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Quantification of Free Coumarin and of Its Liberation From Glucosylated Precursors by Stable Isotope Dilution Assays Based on Liquid Chromatography-Tandem Mass Spectrometric Detection

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ABSTRACT

A stable isotope dilution assay for the quantification of free coumarin and glucosylated coumarin precursors has been developed by using [$^{13}C_2$]-coumarin as the internal standard. The doubly labelled coumarin was synthesized by reacting [$^{13}C_2$]-acetic anhydride with salicylic aldehyde and characterized by means of mass spectrometry and NMR experiments.The specifity of liquid chromatography-tandem mass spectrometry enabled unequivocal determination and sensitive quantitation of the odorant. Due to the very simple extraction procedure, free coumarin could be analysed within one hour. For quantification of total coumarin, the odorant was liberated from its precursors by an incubation with hydrochloric acid or β-glucosidase. In analyses of breakfast cereals, the intra-assay coefficient of variation was 9.9 % (n= 5) for total coumarin. When coumarin was added to butter cookies at a level of 10 µg/kg, a recovery of 94.1 % was found. Further addition studies revealed a detection limit of 2.9 µg/kg and a quantification limit of 8.6 µg/kg. Application of the stable isotope dilution assay to several plants, foods and essential oils revealed high contents in cassia products and those foods in which cassia has been used as an ingredient. In contrast to this, Ceylon cinnamon contained much less coumarin. The odorant was also quantified in woodruff, clover seeds, and the essential oils of lavender, citron, and chamomile. Only trace amounts were detected in carrots and the essential oils of peppermint and dill, whereas in bilberries, black raspberries and Angelica roots coumarin was below detectable levels. In Ceylon cinnamon and cassia the odorant occurred mainly in its free form, whereas in fenugreek seeds and woodruff 68% and 88% of the total coumarin content was liberated from glucosylated precursors, respectively.

Key words: cassia, cinnamon, coumarin, glucosides, liquid chromatography – tandem mass spectrometry, stable isotope dilution assay

INTRODUCTION

The odorant coumarin is a natural component of several spices, the respective essential oils, and other flavoring foods such as *Cinnamomum aromaticum* (cassia bark), *Asperula odorata* (sweet woodruff), *Dipterix odorata* (tonka bean), and species of clover. Besides occurring naturally in these foods, coumarin has been widely used as a flavoring compound due to its sweet and aromatic odor. However, since the early 1950s, the odorant has been found to exert hepatotoxicity and was suspected to be mutagenic and carcinogenic *(1)*. Although coumarin in humans is mainly metabolized to 7-hydroxycoumarin *(2)*, a subpopulation lacks this detoxification pathway and, by contrast, metabolizes the odorant to its 3,4-epoxy derivative, which was suspected to form DNA adducts and may react to hepatotoxic *o*-hydroxyphenylacetaldehyde *(3)*. Whereas the former reaction was found not to be the cause for carcinogenic effects in rodents, the latter product was confirmed to evoke hepatotoxicity by coumarin *(4)*. For these reasons a maximum level of 2 mg/kg for foods generally and 10 mg/kg in alcoholic beverages has been set in the European Union *(5)*. Moreover, coumarin is not allowed to be used as flavoring additive to foods.

In the last decades cassia bark increasingly substituted true cinnamon in baked goods, particularly in seasonal products such as gingerbread or cinnamon star cookies. Moreover, indications to use cassia bark powder as a supplement and remedy against type 2 diabetes mellitus *(6)* increased consumption of this spice in the Western countries. As a tolerable daily intake of 0.1 mg/kg body weight has been established by the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC) *(7)*, a longer lasting consumption of products high in cassia can be expected to provoke hepatotoxic effects. Therefore, supplements containing cassia have been classified as drugs and coumarin provisionally had been restricted to 67 mg/kg in cinnamon star cookies and to 50 mg/kg in gingerbread in Germany during the winter season in 2006 until November 1st . Moreover, the consumption of cinnamon star cookies by children had been recommended not to exceed four cookies per day.

In view of these concerns, analytical methods are required for accurate and sensitive quantitation of coumarin in baked goods or spices.The most frequently used method for quantifying coumarin is a HPLC assay with UV detection *(8, 9)*.However, as the detection limit of HPLC has been reported to be as high as approximately 2 mg/kg *(10)*, the latter method appeared not sensitive enough to verify the compliance of foods with the legal limit of 2 mg/kg. Moreover, complex matrices such as baked goods require more accurate methodologies for quantitation. GC-MS methods are also known *(11)*, but have been little used until now due to the need for extraction with organic solvents and for separating the odorant from non-volatile matrix compounds (*12*).

For a convenient cleanup and unambigueous detection of many low-volatile compounds, LC coupled to mass detection has gained increasing importance. However, it is generally accepted that clean-up is likely to cause losses of the analyte and ionization efficiency in LC-MS strongly depends on coeluting matrix compounds *(13)*. Therefore, quantitation of food samples is more accurate if an internal standard (IS) is used, which has very similar chemical and physical properties and behaves nearly identically throughout the whole analytical procedure. Therefore, stable isotopologues of the analytes are considered the best IS in LC-MS. Since we reported on the quantitation of vitamins *(14)* and trichothecene mycotoxins *(15),* the use of labelled analogues furthermore allows compensation for losses and enables the most accurate quantitation.

Therefore, the aim of the present investigation was to synthesize an isotopologue of coumarin, and to apply it as internal standard (IS) for quantitation of coumarin using LC-MS detection. Furthermore, as coumarin is known to occur in some plants mainly bound as a glucosylated precursor, an additional aim of this study was to quantify bound coumarin as well.

MATERIALS AND METHODS

Chemicals

The following chemicals were obtained commercially: [$^{13}C_2$]-acetic anhydride, chloroform, coumarin, salicylic aldehyde (Aldrich, Steinheim, Germany); acetonitrile, $CaCl_2$, diethyl ether, formic acid, hydrochloric acid, methanol, pentane, sodium sulfate, sulfuric acid (Merck, Darmstadt, Germany); β-glucosidase from almonds (Sigma, Deisenhofen, Germany).

Synthesis of [$^{13}C_2$]-coumarin

[^{13}C]-labelled coumarin was prepared by a modification of the synthetic procedure to unlabelled coumarin by Perkin *(16)*. Salicylic aldehyde (30 mg, 246 µmol), aqueous sulfuric acid (60% w:w, 1 mL), and [$^{13}C_2$]-acetic anhydride (13 mg, 123 µmol) were mixed in a closable vial, the latter was purged with nitrogen and heated for 6 h at 150 °C. Subsequently, water (2 mL) was added to the mixture, which was then transferred in a separation funnel. The resulting solution was extracted with chloroform (3 x 5 mL) and the organic phases were dried over anhydrous sodium sulfate. After evaporating the solvent, the residue was dissolved in diethyl ether (1 mL) and purified by preparative thin layer chromatography (TLC) using silica gel with fluorescence detection as the stationary phase (µm, Merck, Darmstadt, Germany) and a mixture of diethyl ether and pentane (1:1, v:v) as the mobile phase. Three fractions at R_F of 0.45, 0.56, and 0.83 were visible on the TLC plate and the zone with R_F = 0.45, representing [$^{13}C_2$]-coumarin, was scratched from the plate. The resulting powder was suspended in diethyl ether (2 mL) and the suspension was filtered yielding the pure product. The two fractions at R_F 0.56 and 0.83 were suspended in water (5 mL), the suspension filtered and, after addition of sulfuric acid (60% w:w, 1mL), the resulting solution heated for 5 h at 150 °C. Separation of [$^{13}C_2$]-coumarin was performed as described above and this procedure repeated another two times until no product was generated. Purity of the product (4.2 mg; 11.5 %) was checked by GC-MS and LC-MS/MS.

