.
[9] M.G.L. Hertog, P.C.H. Hollman, M.B. Katan, J. Agric. Food Chem. 40 (1992) 2379.
[10] M.G.L. Hertog, P.C.H. Hollman, B. Vande Pute, J. Agric. Food. Chem. 41 (1993) 1242.
[11] A. Crozier, M.E.J. Lean, M.S. McDonald, C. Black, J. Agric. Food Chem. 45 (1997) 590.
[12] M.S. McDonald, M. Hughes, J. Burns, M.E.J. Lean, D. Matthews, A. Crozier, J. Agric. Food Chem. 46 (1998) 368.
[13] J. Burns, P.T. Gardner, D.B. McPhail, J. O'Neil, S. Crawford, I. Morecroft, C. Lister, D. Matthews, M.R. MacLean, M.E.J. Lean, G.G. Duthie, A. Crozier, J. Agric. Food Chem. 48 (2000) 220.
[14] A.J. Stewart, S. Bozonnet, W. Mullen, G.I. Jenkins, M.E.J. Lean, A. Crozier, J. Agric. Food Chem. 48 (2000) 2663.
[15] C. Ewald, S. Fjelkner-Modig, K. Johansson, I. Sjöholm, B. Åkesson, Food Chem. 64 (1999) 231.
[16] I. Erlund, G. Alfthan, H. Siren, K. Ariniemi, A. Aro, J. Chromatogr. B 727 (1999) 179.
[17] P.C.H. Hollman, J.M.P. van Trijp, M.N.C. P Buysman, Anal. Chem. 68 (1996) 3511.
[18] P. Ader, A. Wessmann, S. Wolffram, Free Radic. Biol. Med. 28 (2000) 1056.
[19] A.A. Aziz, C.A. Edwards, M.E.J. Lean, A. Crozier, Free Radic. Res. 29 (1998) 257.

[20] T. Tsushida, M. Suzuki, Nippon Shokuhin Kagaku Kogaku Kaishi 42 (1995) 100.
[21] G. Williamson, A.J. Day, G.W. Plumb, D. Coteau, Biochem. Soc. Trans. 28 (2000) 16.
[22] A.L.A. Sesink, K.A. O'Leary, P.C.H. Hollman, J. Nutr. 131 (2001) 1938.
[23] A. Day, G. Williamson, Br. J. Nutr. 86 (2001) S105.
[24] J. Burns, W. Mullen, N. Landrault, P.-L. Teissedre, M.E.J. Lean, A. Crozier, J. Agric. Food Chem. 50 (2002) 4096.
[25] W. Mullen, J. McGinn, M.E.J. Lean, M.R. MacLean, P. Gardner, G.G. Duthie, T. Yokota, A. Crozier, J. Agric. Food Chem. 50 (2002) 5191.
[26] W. Mullen, B.A. Graf, S.T. Caldwell, R.C. Hartley, G.G. Duthie, C.A. Edwards, M.E.J. Lean, A. Crozier, J. Agric. Food Chem. 50 (2002) 6902.
[27] S.T. Caldwell, A. Crozier, R.C. Hartley, Tetrahedron 56 (2000) 4101.
[28] D.R. Reeve, A. Crozier, J. Chromatogr. 137 (1977) 217.
[29] J. Wittig, M. Herderich, E.U. Graefe, M. Veit, J. Chromatogr. B 753 (2001) 237.
[30] N. Fang, S. Yu, T.M. Badger, J. Agric. Food Chem. 50 (2002) 2707.
[31] M.M. Giusti, L.E. Rodriguez-Saona, D. Griffin, R.E. Wrolstad, J. Agric. Food Chem. 47 (1999) 4657.
[32] M.M. Abd El Mohsen, G. Kuhnle, A.R. Rechner, H. Schroeter, S. Rose, P. Jenner, C.A. Rice-Evans, Free Radic. Biol. Med. 33 (2002) 1693.