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應用手冊

A Metabolomic Approach to Analyzing Plant Flavonoids Using Constant Neutral Loss Acquisitions

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Abstract

The aim of this application note is to show the application of exact mass neutral loss mass spectrometry to the analysis of the flavonoids produced in plant species as a result of either mutation or genetic modification.

Benefits

- Selective (±20 mDa window) and sensitive tool for screening for glycosylated metabolites in complex extracts (19 putative sugar losses monitored)
- Fast screening method for differentially produced glycosides in plants, containing MS and MS/MS information from one single chromatographic run
- Exact mass measurements (<5 ppm RMS) and elemental composition reports provide information on the putative metabolites and their aglycon fragments

Introduction

Metabolomics is the term coined for essentially comprehensive, nonbiased, analysis of complex metabolite mixtures typical of plant extracts.

Flavonoids are a group of polyphenolic substances found in most plants, concentrating in seeds, fruit skin or peel, bark, and flowers. Examples of typical flavonoid aglycon structures found in plants are shown in Figure 1. Flavonoids have been shown to have antibacterial, anti-inflammatory, anti-allergic, anti-mutagenic, anti-viral, anti-thrombotic, and vasodilatory activity. The potent antioxidant activity and their ability to scavenge free radicals is a major function of flavonoids, and may underlie many of the above actions in the body. Studies have indicated that their consumption is associated with a reduced risk of cancer and cardiovascular disease. Vegetables, fruits and beverages are the main dietary source of flavonoids.

A great number of plant medicines contain flavonoids.

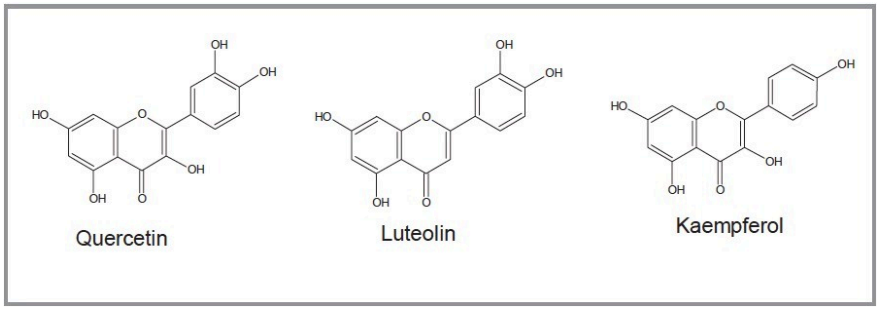

Figure 1. Typical plant flavonoid aglycon structures.

Waters Micromass Q-Tof Ultima

Neutral Loss Acquisitions

Apart from the aglycon, many variations exist on this basic structure by the addition of various sugar moieties to free hydroxyl groups, resulting in a large group of related molecules. These sugars are fairly readily lost from the parent molecule by collision induced dissociation (CID) and can therefore be readily analyzed by neutral loss acquisitions. This can be a valuable diagnostic tool for a particular compound group such as flavonoid glycosides.

Until recently this work has been carried out with tandem quadrupole technology using nominal masses. The advantage of the hybrid quadrupole-time of flight mass spectrometer is the selectivity that can be achieved using the exact mass capabilities for both precursor ion and neutral loss acquisitions. This enables survey functions to be carried out with a very high level of specificity and at the same time eliminating matrixrelated interference, which may occur when analyzing complex biological matrices.

Exact neutral loss detection is achieved via sequential high and low energy MS acquisitions. When the loss of a sugar or combination of sugars is detected from a predefined list within a mass tolerance window of typically ±20 mDa, data directed MS/MS is automatically performed on the related precursor. The continual acquisition of data using the high and low energy mode allows further interrogation of the data for full scan MS information.

The characterization of flavonoid glycosides in plant extracts was applied to the analysis of tomato fruits and *Arabidopsis thaliana*.

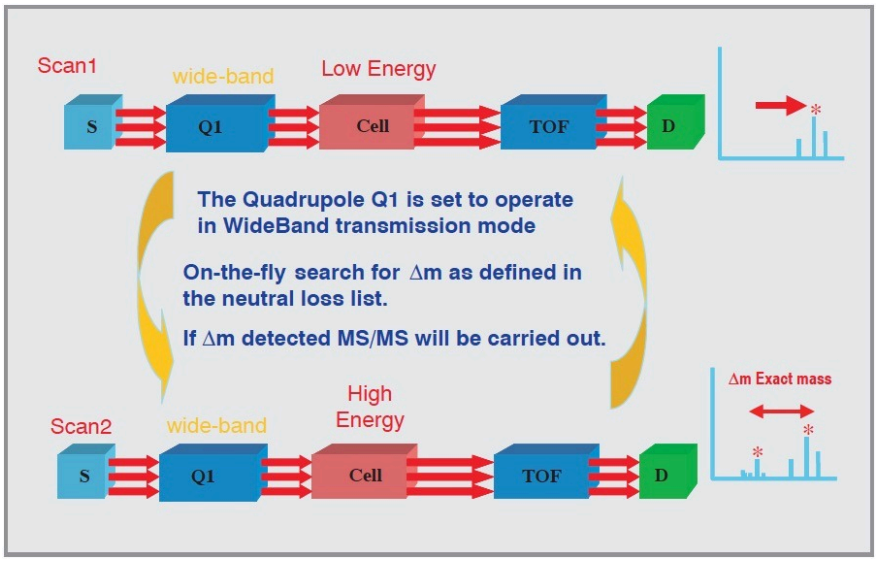

Figure 2. Neutral loss schematic.

Analysis of Transgenic Tomatoes

Since some major crops, e.g. tomato, contain only small amounts of flavonoids in their edible parts the aim is to up-regulate the flavonoid biosynthesis by means of genetic engineering and hence increase the health promoting properties. This study concentrated on the identification of new flavonoid-glycosides in transgenic tomatoes compared to control fruits.

Analysis of Arabidopsis thaliana

Arabidopsis thaliana is a small flowering plant that is widely used as a model organism in plant biology. It is a member of the mustard (Brassicaceae) family, which includes cultivated species such as cabbage and radish, and it grows throughout the Northern Hemisphere as a harmless weed. *Arabidopsis thaliana* is ideal for biological research because the plants mature rather quickly, going from germinating seeds to flowers in just 4-6 weeks. It also undergoes self-fertilization that gives homozygous plants, thus allowing genetic traits to more easily be propagated.