Mass spectra in electron impact ionization and positive electrospray LC-MS/MS are shown in **Figures** 1A and 2B, respectively.

^{13}C-NMR ($CDCl_3$): δ=116.1 (d, $^{1}J_{CC}$ 70.2, C-3), 166.2 (d, $^{1}J_{CC}$ 71.2, C-2).

^{1}H-NMR ($CDCl_3$): δ=6.42 (ddd, $^{1}J_{HC}$ 170, $^{2}J_{HC}$ 8, $^{3}J_{HH}$ 10, H-3), 7.33 (m, H-5 – H-8), 7.60 (m, H-5 – H-8), 7.93 (dd, $^{2}J_{HC}$ 7, $^{3}J_{HH}$ 10, H-4).

Stable isotope dilution assay (SIDA) for the determination of free coumarin in foods

Baked goods, spices or herbs were minced in a blender (Privileg, Quelle, Fürth). Samples (0.1 g) with considerable amounts of glucosylated coumarin were homogenized in a mixture of methanol/ saturated $CaCl_2$ (2 mL, 80:20, v:v) by means of an Ultraturrax. The resulting powders (0.5 g – 0.01g) or homogenates (2 g) were stirred for 1 h at 20 °C in aqueous methanol (80%, 5 mL) or a mixture of methanol/ saturated $CaCl_2$ (80:20, v:v, 5 mL), respectively, containing [$^{13}C_2$]-coumarin (20 ng – 10 µg).

The extracts were filtered and, after passing through a 0.4 µm syringe filter (Millipore, Bedford, MA, USA), analyzed by LC-MS/MS.

Hydrolysis of glucosides for quantification of total coumarin

For liberation of bound coumarin precursors, the homogenized samples were stirred either in hydrochloric acid (2.5 mol/L, 5 mL) at 80 °C for 90 min or in a solution of β-glucosidase from almonds (1 mg/mL, 5 mL) at 37 °C for 60 min. Subsequently, the extracts were filtered and, after passing through a 0.4 µm syringe filter (Millipore, Bedford, MA, USA), analyzed by LC-MS/MS

Liquid chromatography / tandem mass spectrometry (LC-MS/MS)

LC-MS/MS was performed by means of a triple quadrupole Finnigan TSQ Quantum Discovery (Thermo Electron Corporation, Waltham, USA) coupled to a Finnigan Surveyor Plus HPLC System (Thermo Electron Corporation, Waltham, USA) equipped with a 150 x 2 mm i. d., 5 µm, Aqua C-18 reversed phase column (Phenomenex, Aschaffenburg, Germany). 10 µL of the sample solutions were chromatographed using gradient elution with variable mixtures of aqueous formic acid (0.1%, eluent A) and formic acid in acetonitrile (0.1%, eluent B), at a flow of 0.2 mL/min. A 20-min linear gradient was programmed from 0 to 100% B. Then, 100% B was maintained for 3 min and subsequently brought back within 1 min to 0 % B and held for another 15 min to allow for column equilibration. During the first 14 min of the gradient programme, the column effluent was diverted to waste to ensure an adequate spray stability. For [$^{13}C_2$]-coumarin, the mass transition (*m/z* precursor ion/ *m/z* product ion) 149/104 and for unlabelled coumarin, the mass transition 147/103 were chosen. Spray voltage was set to 3500 V, sheath gas pressure was 35 mTorr and auxiliary gas pressure 5 mTorr. Capillary temperature was 350 °C and capillary offset 35 V. Source CID (collision induced dissociation) was used with the collision energy set at 12 V. For LC-MS/MS-experiments, the collision gas pressure in quadrupole 2 was 1.5 mTorr, scan time 0.20 s and peak width in quadrupole 1 and 3 were adjusted to ± 0.7 amu. The collision energy for coumarin isotopologues was set to 16 V.

GC/MS Analysis

Mass chromatograms in the electron impact (EI) mode were recorded by means of a MD 800 quadrupole mass spectrometer (Fisons Instruments, Manchester, UK) coupled to a type 8000 gas chromatograph (ThermoQuest, Egelsbach, Germany) equipped with a 30 m x 0.32 mm i. d., 0.25 μm, fused silica capillary DB-5 (Fisons Instruments, Mainz, Germany). The samples were applied by split injection at 230 °C and a split ratio of 1: 20. After injecting the sample (2µL), the temperature of the oven was raised from 40 °C to 250 °C at a rate of 10 °C/min and held at this temperature for 5 min. The flow rate of the carrier gas helium was 2 mL/min. Ionisation energy in the electron impact mode was 70 eV.

Determination of response factors for LC-MS/MS

Solutions of coumarin and [$^{13}C_2$]-coumarin in aqueous formic acid (0.1%) were mixed in molar ratios ranging from 0.1 to 9 to give a total volume of 10 mL and a total coumarin content of 3 µg. Subsequently, the coumarin mixtures were subjected to LC-MS/MS as outlined before. Response factors R_f were calculated as reported recently (*17)* and gave a response factor of 0.90 for the SRM transition 147/103 and 149/104 for unlabelled and labelled coumarin, respectively.

Determination of detection and quantification limits

2, 5, 10 and 20 ng of coumarin were added to ground butter cookies (0.5 g) and analyzed as detailed before. Each sample was analyzed in triplicate. Detection (DL) and quantification limits (QL) were calculated according to Hädrich and Vogelgesang *(18)*.

Precision and recovery

Intra-assay precision was evaluated by analyzing breakfast cereals in five extracts as detailed before.

Recovery was determined by adding 5 ng of coumarin to ground butter cookies (0.5 g) and performing SIDA as detailed before in quadruplicate analysis.

NMR

^{13}C-NMR spectra were recorded with an AM 360 spectrometer (Bruker, Karlsruhe, Germany): transmitter frequency 90.56 MHz; spectral width 23809 Hz; repetition time 2.5 s; 256 scans; 64 K data set; 1-Hz line broadening. Processing was performed by multiplication with a Lorentz-Gaussian function prior to transformation. Chemical shifts are expressed in ppm downfield from tetramethylsilane and *J* -values are in Hz.

RESULTS AND DISCUSSION

Synthesis of [$^{13}C_2$]-coumarin

A previous study on the synthesis and application of [^{13}C]-labelled coumarin has been reported by Christakopoulos et al. *(12)*. However, the latter authors prepared only singly labelled coumarin, which suffers from a spectral overlap of 10% in the molecular ions of labelled and unlabelled coumarin due to natural isotopic abundance in unlabelled coumarin. To overcome this lack and, due to the commercial availability of fully labelled [^{13}C]-acetic anhydride, we prepared doubly labelled coumarin according to a modification of Perkin's initial route to unlabelled coumarin *(16)* (**Figure 3**). We obtained [$^{13}C_2$]-coumarin in 11.5 % yield, showing in electron impact ionization a negligible overlap in its molecular ion signal at *m/z* 148 with unlabelled coumarin, as low as 2%. The correct incorporation of the labels was verified by ^{13}C NMR spectrometry, which revealed two doublet signals corresponding to the labelled positions C-2 and C-3 of coumarin in comparison to the reference spectrum of unlabelled coumarin.

