**Genetic and Phytochemical Characterization of Lettuce Flavonoid Biosynthesis Mutants**

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**Supplementary Figure S1.** Naringenin chalcone hesoxide and malonylhexoside are identified in *nco* lettuce extract based on MS and UV spectra.

**Supplementary Figure S2.** Chromatograms of non-hydrolyzed lettuce extracts from 18-week old plants.

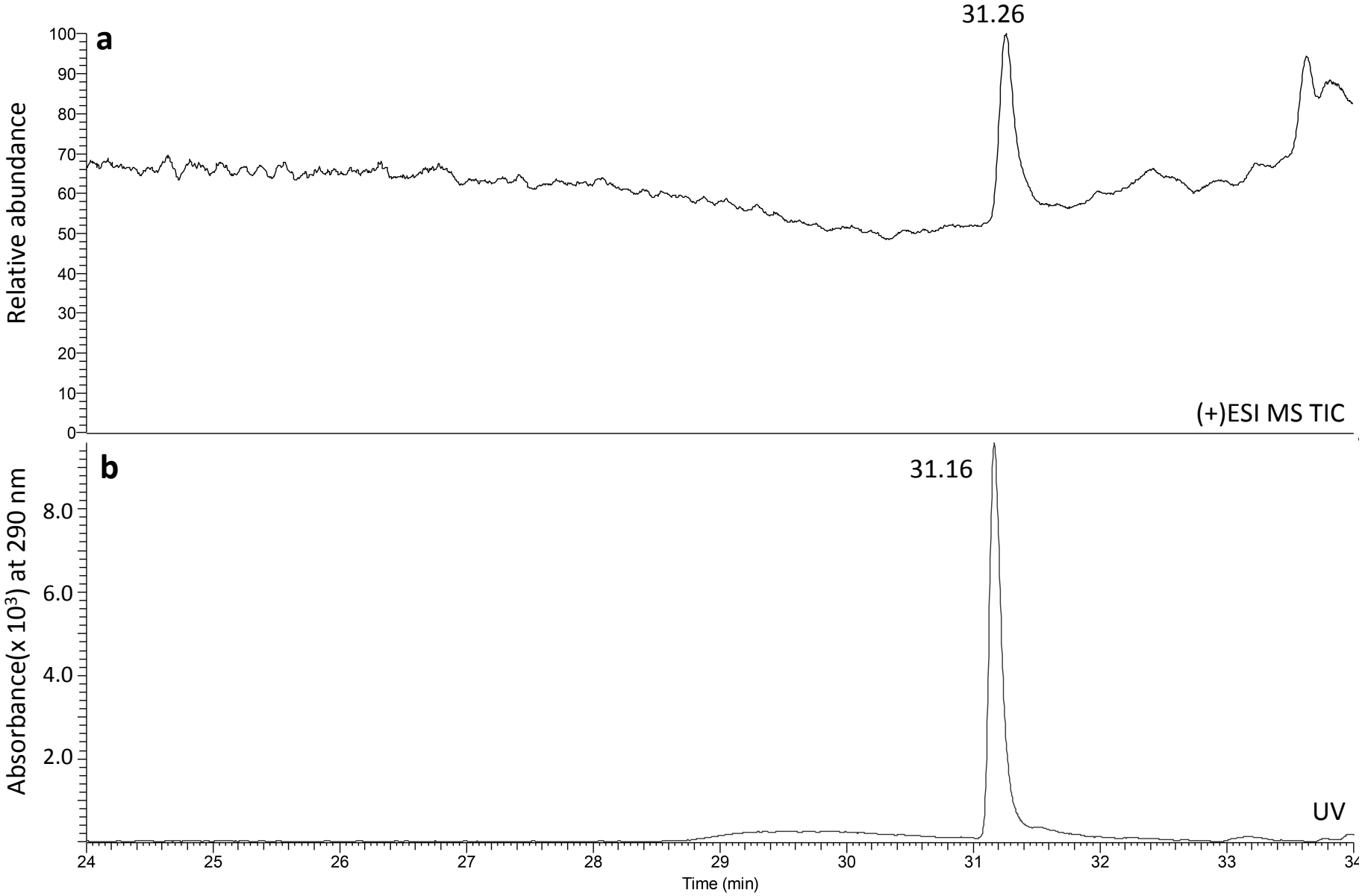
**Supplementary Figure S3.** Chromatograms of acid hydrolyzed lettuce extracts from 18-week old plants.

**Supplementary Table S1.** Phenotype segregation ratios in individual *kfoA*, *kfoB* and *nco* lines.

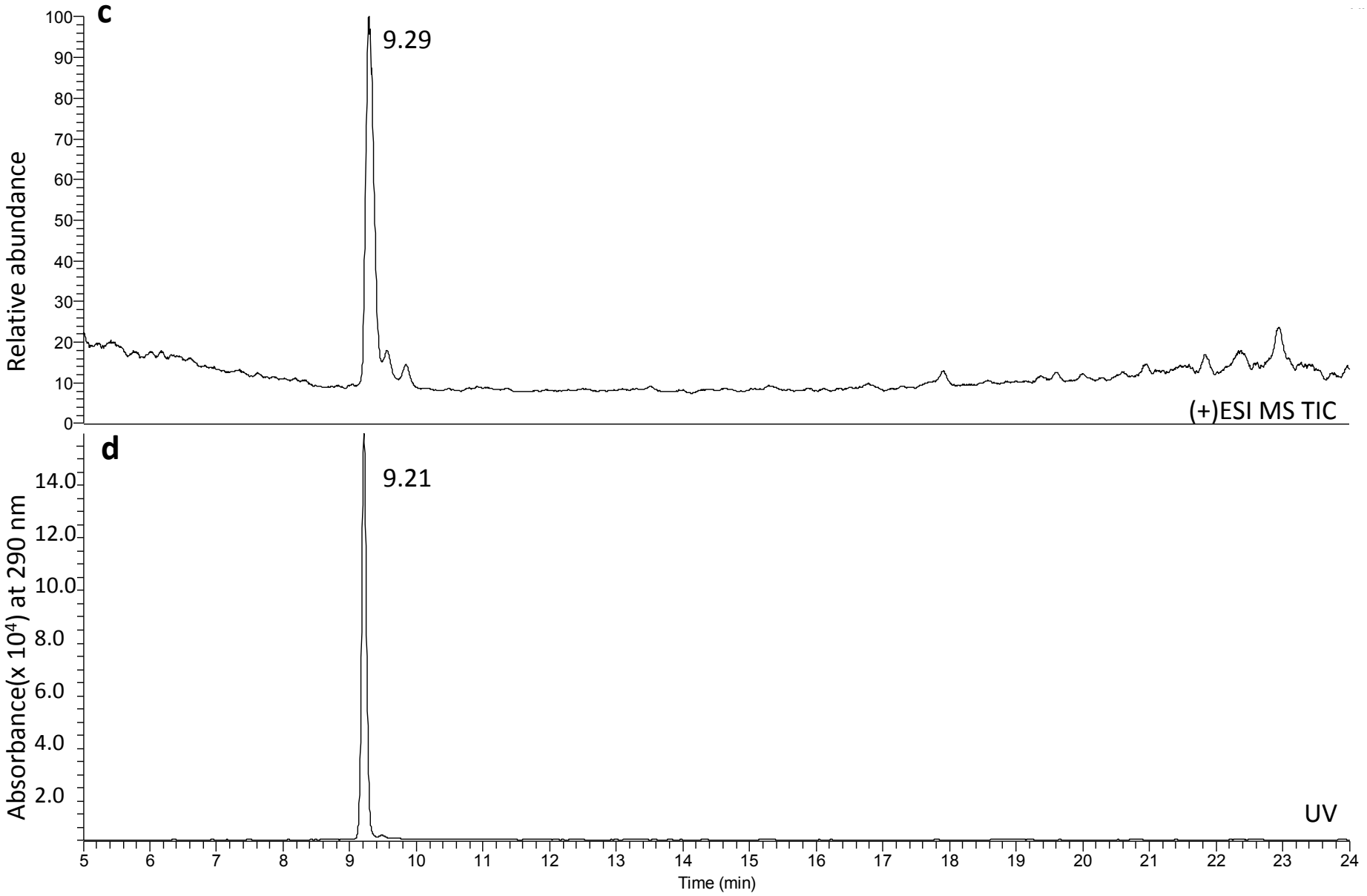
**Supplementary Table S2.** Primers for PCR amplification and sequencing of *CHI*, *F3H* and *F3'H* in lettuce.