Unlike most crop plants, it has a remarkably small genome (the sum total of the genetic material found in each of its cells), and this makes the job of identifying all of its genes much easier. Although the genome of Arabidopsis is compact, the plant's life processes are similar to those of more complex crop plants of economic importance such as maize, soybean, wheat or oilseed rape. By decoding the complete sequence, scientists are in a better position to modify and improve other plants to help make them less prone to disease and more resilient in growing conditions that are less than favorable. *Arabidopsis thaliana* was analyzed as part of a gene function study. Extracts were studied of two different tissues from the same type of plant (cauline and rosette leaves) as well as

mutant cauline leaves.

Experimental

Sample Preparation

Tomatoes

The plants were grown simultaneously in a greenhouse and the tomato fruits harvested into liquid nitrogen and ground. 0.5 g of the material was extracted in 2 mL of 62.5% methanol: 37.5% water + 0.125% formic acid, sonicated for 15 min then filtered.

Arabidopsis

Sample 1 - a mixture of 10 wild-type rosette leaves ecotype WS

Sample 2 - a mixture of 3 mutant WS plants, cauline leaves

Sample 3 - a mixture of 3 wild type WS plants, cauline leaves

Plant material was taken from plants grown in a climate-controlled greenhouse, directly frozen in liquid nitrogen and ground to a fine powder. 150 mg fresh weight of frozen material was weighed and extracted in 0.75 mL of 62.5% methanol in Milli-Q water acidified with 0.125% formic acid. After sonication for 30 minutes, the extract was filtered through a 0.45 µm PTFE filter that was prewashed to remove polyethyleneglycol, before injection into the LC-MS system.

LC-MS Conditions

The plant extracts were analyzed on a Alliance HT 2795 Separations Module coupled to a Waters Micromass Q-Tof Ultima API Mass Spectrometer fitted with a LockSpray source and operating in electrospray positive ionization mode. The instrument was calibrated in MS/MS mode from an infusion of GFP (Glufibrinopeptide) at 0.5 pmol/µL in 1:1 MeCN: water + 0.1% formic acid.

The following conditions were used:

Column:	Waters XTerra® MS C_{18} 3.5 m 2.1x150 mm
Mobile Phase A:	Water +0.1% formic acid
Mobile Phase B:	MeCN +0.1% formic acid
Gradient:	0 min 95%A, 25 min 75%A, 35 min 65%A, 37 min 50%A, 40 min 50%A, 42 min 95%A
Flow Rate:	0.25 mL/min
Injection Volume:	20 µL
Ionization Mode:	Positive ion electrospray
Cone Voltage:	35 V
Source Temp:	120 °C
Desolvation Temp:	250 °C
Lock Reference:	leucine enkephalin, 0.2 ng/µL 1:1 MeCN: water + 0.1% formic acid
Lock Flow:	10 µL/min
Lock Mass:	m/z 556.2771

The samples were analyzed in Exact Neutral Loss mode using centroid acquisitions as follows:

MS Acquisitions:	m/z 50-900, 0.4 sec, 0.1 sec inter scan delay
Low Energy Survey Spectra:	5 eV
High Energy Survey Spectra:	25 eV
MS/MS Spectra:	m/z 50-1500, 0.9 sec, 0.1 sec inter scan delay
Collision Energy:	CE1= 15 eV, CE2= 25 eV
Collision Gas:	argon

The neutral losses monitored with a window of ±20 mDa were:

Pentose $C_5H_{10}O_5$	monoisotopic neutral loss 132.0422Da
Deoxyhexose $C_6H_{12}O_5$	monoisotopic neutral loss 146.0579Da
Hexosamine $C_6H_{13}NO_5$	monoisotopic neutral loss 161.0688Da
Hexose $C_6H_{12}O_6$	monoisotopic neutral loss 162.0528Da
Hexuronic acid $C_6H_{10}O_7$	monoisotopic neutral loss 176.0321Da

Different combinations of all of these were also monitored for neutral losses with 19 individual masses and/or combined masses being analyzed.