Mass spectrometry of coumarin in electron impact ionization

In accordance with the two [^{13}C]-labels introduced, labelled coumarin showed a shift of the molecular ion signal to *m/z* 148 compared to the respective signal at *m/z* 146 of unlabelled coumarin (**Figure 1**). The two further intense signals of [$^{13}C]_2$-coumarin at *m/z* 119 and 90 were consistent with the loss of two molecules of ^{13}CO from the lactone ring to form the radical cation (**3**) (**Figure 4A**). In the case of unlabelled coumarin these losses result in signals at *m/z* 118 and 90, the latter of which is identical with the respective ion of the labelled compound. This pathway is in good accordance with the assumptions of Christakopoulos et al. *(12)* and Porter *(19)*.

Mass spectrometry of coumarin in electrospray ionization

For the anticipated stable isotope dilution assay (SIDA) with LC-MS detection, mass spectrometry after atmospheric pressure ionization was applied for coumarin detection. Most suitable was the positive electrospray mode giving an intense signal of the protonated molecule at *m/z* 149 and 147 for [$^{13}C_2$]-coumarin and unlabelled coumarin, respectively.

As the recently developed SIDAs of mycotoxins and vitamins were based on LC-MS/MS due to matrix interferences *(14, 15)* , tandem MS was also applied to coumarin isotopologues, being necessary for unequivocal quantification. By employing collision-induced dissociation (CID) to $[M+H]^+$ of isotopologic coumarins, the spectra shown in **Figure 2** were obtained. Besides residual $[M+H]^+$, two intense signals at *m/z* 103 as well as *m/z* 91 and at *m/z* 104 as well as *m/z* 91 for coumarin and [$^{13}C_2$]-coumarin, respectively, are discernable. The two signals for each coumarin isotopologue can be assigned to a) loss of one CO_2 retaining one ^{13}C label in [$^{13}C_2$]-coumarin and b) loss of two CO resulting in unlabelled benzylium (**4**) or tropylium (**5**) for both labelled and unlabelled coumarin (**Figure 4B**). As the product ion of path a) retains one label, the latter signal was used for differentiation and quantification of the isotopologues.

The calibration by measuring different ratios of the isotopologues revealed a constant response factor for the product ions at *m/z* 103 and at *m/z* 104 of $[M+H]^+$ in MS/MS over two decades of isotope ratios.

Extraction and analysis of free and total coumarin by LC/MS/MS

As LC-tandem MS was specific enough to differentiate coumarin isotopologues from sample interferences, sample preparation for LC-MS/MS of free coumarin proved to be very straightforward. After homogenizing the samples in 80% methanol containing definite amounts of [$^{13}C_2$]-coumarin, the extracts only had to be filtered and passed through a syringe filter. LC-MS/MS of cinnamon star cookies (**Figure 5**), Ceylon cinnamon powder (**Figure 6**), Ceylon cinnamon bark essential oil (**Figure 7**) and curry spice (**Figure 8**) displayed in ESI-MS conceivable signals for the isotopologues in their respective selected reaction monitoring (SRM) traces devoid from matrix peaks. In particular when regarding the chromatograms of Ceylon cinnamon (**Figure 6**) and curry spice (**Figure 8**), the UV signal at 275 nm reveals a myriad of signals, which hardly can be differentiated from the small coumarin signal.

Generally, plants have been reported to liberate coumarin from its precursor coumarinyl glucoside *(20)* by enzyme action after disrupture of cells. For example, in woodruff, only a minor part of the sum (henceforth referred to as “total” coumarin) of free and liberable coumarin is assumed to occur in its free form. To prove this assumption, we examined two different methods to analyze total coumarin. The chemical alternative included treatment with hydrochloric acid at 90 °C, whereas the enzymatic counterpart consisted of treatment with β-glucosidase from almonds (emulsin) after extraction. By applicating these methods to various foods containing coumarin we obtained the results depicted in **Table 1**. For both cassia and cinnamon we found no significant amounts of bound coumarin. However, in cinnamon star cookies, only 90% of coumarin occurred in its free form, thus indicating that the cassia used as ingredient contained small amounts of the bound precursor. In contrast to this, for fenugreek seeds higher coumarin contents were found after both hydrochloric acid and β-glucosidase treatment. Obviously, about 68% were present as bound precursor, which was hydrolyzed effectively both by acidic and enzymatic hydrolysis. However, in curry spice, the coumarin content of which originated from fenugreek seeds as ingredients, β-glucosidase was more effective in liberating the precursor. Contrary to the seeds, the percentage of free coumarin in curry spice was as high as 63%. The highest amount of 88% bound coumarin was found in woodruff. As liberation of coumarin was easily initiated during homogenizing the tissue and instant activity of endogenous beta-glucosidase, the free content was only quantifyable when inactivating the enzyme by $CaCl_2$ treatment during sample homogenization.

Performance criteria

To evaluate whether sensitivity of LC-MS/MS was sufficient for quantifying coumarin in foods, and especially in baked goods, the detection limit (DL) was determined in butter cookies according to the method of Hädrich and Vogelgesang *(15).* The calculations resulted in a DL of 2.9 µg/kg and a quantification limit of 8.6 µg/kg in starch containing foods, which proved to be sufficient as the legal limit for coumarin content in foods is as high as 2 mg/kg. In comparison to HPLC-UV, the new method appeared to be three orders of magnitude more sensitive. Recovery was evaluated by adding 10 µg/kg to butter cookies and was found to be 94.1%.

Intra-assay precision was determined by analyzing coumarin in breakfast cereals and revealed a coefficient of variation of 9.9 % for total coumarin (n=5).

Coumarin contents in foods

To prove the suitability of the new method and to verify or disprove coumarin occurrence in different foods, we applied it to a variety of products and acquired the data listed in **Table 2**. The scope of this survey was not to obtain a representative range of contents in the single foods, but to get an insight as to whether previous studies might have reported inaccurate data due to insufficient methodology.

The highest total coumarin content exceeding 4 g/kg was found in cassia bark oil, which is well in line with the high abundance of the odorant reported in the literature (*10*). However, our data are substantially lower than the published high contents ranging between 16 and 25 g/kg. Moreover, concentrations exceeding the g/kg range were detected in cassia, which is in agreement with data obtained by He et al. *(19)*. In contrast to this, we corroborated the findings of Miller et al. *(20)* and Ehlers et al. *(10)*, who found far lower contents in Ceylon cinnamon. In the powdered Ceylon cinnamon powder and the bark essential oil we quantified coumarin concentrations of about three orders of magnitude below those of the respective cassia products. In many foods that are labelled to contain cinnamon, coumarin was detected in the mg/kg range. Therefore, in these products cassia must have been used to generate the flavor of cinnamon. Particularly in cinnamon star cookies coumarin was highly abundant with concentrations amounting to 65 mg/kg. In cinnamon-flavoured tea and in mulled wine the coumarin concentration in the beverages was below 1 mg/kg indicating that only small amounts of cassia have been included as an ingredient.