**Supplementary Figure S1.** Naringenin chalcone hesoxide and malonylhexoside are identified in *nco* lettuce extract based on MS and UV spectra. Shown are (+) ESI MS Total Ion Current (TIC); m/z 100-1,000 and corresponding UV spectra, and (+) ESI MS and relative absorbance spectra of select peaks. (+) ESI MS TIC of (**a**) naringenin, (**c**) prunin (naringenin-7-O-glucoside), (**e**) naringenin chalcone, (**g**) *nco* methanolic extract; UV absorbance spectra of (**b**) naringenin at 290 nm, (**d**) prunin at 290 nm, (**f**) naringenin chalcone at 365 nm, (**h**) *nco* methanolic extract at 290 nm, (**i**) *nco* methanolic extract at 365 nm; (+) ESI MS of (**j**) naringenin (retention time (RT) 31.26), (**k**) prunin (RT 9.29), (**l**) naringenin chalcone (RT 31.16), (**m**) *nco* methanolic extract (RT 19.12), (**n**) *nco* methanolic extract (RT 22.52); absorbance spectra of (**o**) naringenin (RT 31.16), (**p**) prunin (RT 9.21), (**q**) naringenin chalcone (RT 31.07), (**r**) *nco* methanolic extract (RT 19.12), (**s**) *nco* methanolic extract (RT 22.52). Note that (+) ESI MS spectra of naringenin (**j**) and naringenin chalcone (**l**) share an identical m/z 273.075 [M+H]. This [M+H] value is also observed in prunin (**k**) and two separate peaks (**m,n**) of the *nco* extract. Therefore, naringenin and naringenin chalcone glycosides cannot be distinguished based on (+) ESI MS spectra. However, naringenin (**o**) and naringenin chalcone (**q**) differ in their UV absorbance spectra. Prunin (**p**) has a similar absorbance spectrum to naringenin. Both peaks of the *nco* extract that share the m/z 273.075 (**r,s**) have an absorption spectrum almost identical to naringenin chalcone, and different from naringenin. Based on their UV and mass spectra as well as literature data [27], we identify the RT19.12 peak as naringenin chalcone hexoside, and the RT22.52 peak as naringenin chalcone malonylhexoside.