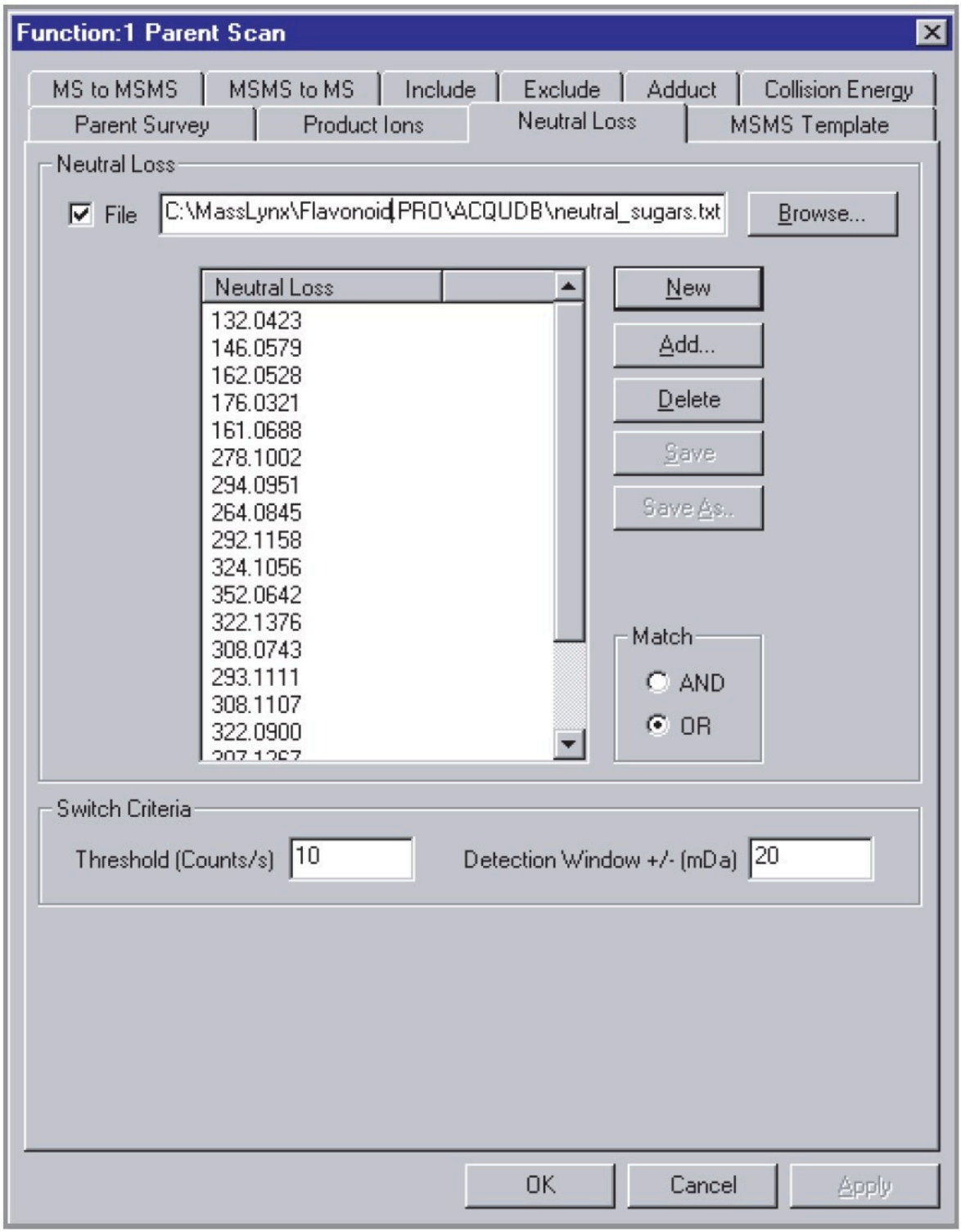

Figure 3. Screen capture of neutral losses monitored.

Results and Discussion

The low and high energy MS survey mode chromatograms and the MS/MS product ion chromatograms from both the control and transgenic fruits are shown in Figure 4. These show that 2 significant new flavonoid-glycosides have been detected in the transgenic fruit compared to the control at 12.3 and 19.7 minutes. The instrument was set to switch back from MS/MS to MS mode after either 6 seconds or in the absence of one of the neutral losses from the predefined list. Due to the expected presence of isomeric species the detected parent ion masses were not put into an exclude list

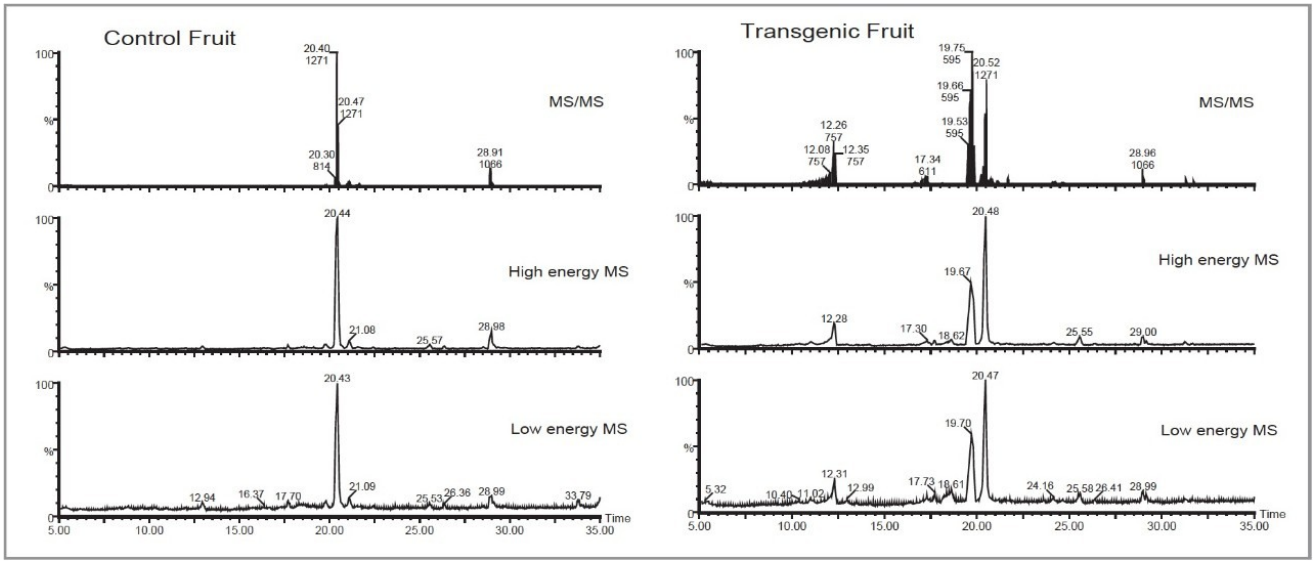

Figure 4. Chromatograms from control and transgenic fruit.

The spectra associated with the new flavonoid glycoside detected at 12.3 min are shown in Figure 5.