One sample of rice pudding with cinnamon contained only 0.17 mg/kg coumarin and proved that the manufacturers have changed their recipe towards an omission of cassia.

Further foods containing substantial amounts of coumarin were woodruff, lavender essential oil, and blue-white clover seeds. In contrast to this, the levels in fenugreek seeds as the ingredient of many spices was smaller than 1 mg/kg and, in consequence, curry spice was also low in coumarin. The occurrence of the odorant in chamomille and citron essential oil was confirmed, whereas in green tea powder, carrots and the essential oils of coriander, dill and peppermint coumarin was present only in minute amounts. Moreover, earlier reports on coumarin occurring in bilberries, raspberries, carrots and Angelica roots were not corroborated (*21-23*). Presumably, coumarin in Angelica was mixed up with coumarin derivatives such as umbelliferon and its glucosides.

In flavor research, many successful applications of SIDAs based on GC/MS detection have been reported. However, gas chromatography requires separation of the odorants from non-volatile compounds, which demands at least one further distillation step. The present study confirmed the merits of SIDA methodology in respect to specifity, sensitivity and accuracy also in an LC-MS instrumentation. What is more, the simple and fast sample preparation points to the superiority of the present assay, which is the first report on accurate coumarin quantitation in trace amounts and in complex matrices such as baked products.

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Table 1. Free and Total Content of Coumarin in Foods

Sample	Coumarin (mg/ kg)		
	without hydrolysis	after treatment with hydrochloric acid	after glycosidase treatment
Cassia	1380	1450	1490
Ceylon cinnamon	2.5	2.4	2.4
Cinnamon star cookies	61	68	72
Fenugreek seeds	0.11	0.31	0.34
Curry spice	0.29	0.33	0.46
Woodruff	24	n.a.	203

n.a. not analyzed

Table 2. Total Content of Coumarin in Foods

Food (no. of samples)	Range of total coumarin (mg/kg)
Cassia cinnamon (*Cinnamomum aromaticum)*, powdered (3)	1250 - 1490
Ceylon cinnamon (*Cinnamomum ceylanicum)* , powdered (1)	2.4
Ceylon cinnamon (*Cinnamomum ceylanicum)*, bark (1)	0.86
Cassia (*Cinnamomum aromaticum),* bark essential oil (1)	4370
Cinnamon (*Cinnamomum ceylanicum),* bark essential oil (1)	40
Cinnamon capsule (1)	292
Cinnamon star cookies (2)	19 - 65
Pastries with cinnamon except cinnamon star cookies (2)	7.8 - 23
Breakfast cereals with cinnamon (1)	7.3
Rice pudding with cinnamon (1)	0.17
Cinnamon tea (2)	0.736 – 0.94
Mulled wine (1)	0.036
Woodruff (*Asperula odorata*) (1)	203
Blue-white clover seeds (*Trigonella caerulea)* (1)	37
Fenugreek seeds (*Trigonella foenicum graecum)* (1)	0.34
Curry spice (1)	0.46
Cookies without cinnamon, commercial products (2)	n.d. – 0.03
Green tea (*Thea sinensis*), powder (1)	0.21
Soft drink with green tea (1)	0.07
Lavender (*Lavandula angustifolia*), essential oil (1)	124
Chamomille (*Matricaria recutita*), essential oil (1)	3.23
Citron (*Citrus limon*), essential oil (1)	3.86
Coriander (*Coriandrum sativum*), essential oil (1)	0.30
Dill (*Anethum graveolens*) seeds, essential oil (1)	0.21
Peppermint (*Mentha x piperita*), essential oil (1)	0.30
Bilberries (*Vaccinium myrtillus*) (1)	n. d.
Raspberries, black (*Ribes nigrum*) (1)	n. d.
Carrots (*Daucus carota*), raw (1)	n. q.
Angelica roots (*Angelica archangelica*) (1)	n. d.

n.d. below limit of detection, n.q. below limit of quantitation

Legends to the Figures

Figure 1. Mass spectrum of A. coumarin and B. [$^{13}C_2$]-coumarin in electron impact ionization.

Figure 2. LC-MS/MS spectrum of A. coumarin and B. [$^{13}C_2$]-coumarin after collision-induced dissociation (CID) of the protonated molecules in positive electrospray ionization mode.

Figure 3. Synthetic route to [$^{13}C_2$]-coumarin

Figure 4. Hypothetical fragmentation pathways of coumarin isotopologues in electron impact ionization (above) and collision-induced dissociation (CID) of the protonated molecules in positive electrospray ionization mode (below).

Figure 5. LC-MS/MS chromatograms of cinnamon star cookies in positive electrospray ionization mode after collision-induced dissociation (CID) of the protonated molecules. The internal standard [$^{13}C_2$]-coumarin is detected in the trace MS/MS 149/104 and unlabeled coumarin in trace MS/MS 147/103. UV: UV absorption.

Figure 6. LC-MS/MS chromatograms of Ceylon cinnamon.

Figure 7. LC-MS/MS chromatograms of Ceylon cinnamon bark oil

Figure 8. LC-MS/MS chromatograms of curry spice after treatment with glucosidase.