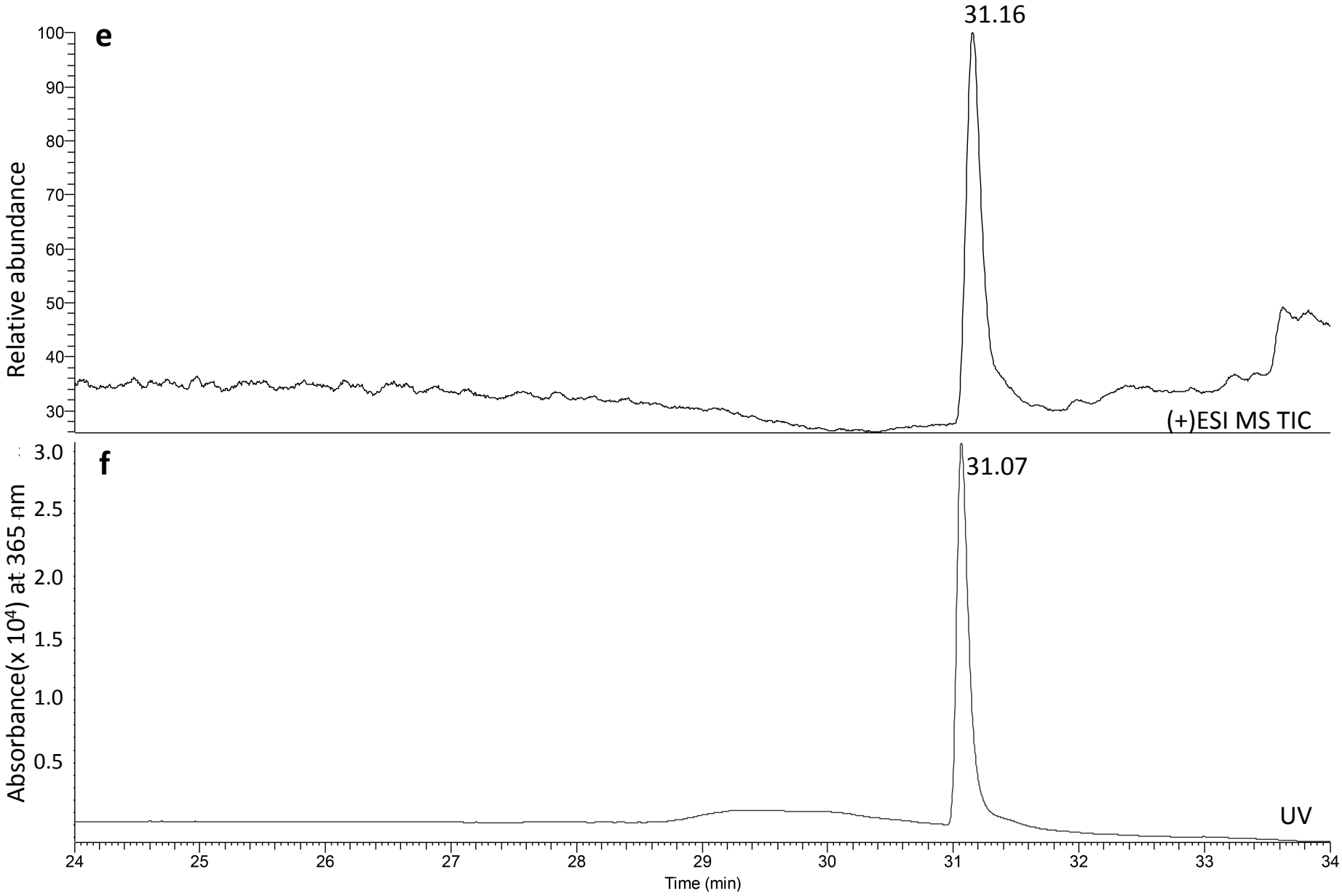
# Naringenin 20 ng



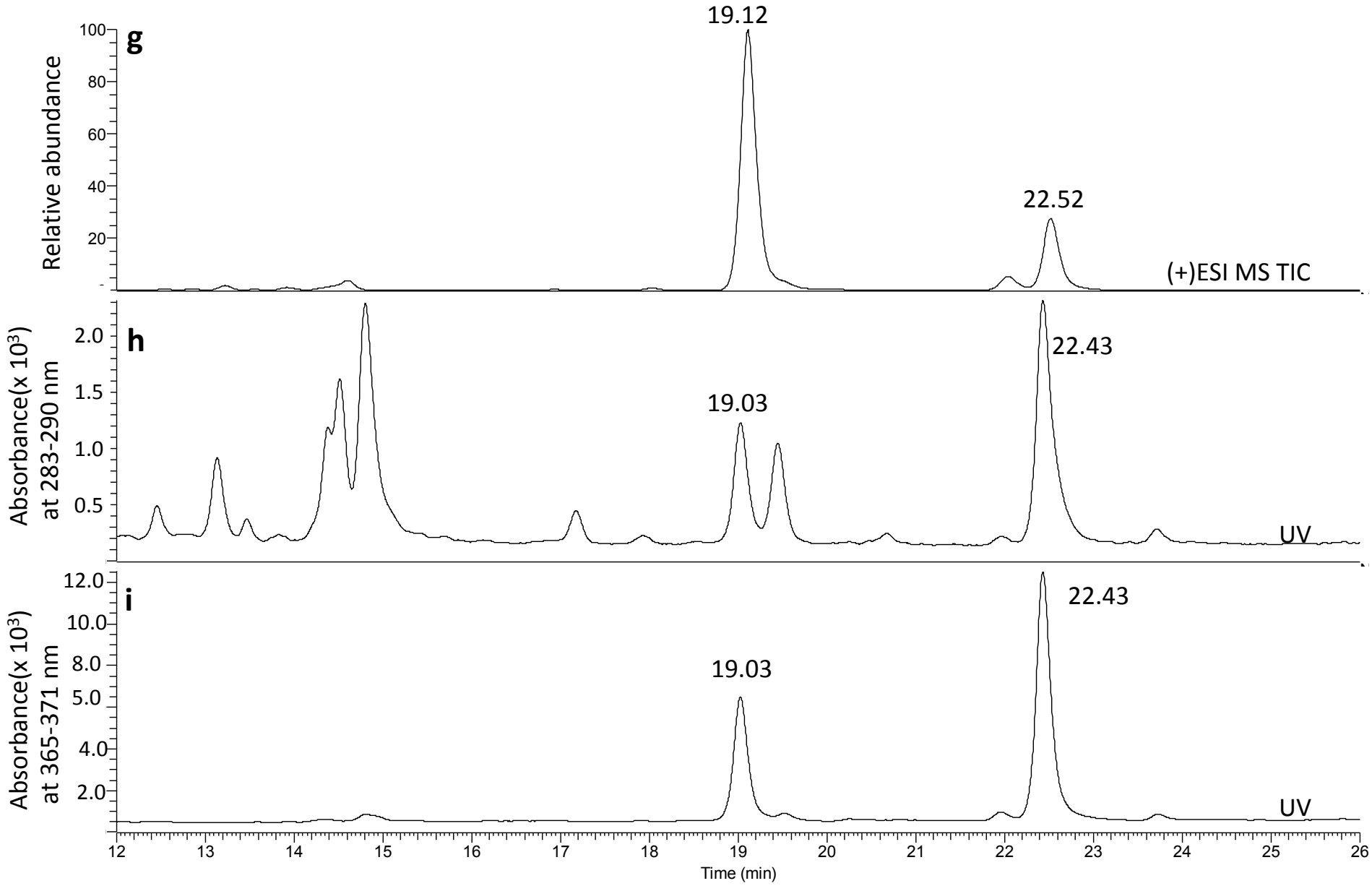
# Prunin 100 ng



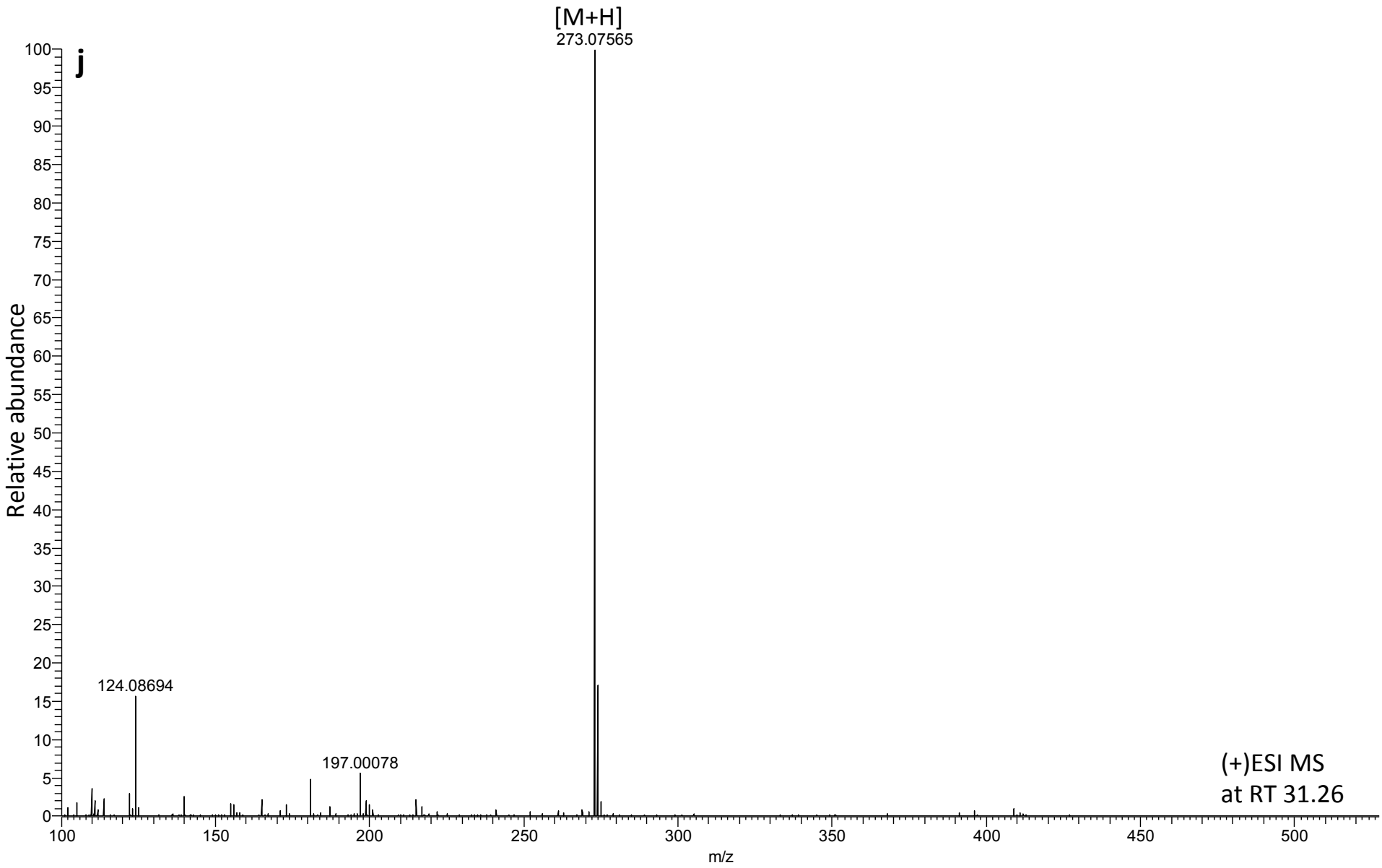
# Naringenin chalcone 20 ng



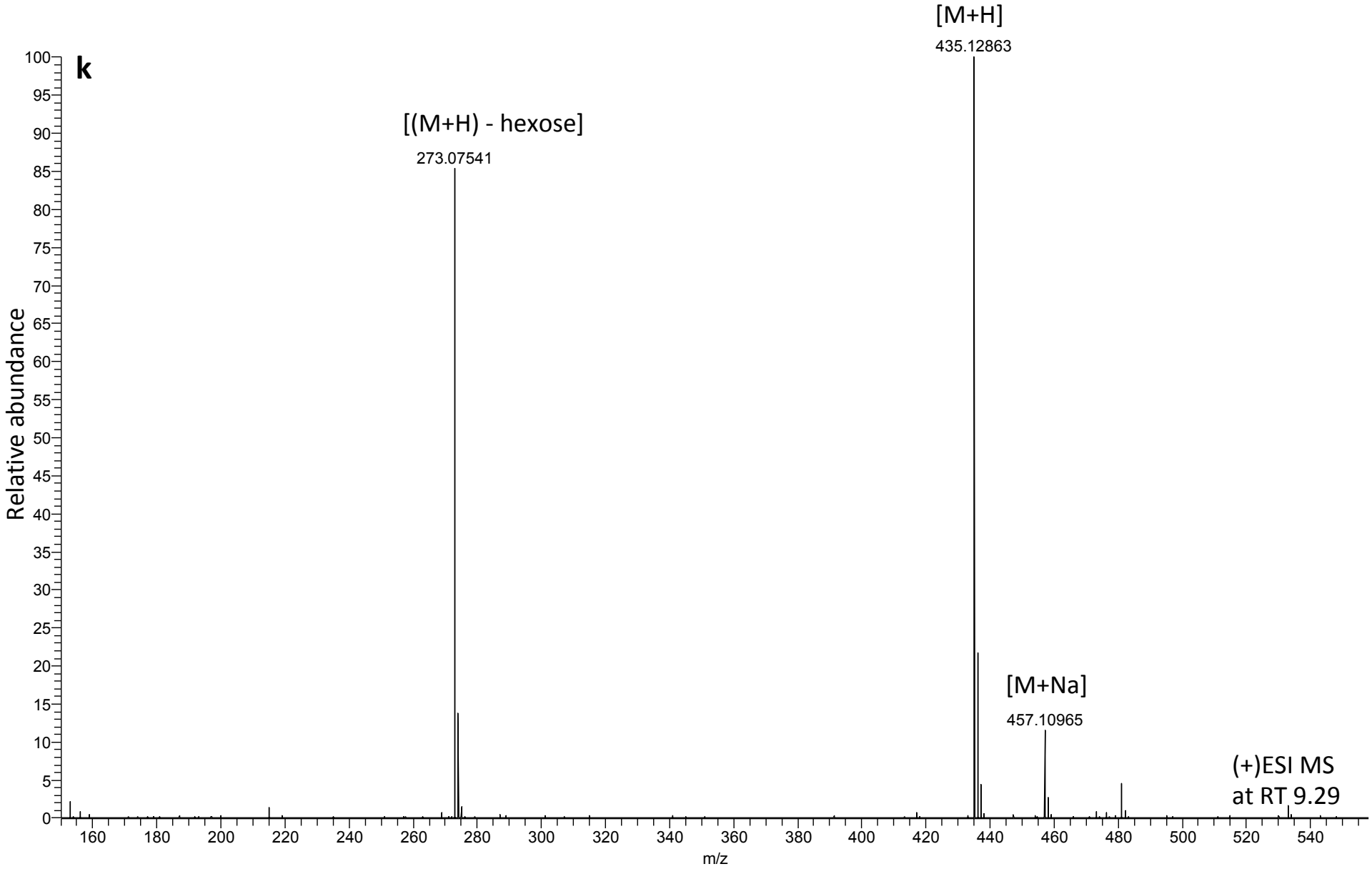
# *Nco* extract


g
19.12
22.52
(+)ESI MS TIC
Relative abundance
h
19.03
22.43
UV
Absorbance(x 10³) at 283-290 nm
i
19.03
22.43
UV
Absorbance(x 10³) at 365-371 nm
Time (min)