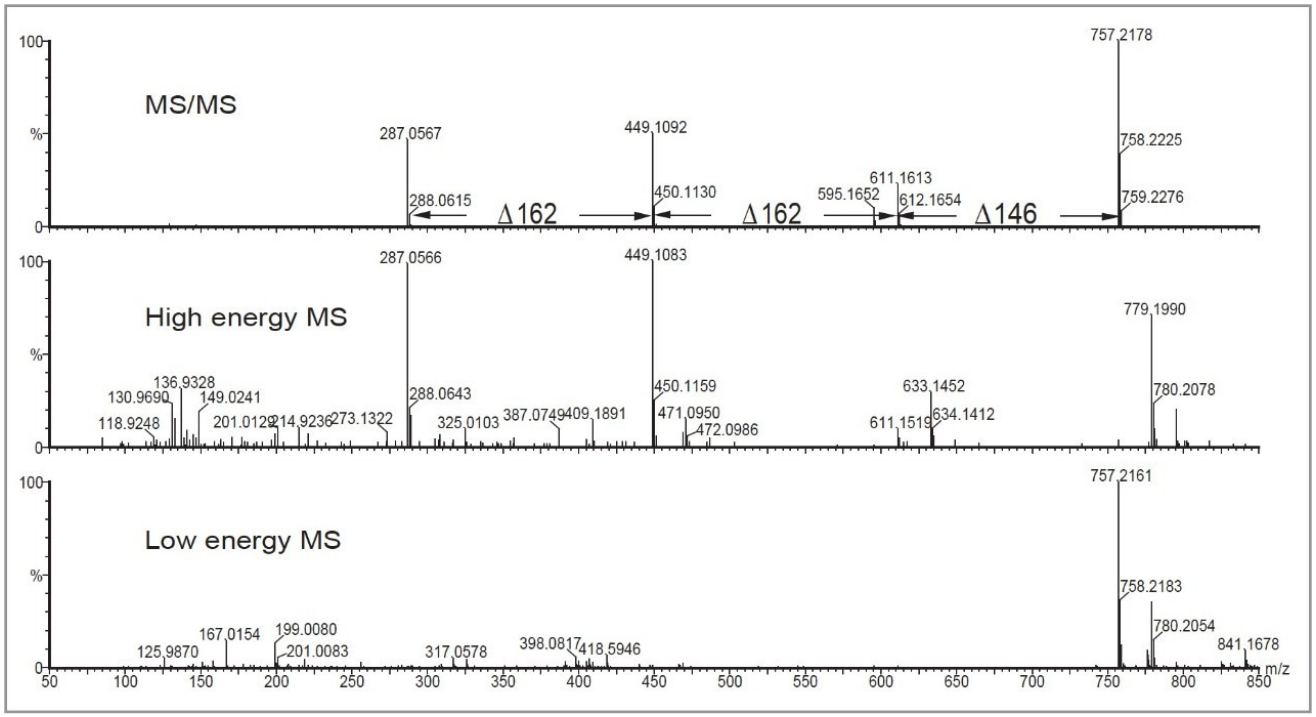

Figure 5. Low energy, high energy, and MS/MS product ion spectra associated with the new flavonoid glycoside detected in the transgenic fruit at 12.3 min.

The peak at retention time 12.3 min shows the neutral loss of 2 hexoses (162) and one deoxyhexose (146) possibly glucose and rhamnose, respectively. Without the availability of retention time standards for all the potential isomeric flavonoid glycosides the position of the glycosilations and structure of the parent aglycon can not be confirmed. The elemental composition report for the MS/MS product ion spectrum and postulated structure are shown in Figure 6.

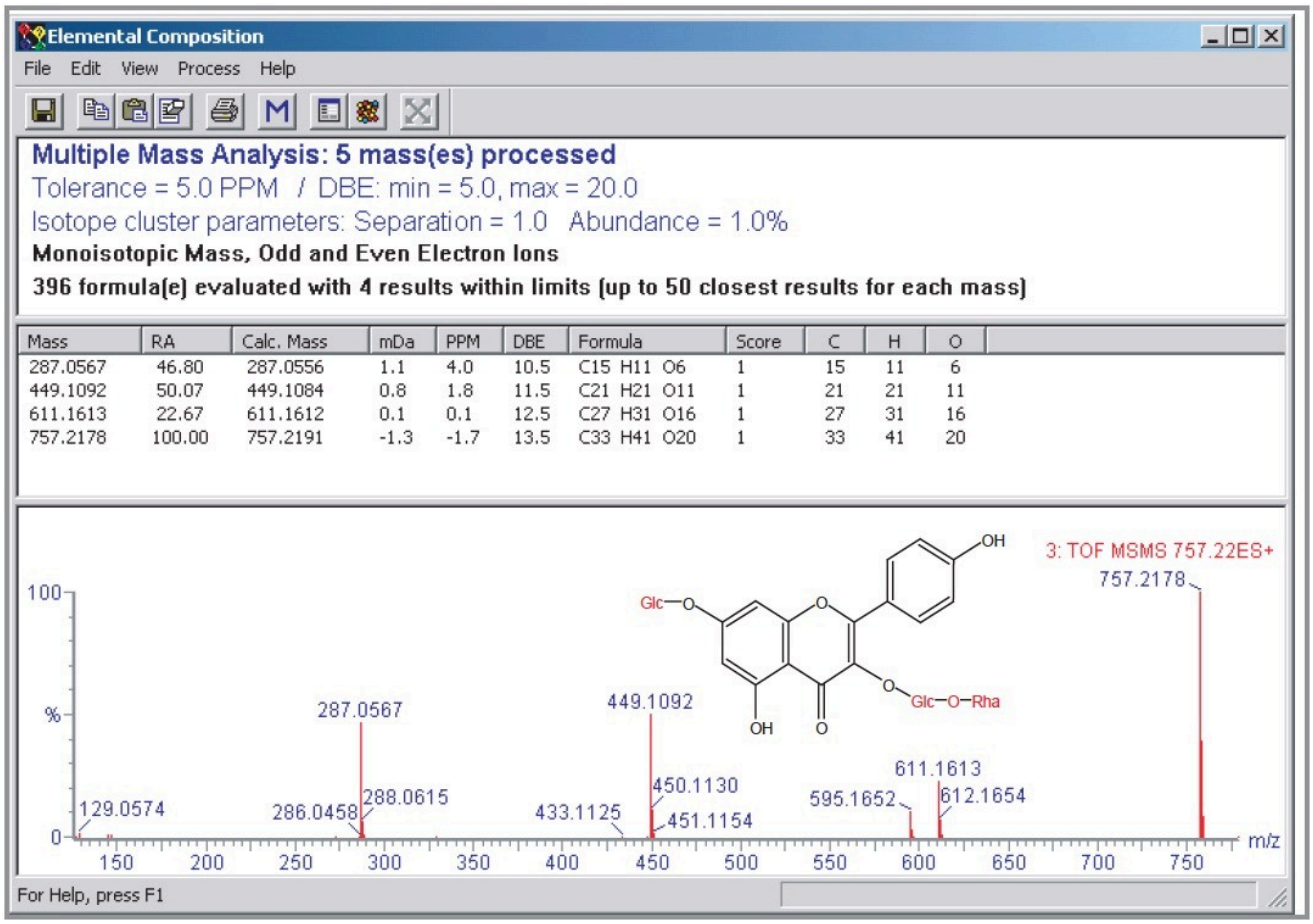

Figure 6. Elemental composition report and postulated structure for the MS/MS product ion spectrum for the glycosylated flavonoid eluting at 12.3 min in the transgenic fruit sample.