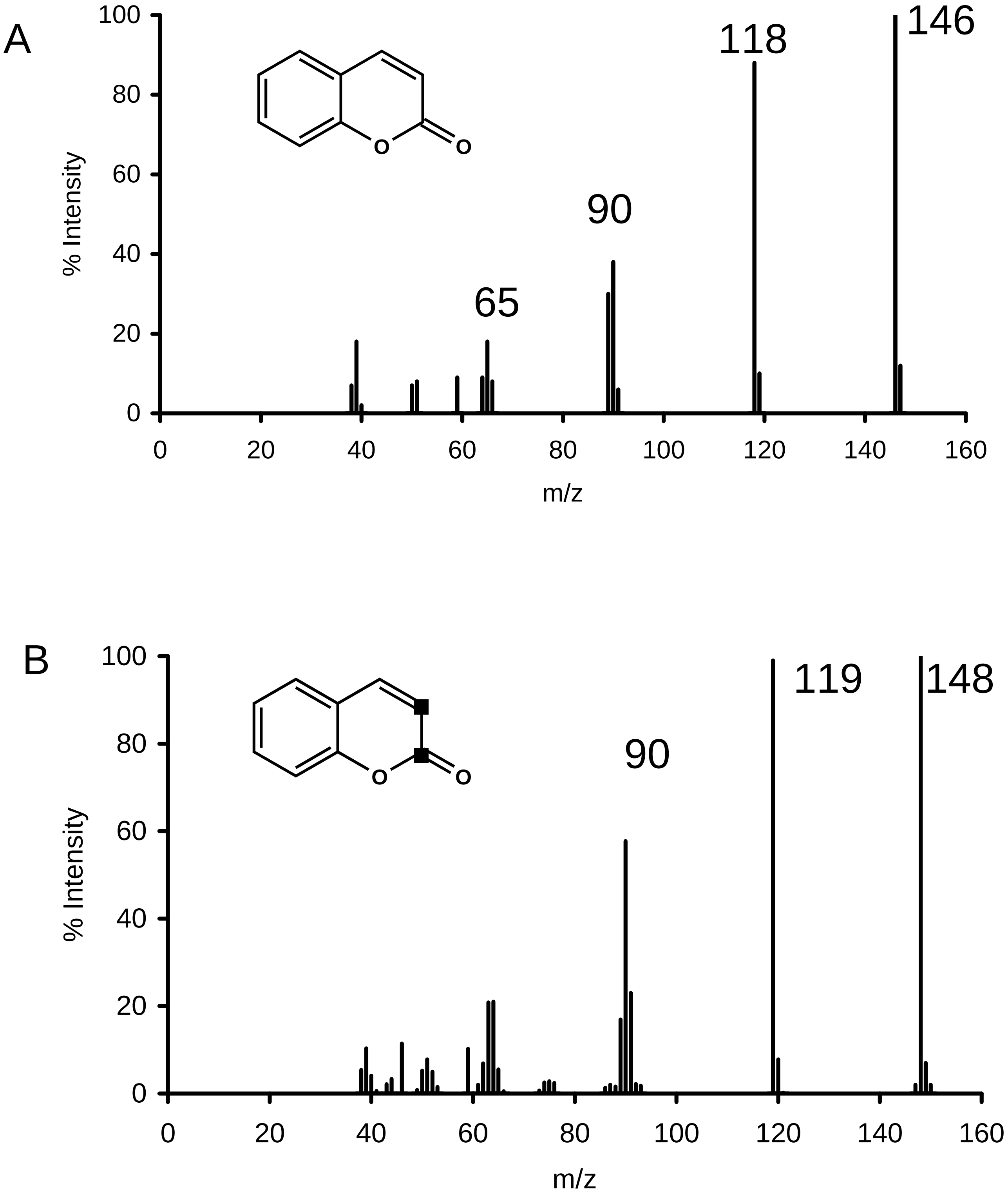

A
100
80
60
40
20
0
% Intensity
O
O
146
118
90
65
0
20
40
60
80
100
120
140
160
m/z
B
100
80
60
40
20
0
% Intensity
O
O
119
148
90
0
20
40
60
80
100
120
140
160
m/z

fig. 1

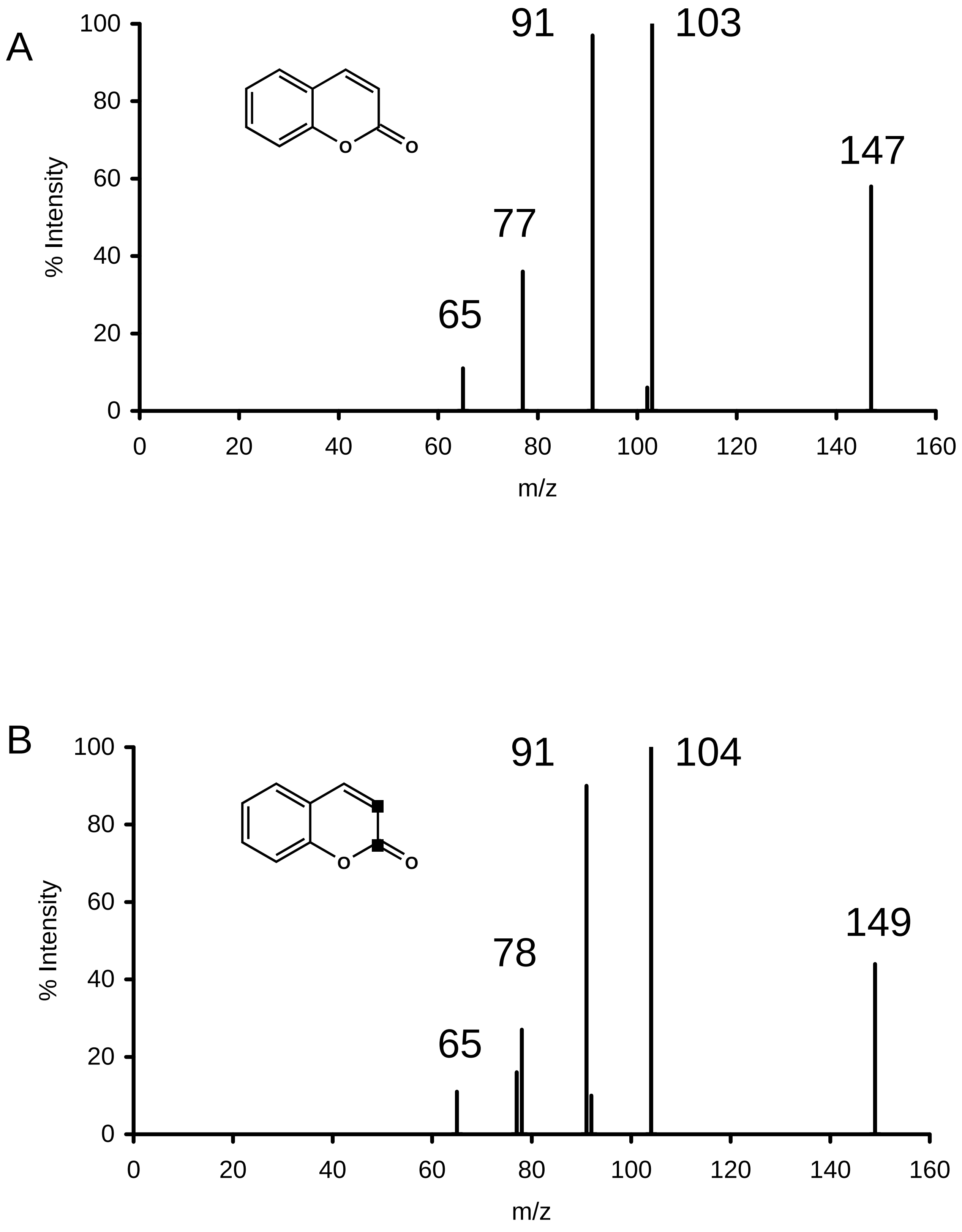
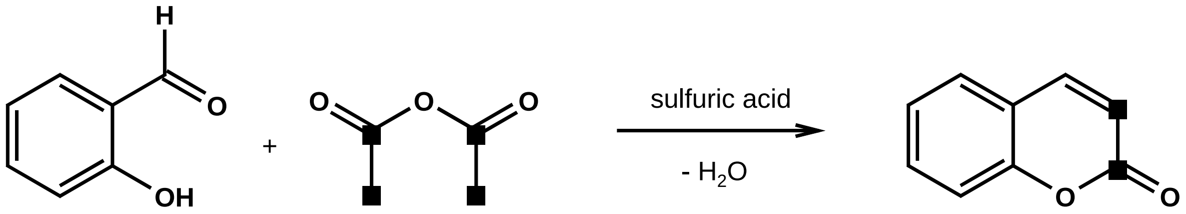

A
% Intensity
0
20
40
60
80
100
m/z
0
20
40
60
80
100
120
140
160
65
77
91
103
147
O
O
B
% Intensity
0
20
40
60
80
100
m/z
0
20
40
60
80
100
120
140
160
65
78
91
104
149
O
O