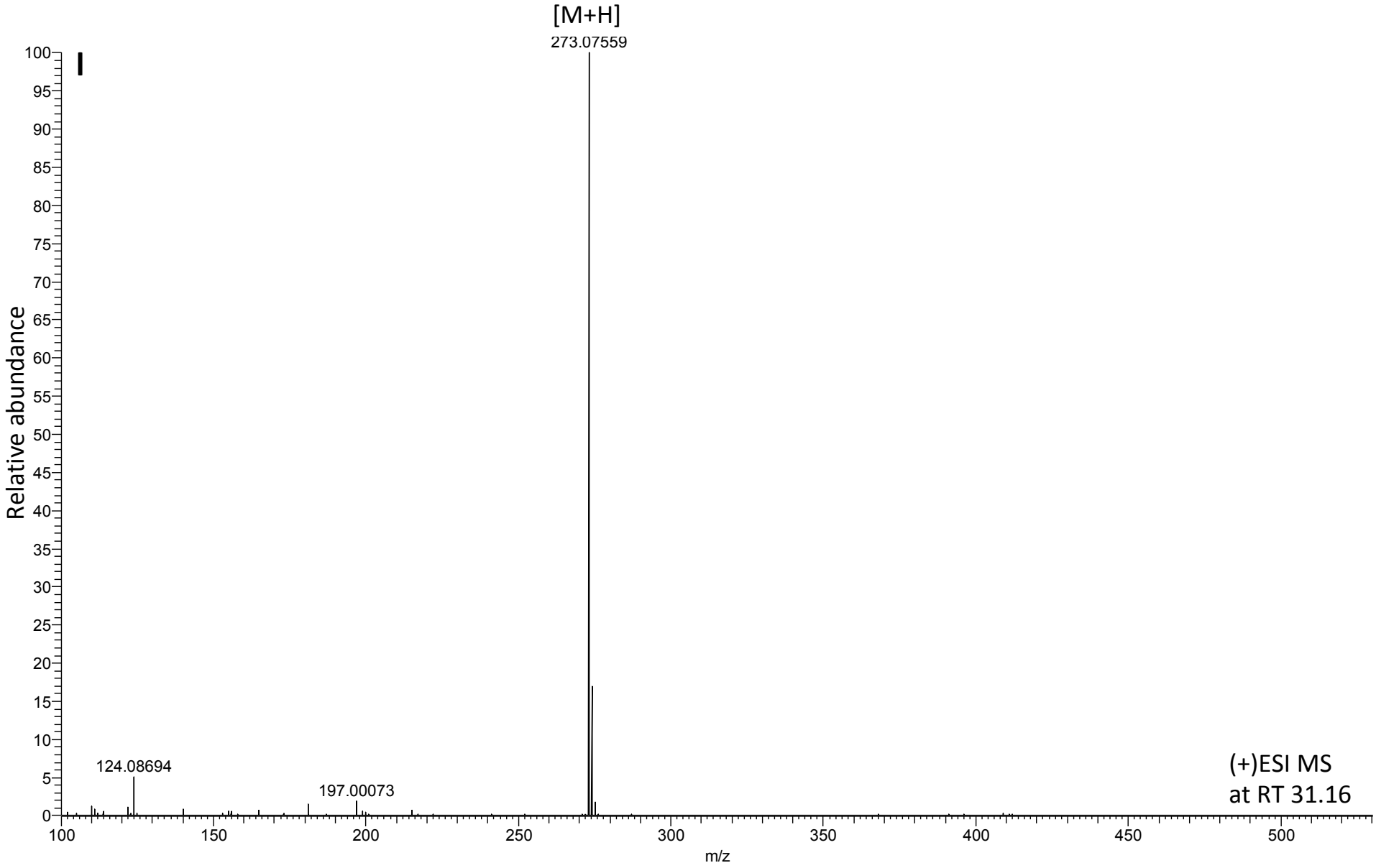
# Naringenin 20 ng



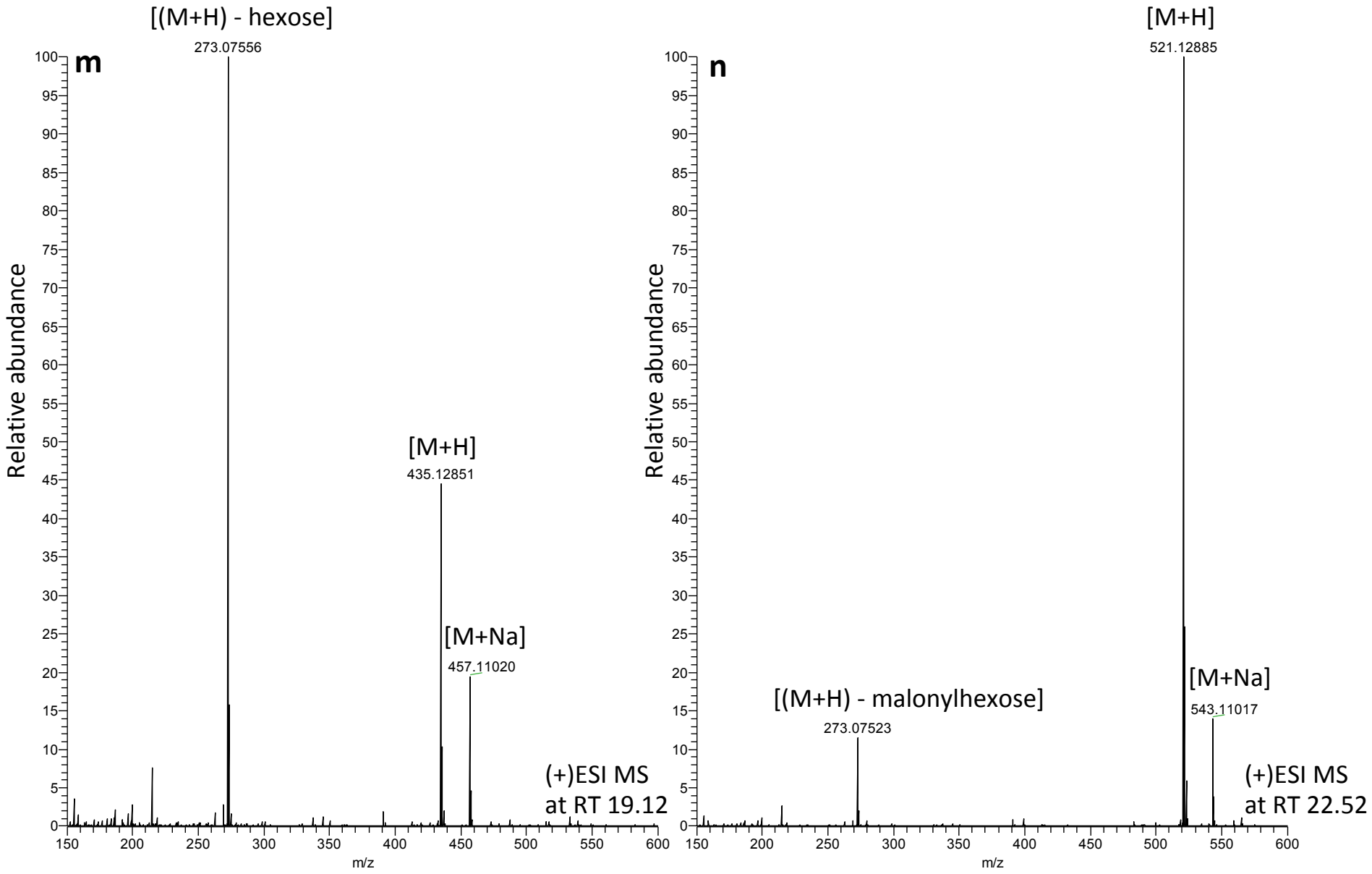
# Prunin 100 ng



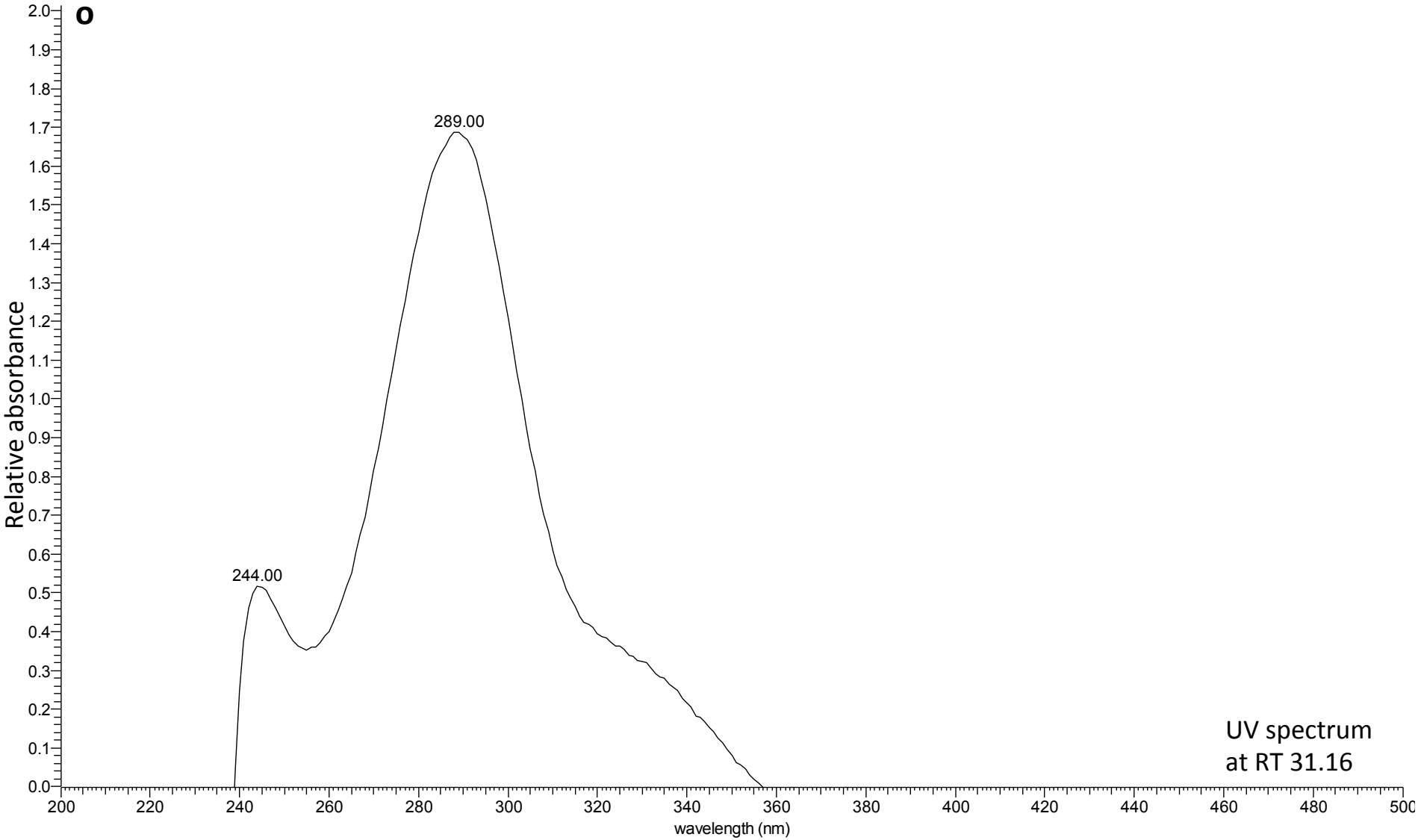
# Naringenin chalcone 20 ng



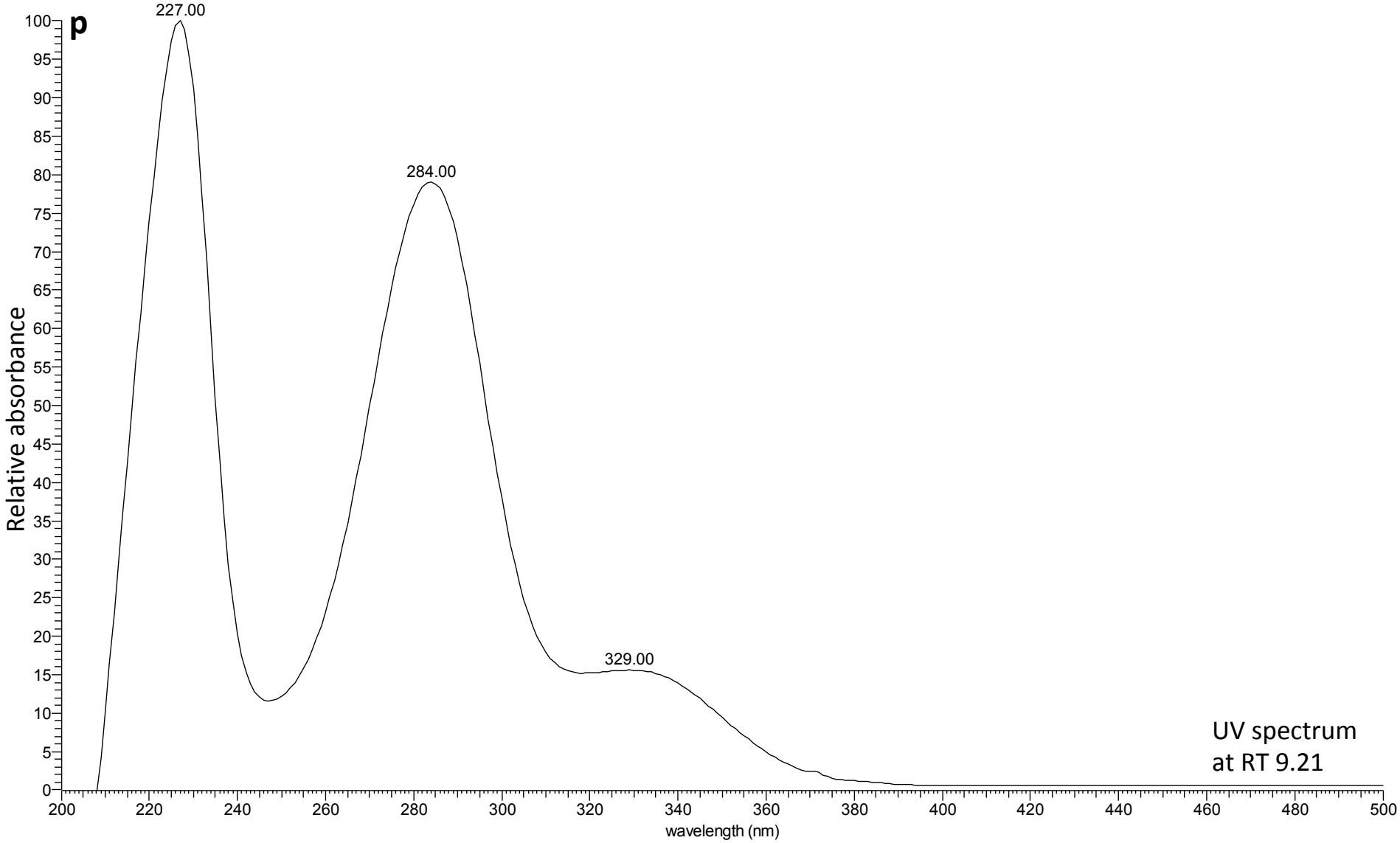
# *Nco* extract


m
[(M+H) - hexose]
273.07556
[M+H]
435.12851
[M+Na]
457.11020
(+)ESI MS
at RT 19.12
Relative abundance
m/z
n
[M+H]
521.12885
[(M+H) - malonylhexose]
273.07523
[M+Na]
543.11017
(+)ESI MS
at RT 22.52
Relative abundance
m/z