The major flavonoid detected in the transgenic fruit showed the loss of a single hexose (162 Da) and a deoxyhexose (146 Da) see Figure 7. This peak has been shown to be kaempferol-3-O-rutinoside, as determined by standards and NMR1. The presence of *m/z* 433 has been postulated to be the result of rearrangement of the algycon with a rhamnoside fragment after fragmentation of the disaccharide.[2]

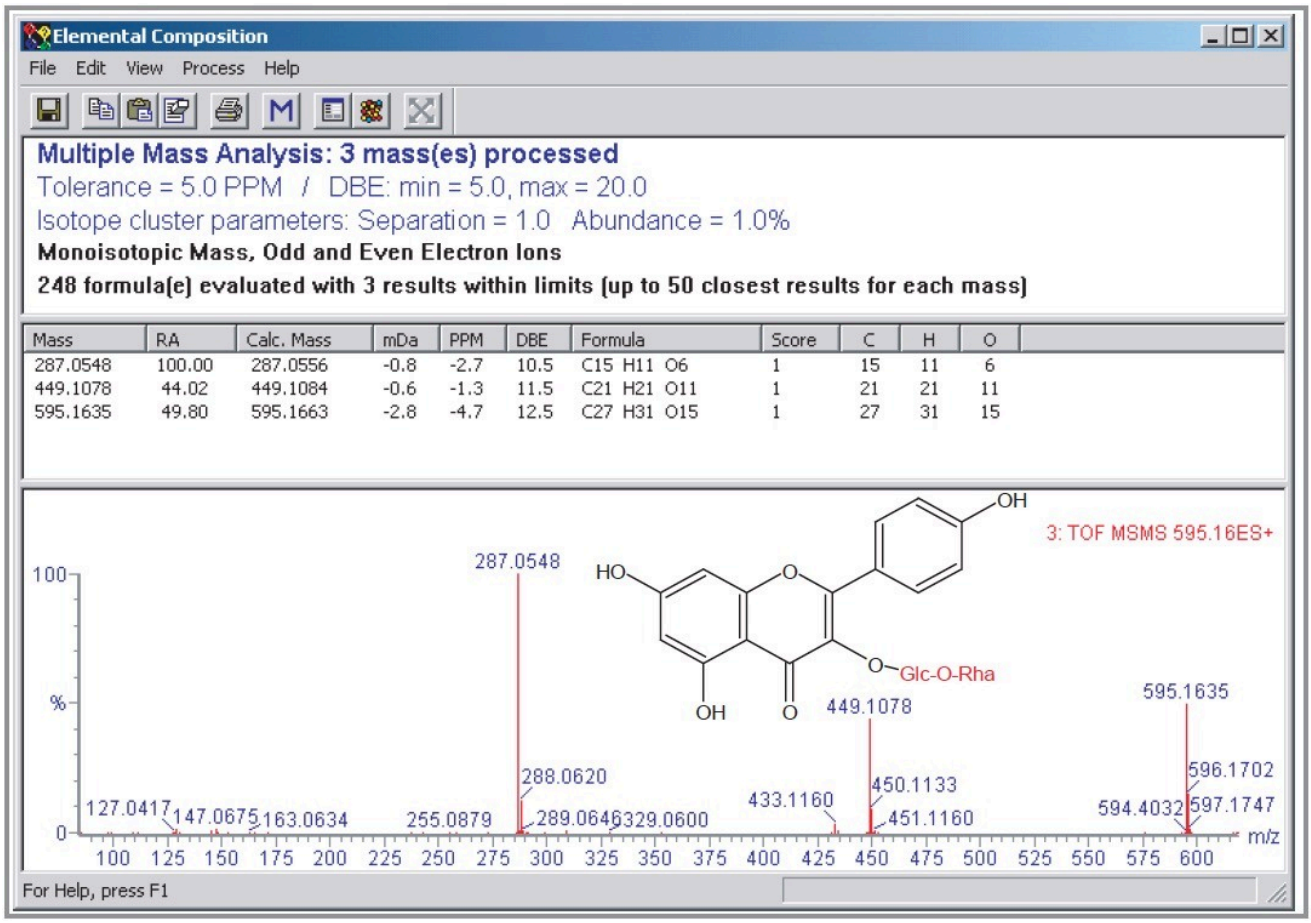

Figure 7. Elemental composition report and postulated structure for the MS/MS product ion spectrum for the glycosylated flavonoid eluting at 19.7 min in the transgenic fruit sample.

Arabidopsis Extracts

The total ion chromatograms obtained from the three arabidopsis extracts are shown in Figure 8. There are visible differences between the TIC chromatograms from the three samples.