Fig 2



1

2

fig. 3

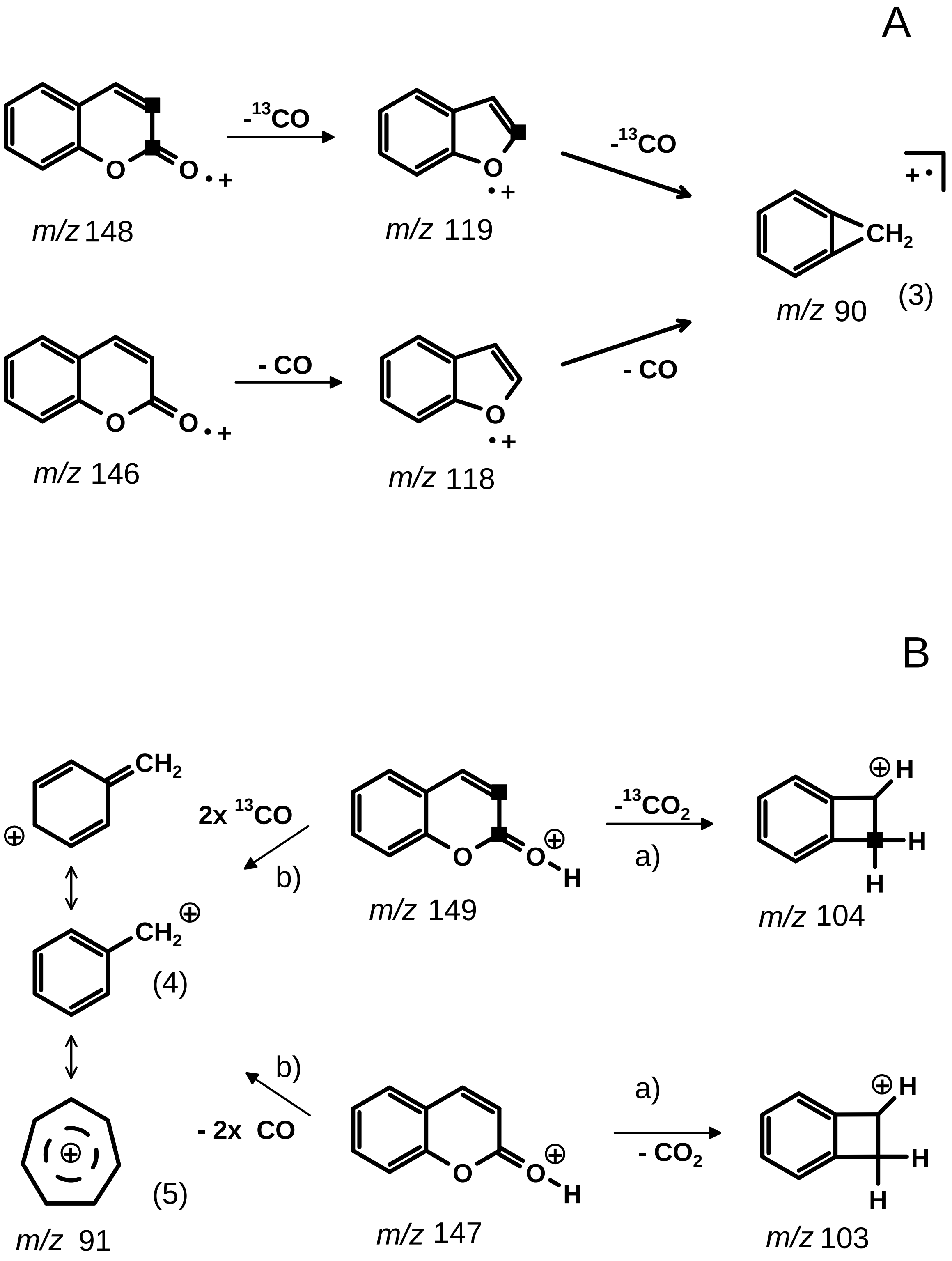

A
- 13CO
O
O • +
m/z 148
O
• +
m/z 119
- 13CO
+ •
CH2
m/z 90
(3)
- CO
O
O • +
m/z 146
O
• +
m/z 118
- CO
B
CH2
⊕
2x 13CO
b)
O
⊕
O
H
m/z 149
- 13CO2
a)
⊕ H
H
H
m/z 104
CH2 ⊕
(4)
b)
- 2x CO
O
⊕
O
H
m/z 147
a)
- CO2
⊕ H
H
H
m/z 103
⊕
(5)
m/z 91