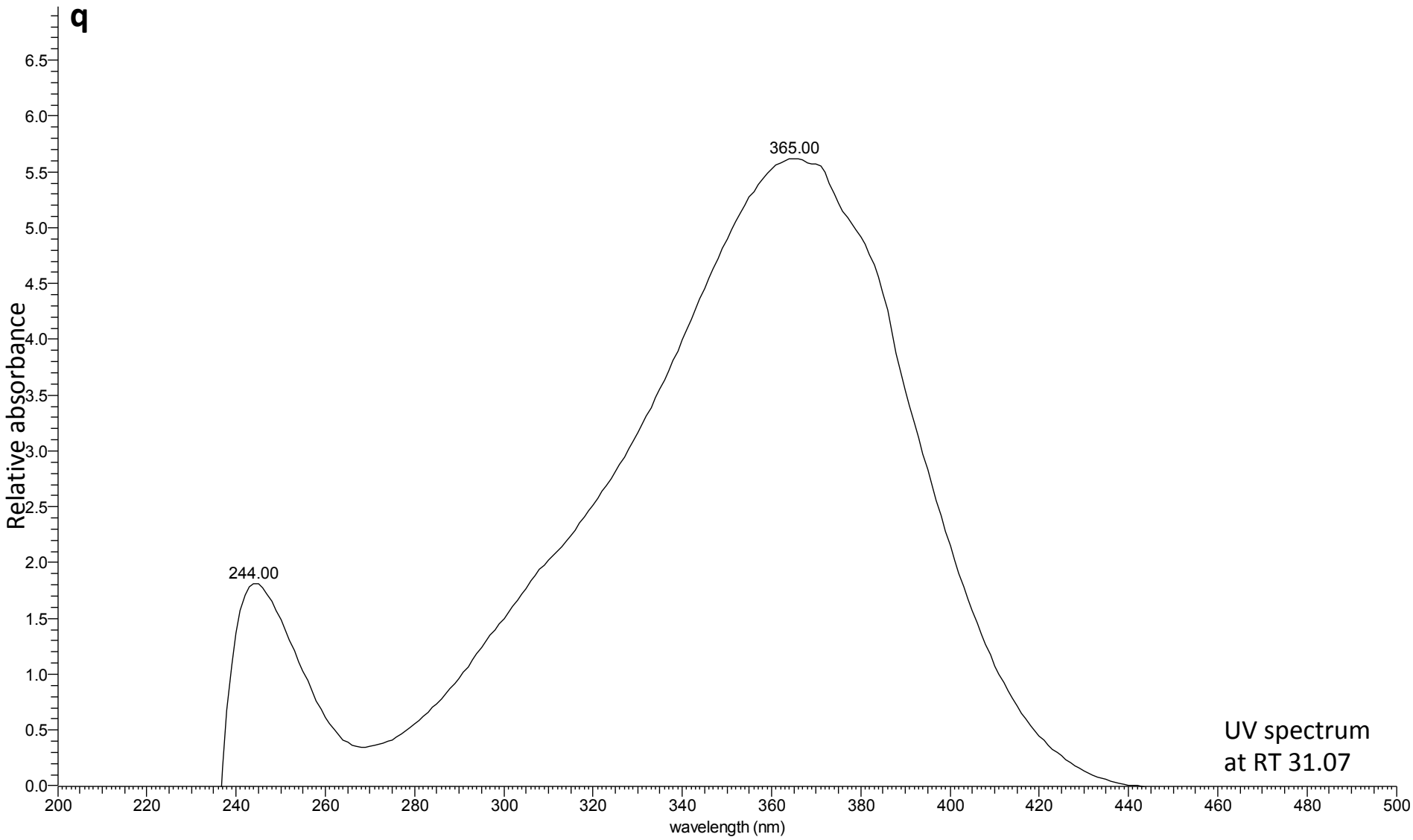
# Naringenin 20 ng