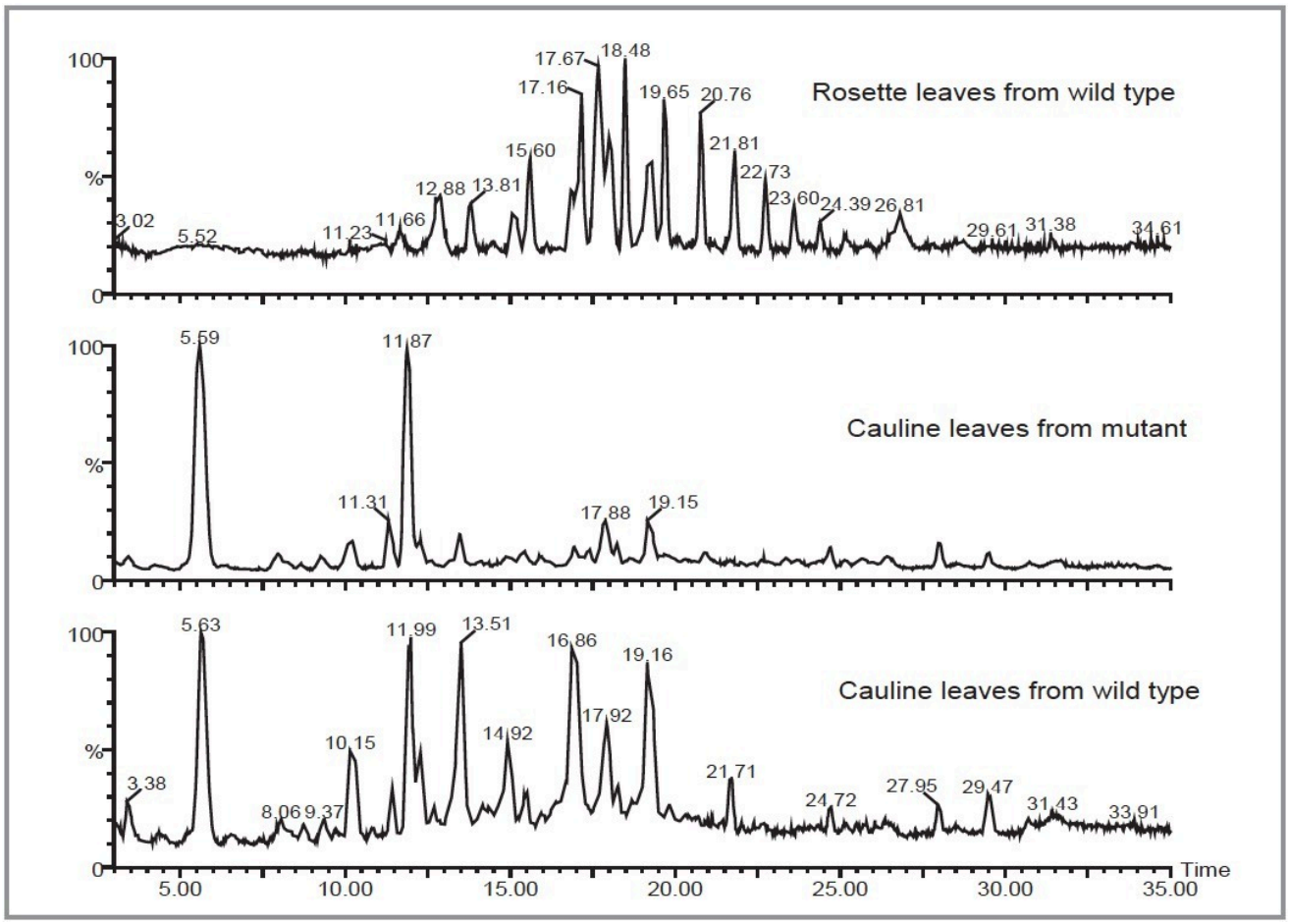

Figure 8. Low energy MS TICs from all 3 samples.

The rosette leaves sample shows a PEG contamination but due to the selectivity (±20 mDa) this was not prohibitive to the analysis as can be seen from the selected spectra (Figure 9B) from the MS/MS product ion chromatogram in Figure 9A.

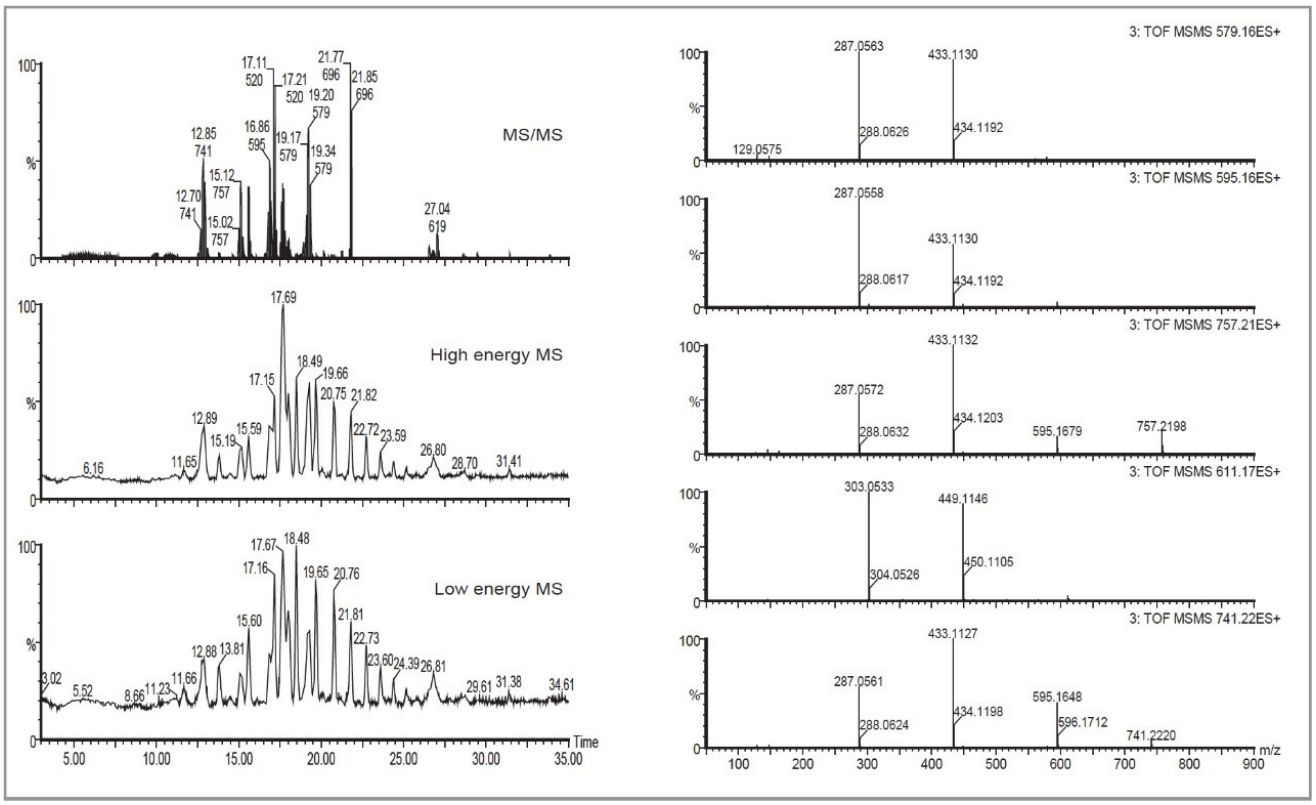

Figure 9. A) Low and high energy MS survey and MS/MS product ion chromatograms from wild type rosette leaves sample. B) Spectra of detected flavonoids.