fig 4

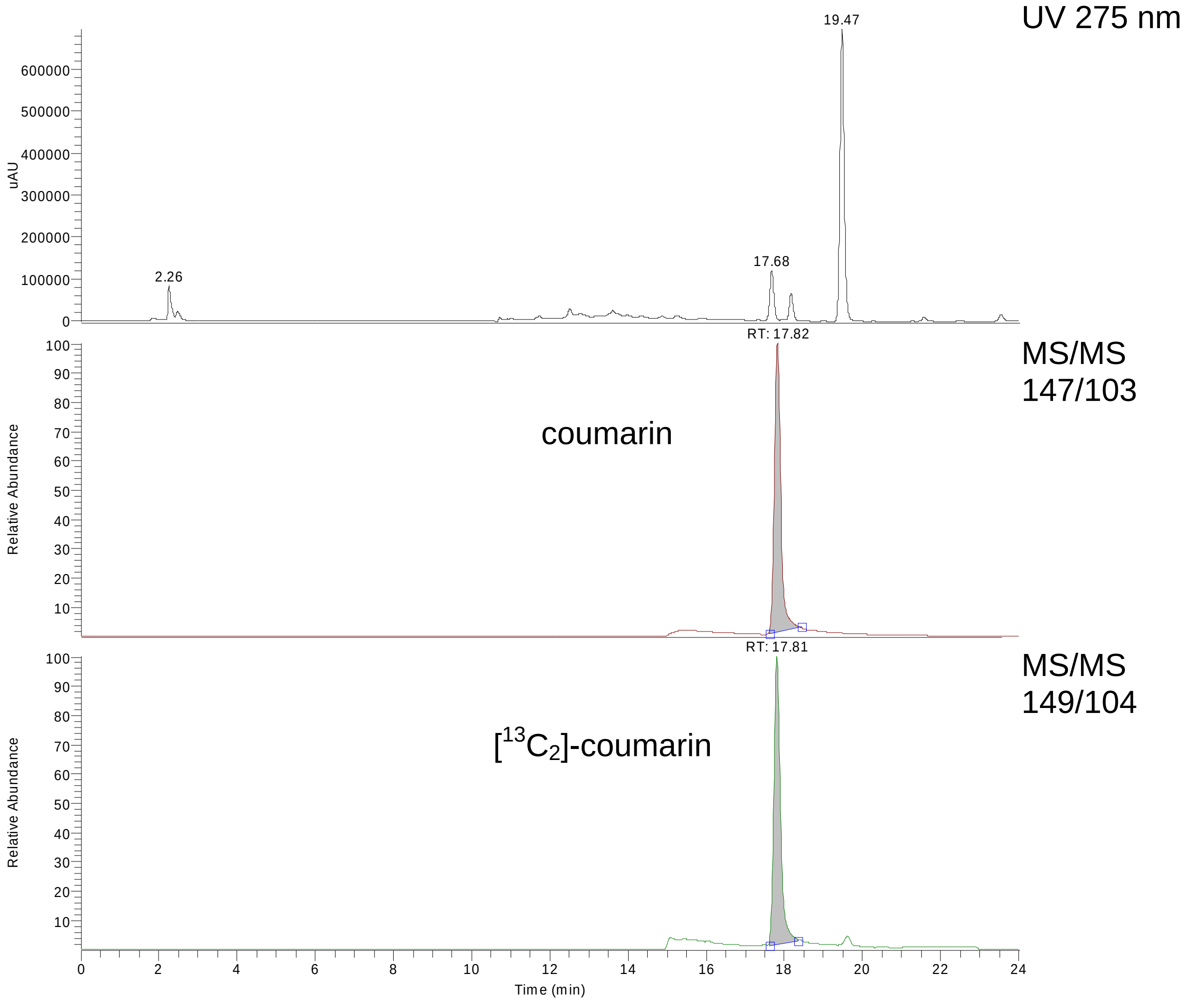

UV 275 nm
19.47
600000
500000
400000
uAU
300000
200000
17.68
100000
2.26
0
RT: 17.82
MS/MS
147/103
coumarin
Relative Abundance
100
90
80
70
60
50
40
30
20
10
RT: 17.81
MS/MS
149/104
[13C2]-coumarin
Relative Abundance
100
90
80
70
60
50
40
30
20
10
0
2
4
6
8
10
12
14
16
18
20
22
24
Time (min)

fig 5

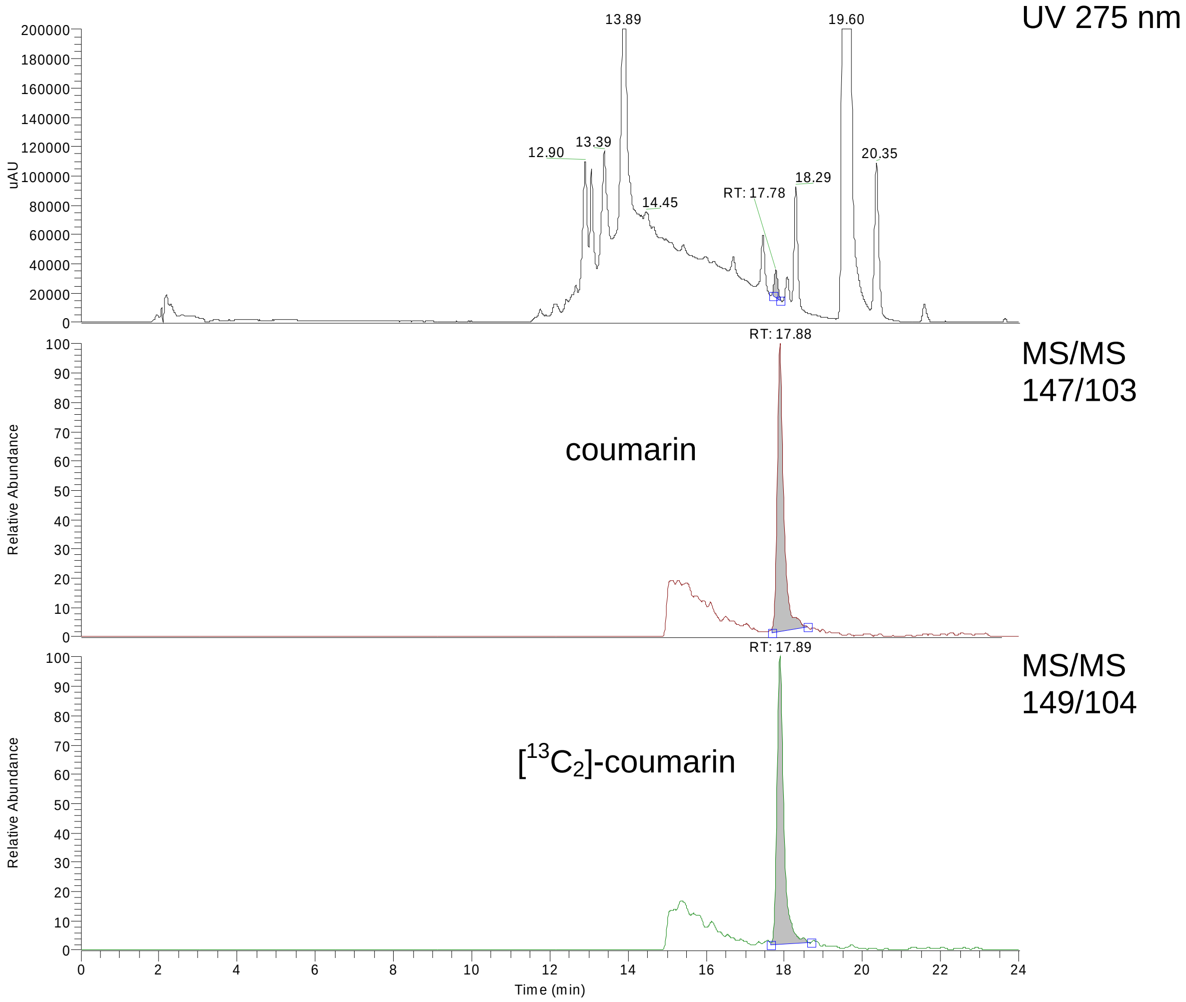

UV 275 nm
uAU
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
13.89
19.60
12.90
13.39
20.35
18.29
14.45
RT: 17.78
MS/MS
147/103
RT: 17.88
coumarin
Relative Abundance
100
90
80
70
60
50
40
30
20
10
0
MS/MS
149/104
RT: 17.89
[13C2]-coumarin
Relative Abundance
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
18
20
22
24
Time (min)