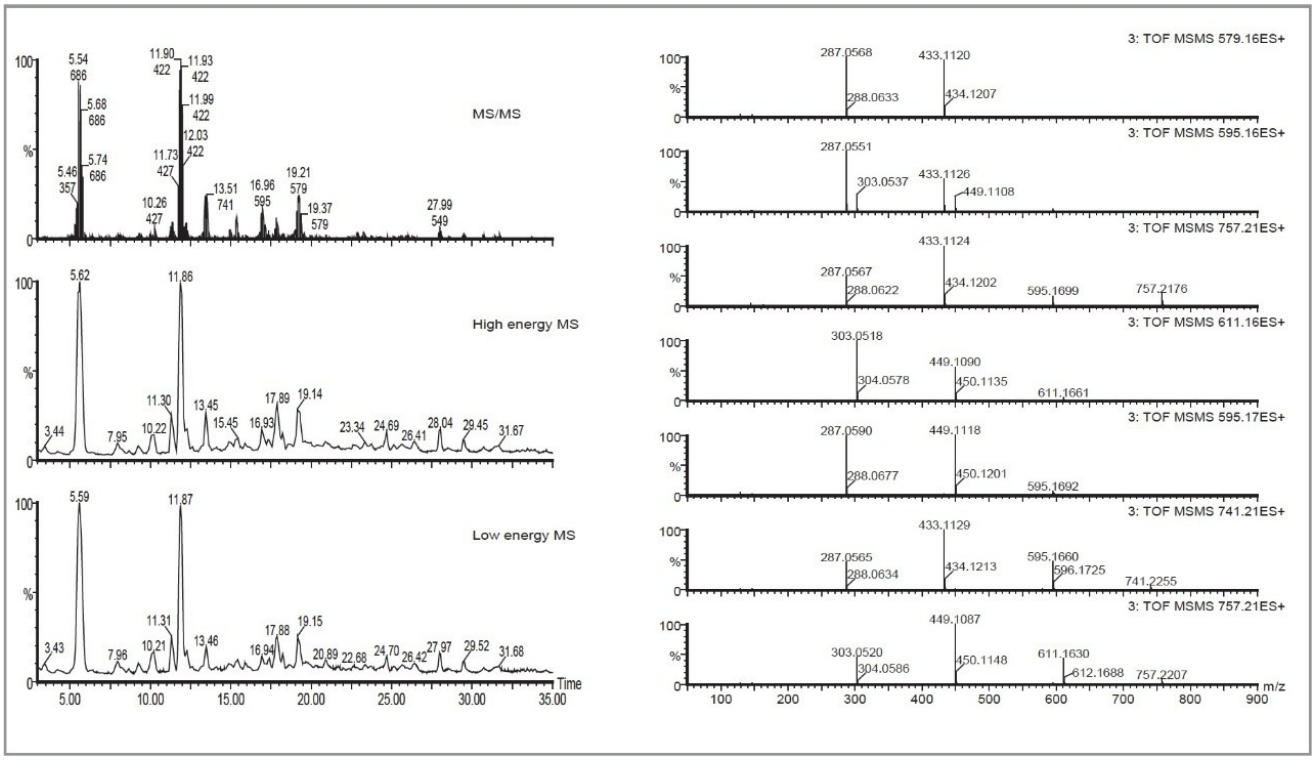

Figure 10. A) Low and high energy MS survey and MS/MS product ion chromatograms from mutant cauline leaves sample. B) Spectra of detected flavonoids.

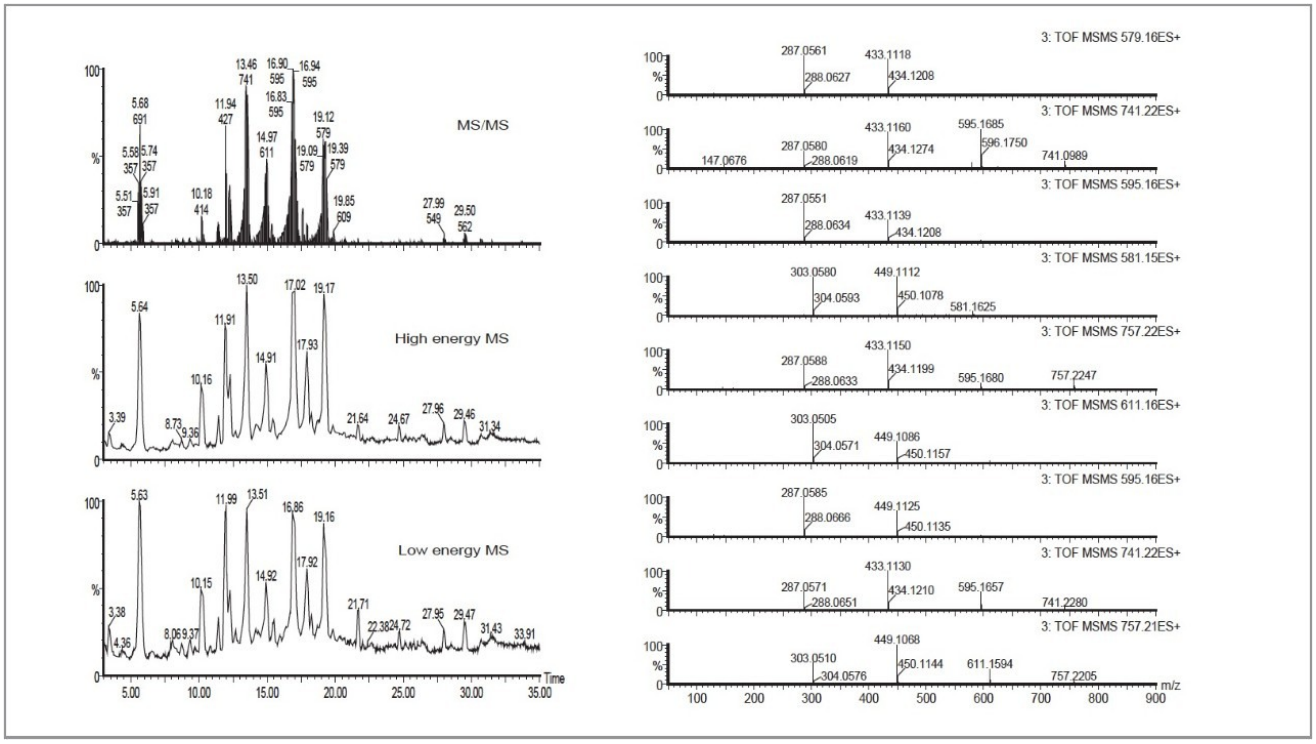

Figure 11. A) Low and high energy MS survey and MS/MS product ion chromatograms from wild type cauline leaves sample. B) Spectra of detected flavonoids.

A table of exact mass measurements for the MS/MS product ion spectra for the arabidopsis samples, with determined elemental composition for each component is shown in Table 1.

The RMS error for all the measurements is < 5 ppm. Some individual errors are higher due to the low ion abundance. Without the availability of standards or NMR analysis, the structures can only be postulated but the 3 and 7 positions are the most likely for the substitutions. The cauline leaves from the arabidopsis samples showed a greater variation in glycosides and a higher flavanoid content than the rosette leaves. The extract from the mutant cauline leaves was pink in coloration indicating the possible production of anthocyanins as a result of stress. The aglycon cyanidin and kaempferol are isomeric so could only be distinguished if a UV or DAD detector was used in line.