fig 6

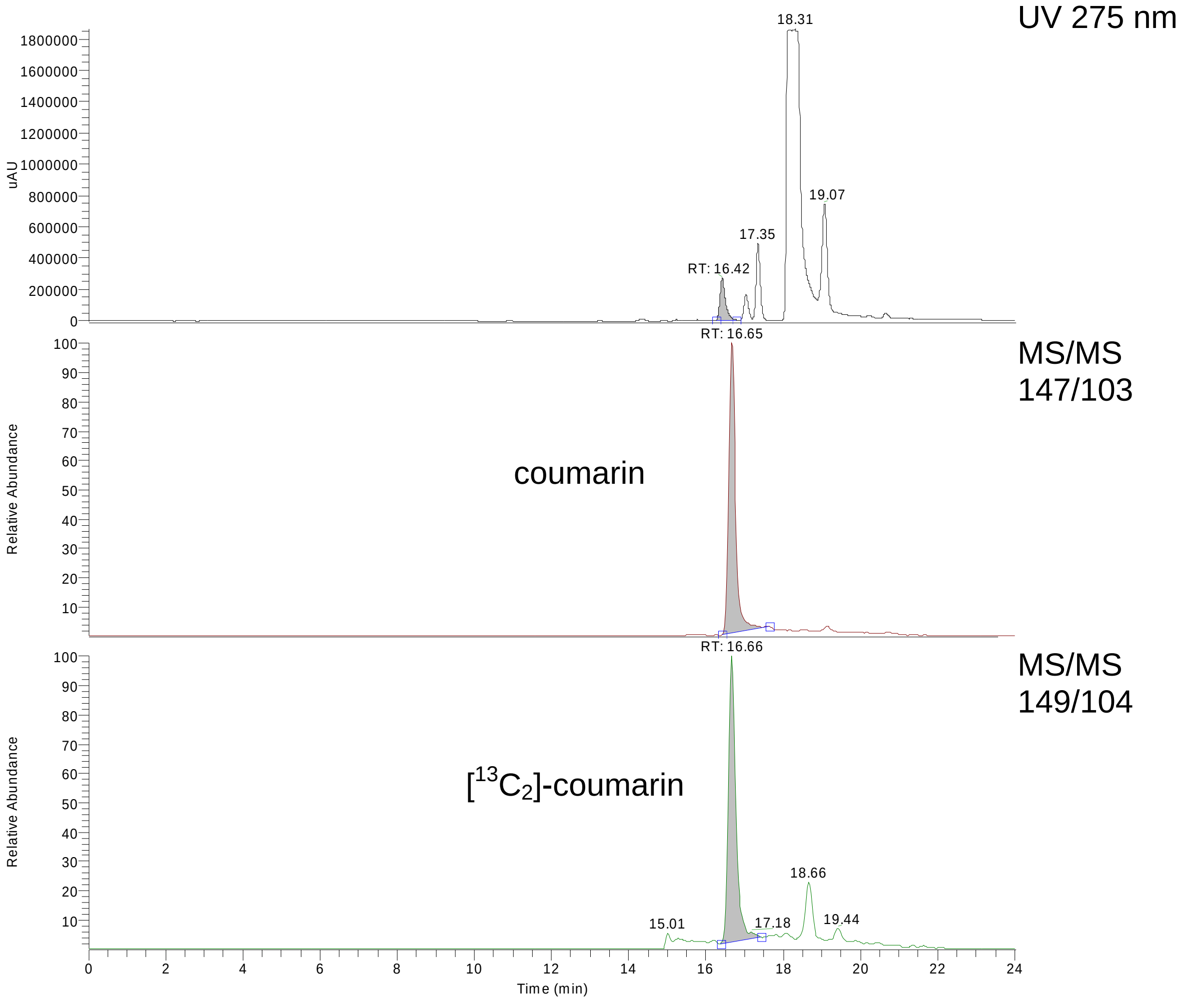

UV 275 nm
18.31
19.07
17.35
RT: 16.42
uAU
1800000
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
MS/MS
147/103
RT: 16.65
coumarin
Relative Abundance
100
90
80
70
60
50
40
30
20
10
MS/MS
149/104
RT: 16.66
[$^{13}C_2$]-coumarin
Relative Abundance
100
90
80
70
60
50
40
30
20
10
18.66
15.01
17.18
19.44
0
2
4
6
8
10
12
14
16
18
20
22
24
Time (min)

fig 7

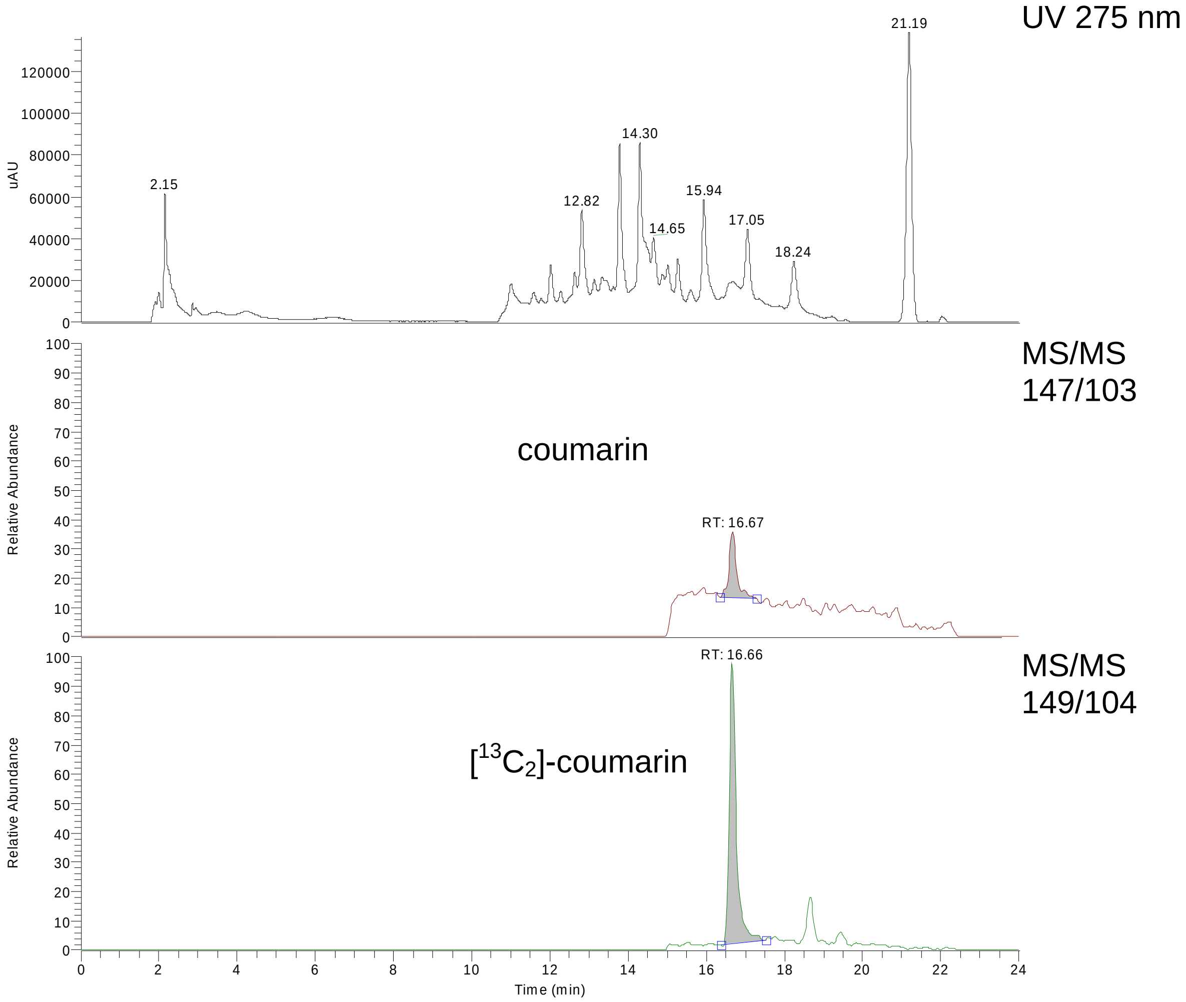

UV 275 nm
21.19
14.30
2.15
12.82
15.94
14.65
17.05
18.24
120000
100000
80000
60000
40000
20000
0
uAU
MS/MS
147/103
coumarin
RT: 16.67
Relative Abundance
100
90
80
70
60
50
40
30
20
10
0
MS/MS
149/104
RT: 16.66
[13C2]-coumarin
Relative Abundance
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
18
20
22
24
Time (min)

fig 8