Wild type rosette leaves				Mutant cauline leaves			Wild type cauline leaves				
Peak RT	Measured Mass	Error mDa	Error ppm	Measured Mass	Error mDa	Error ppm	Measured Mass	Error mDa	Error ppm	Calculated Mass	Elemental Composition
12.3				303.0520	1.5	5.0	303.0510	0.5	1.7	303.0505	$C_{15}H_{11}O_7$
				449.1087	0.3	0.7	449.1068	-1.6	-3.5	449.1084	$C_{21}H_{21}O_{11}$
				611.1630	1.8	2.9	611.1594	-1.8	-3.0	611.1612	$C_{27}H_{31}O_{16}$
							757.2205	1.4	1.8	757.2191	$C_{33}H_{41}O_{20}$
Postulated structure: quercetin dirhamnoside glucoside											
13.5	287.0561	0.5	1.9	287.0565	0.9	3.3	287.0571	1.5	5.4	287.0556	$C_{15}H_{11}O_6$
	433.1127	-0.8	-1.8	433.1129	-0.6	-1.3	433.1130	-0.5	-1.1	433.1135	$C_{21}H_{21}O_{10}$
	595.1648	-1.5	-2.5	595.1660	-0.3	-0.5	595.1657	-0.6	-1.0	595.1663	$C_{27}H_{31}O_{15}$
	741.2220	-2.2	-3	741.2255	1.3	1.7	741.228	3.8	5.1	741.2242	$C_{33}H_{41}O_{19}$
Postulated structure: kaempferol dirhamnoside glucoside											
14.0				287.0590	3.4	12	287.0585	2.9	10.2	287.0556	$C_{15}H_{11}O_6$
				449.1118	3.4	7.6	449.1125	4.1	9.2	449.1084	$C_{21}H_{21}O_{11}$
				595.1692	2.9	4.9				595.1663	$C_{27}H_{31}O_{15}$
Postulated structure: kaempferol rhamnoside glucoside											
14.9	303.0533	2.8	9.3	303.0518	1.3	4.4	303.0505	0	0.1	303.0505	$C_{15}H_{11}O_7$
	449.1146	6.2	14	449.1090	0.6	1.4	449.1086	0.2	0.5	449.1084	$C_{21}H_{21}O_{11}$
				611.1661	4.9	8.0				611.1612	$C_{27}H_{31}O_{16}$
Postulated structure: quercetin rhamnoside glucoside											
15.3	287.0572	1.6	5.7	287.0567	1.1	4.0	287.0588	3.2	11.3	287.0556	$C_{15}H_{11}O_6$
	433.1132	-0.3	-0.6	433.1124	-1.1	-2.5	433.1150	1.5	3.5	433.1135	$C_{21}H_{21}O_{10}$
	595.1679	1.6	2.7	595.1699	3.6	6.1	595.1680	1.7	2.9	595.1663	$C_{27}H_{31}O_{15}$
	757.2198	0.7	0.9	757.2176	-1.5	-2.0	757.2247	5.6	7.4	757.2191	$C_{33}H_{41}O_{20}$
Postulated structure: kaempferol rhamnoside diglucoside											
15.5							303.0580	7.5	24.8	303.0505	$C_{15}H_{11}O_7$
							449.1112	2.8	6.3	449.1084	$C_{21}H_{21}O_{11}$
										581.1506	$C_{26}H_{30}O_{15}$
Postulated structure: quercetin rhamnoside riboside											
16.9	287.0558	0.2	0.8	287.0551	-0.5	-1.6	287.0551	-0.5	-1.6	287.0556	$C_{15}H_{11}O_6$
				303.0537	3.2	10.6				303.0505	$C_{15}H_{11}O_7$
	433.1130	-0.5	-1.1	433.1126	-0.9	-2.0	433.1139	0.4	1.0	433.1135	$C_{21}H_{21}O_{10}$
				449.1108	2.4	5.4				449.1084	$C_{21}H_{21}O_{11}$
	595.1657	-0.6	-1.0							595.1663	$C_{27}H_{31}O_{15}$
Postulated structure: kaempferol rhamnoside glucoside											
18.0							287.0580	2.4	8.5	287.0556	$C_{15}H_{11}O_6$
							433.1160	2.5	5.8	433.1135	$C_{21}H_{21}O_{10}$
							595.1685	2.2	3.7	595.1663	$C_{27}H_{31}O_{15}$
										741.2242	$C_{33}H_{41}O_{19}$
Postulated structure: kaempferol dirhamnoside glucoside											
19.3	287.0563	0.7	2.6	287.0568	1.2	4.3	287.0561	0.5	1.9	287.0556	$C_{15}H_{11}O_6$
	433.1130	-0.5	-1.1	433.112	-1.5	-3.4	433.1118	-1.7	-3.9	433.1135	$C_{21}H_{21}O_{10}$
										579.1714	$C_{27}H_{31}O_{14}$
Postulated structure: kaempferol dirhamnoside											

Table 1. Exact mass measurements for the MS/MS product ion spectra for the arabidopsis samples, with determined elemental composition for each component.

Conclusion

A metabolomic approach was used to screen for metabolites altered in high-flavonoid content genetically modified tomatoes, compared to control fruit and wild and mutant arabidopsis leaves of different tissue type.

The transgenic tomato plant was transformed with a gene construct that enhances flavonoid biosynthesis. Expression of the two genes in the fruit appeared to result in the accumulation of several new flavonoid-glycosides, which were easily resolved by exact mass neutral loss acquisitions. It was confirmed that flavonoid content was >50-fold higher compared with those of common cultivars. Significantly there was no discernible difference in the taste of the tomatoes as shown by a blind testing but there were increased antioxidant properties associated with the flavonoids and an increase in the potential health properties.

The different type of leaves in the arabidopsis samples showed differences in their flavonoid content and the amounts of flavonoid present.

The key benefits of this approach are:

- Selective (±20 mDa window) and sensitive tool for screening for glycosylated metabolites in complex extracts (19 putative sugar losses monitored)
- Fast screening method for differentially produced glycosides in plants, containing MS and MS/MS information from one single chromatographic run
- Exact mass measurements (<5 ppm RMS) and elemental composition reports provide information on the putative metabolites and their aglycon fragments

These results underline the usefulness of exact mass neutral loss acquisitions for the analysis of complex plant extracts.

References

1. LeGall et al 2003, *J. Agric. Food Chem*., 51, (9), 2003.
2. Ma et al 2000, *J. Am. Soc. Mass Spectrum*, 11, (2), 2000.

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