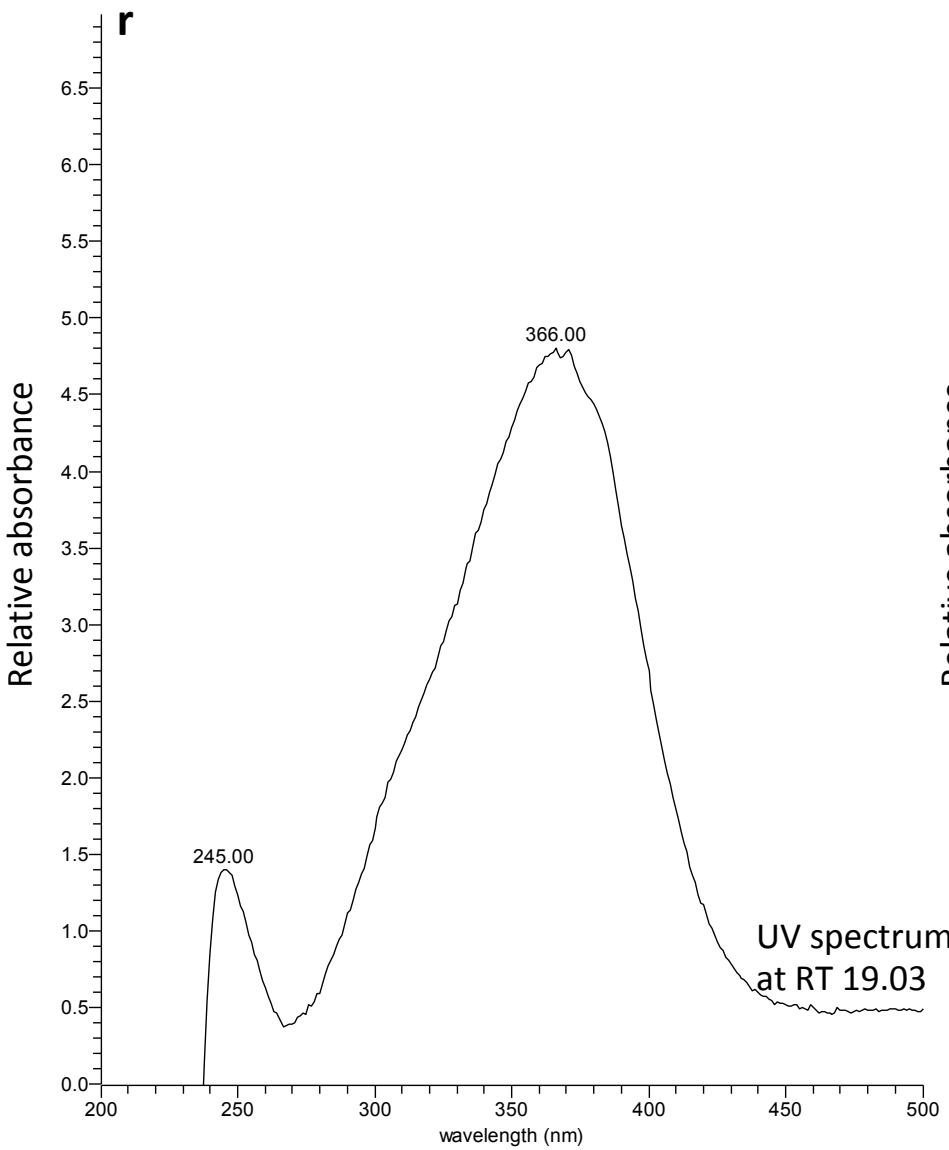
# Prunin 100 ng

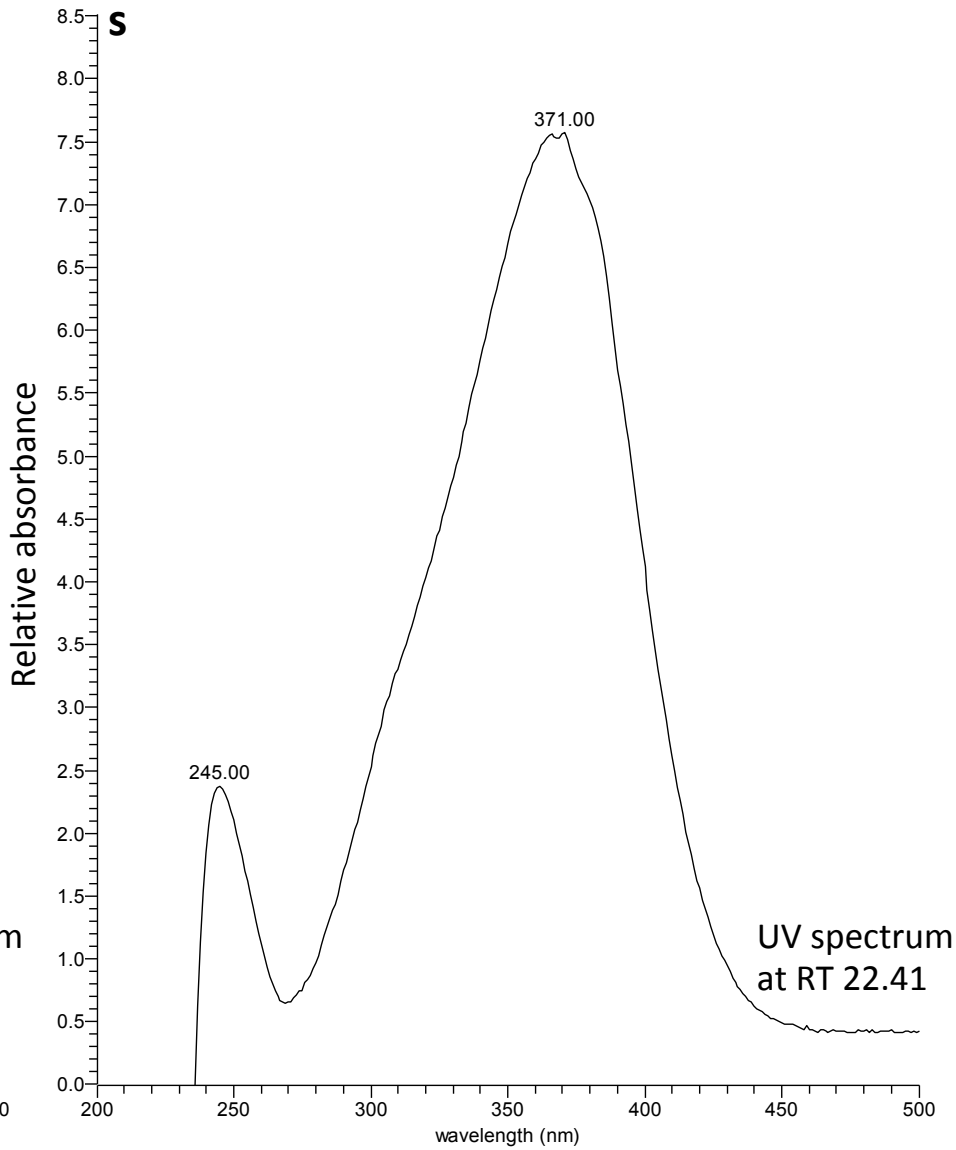


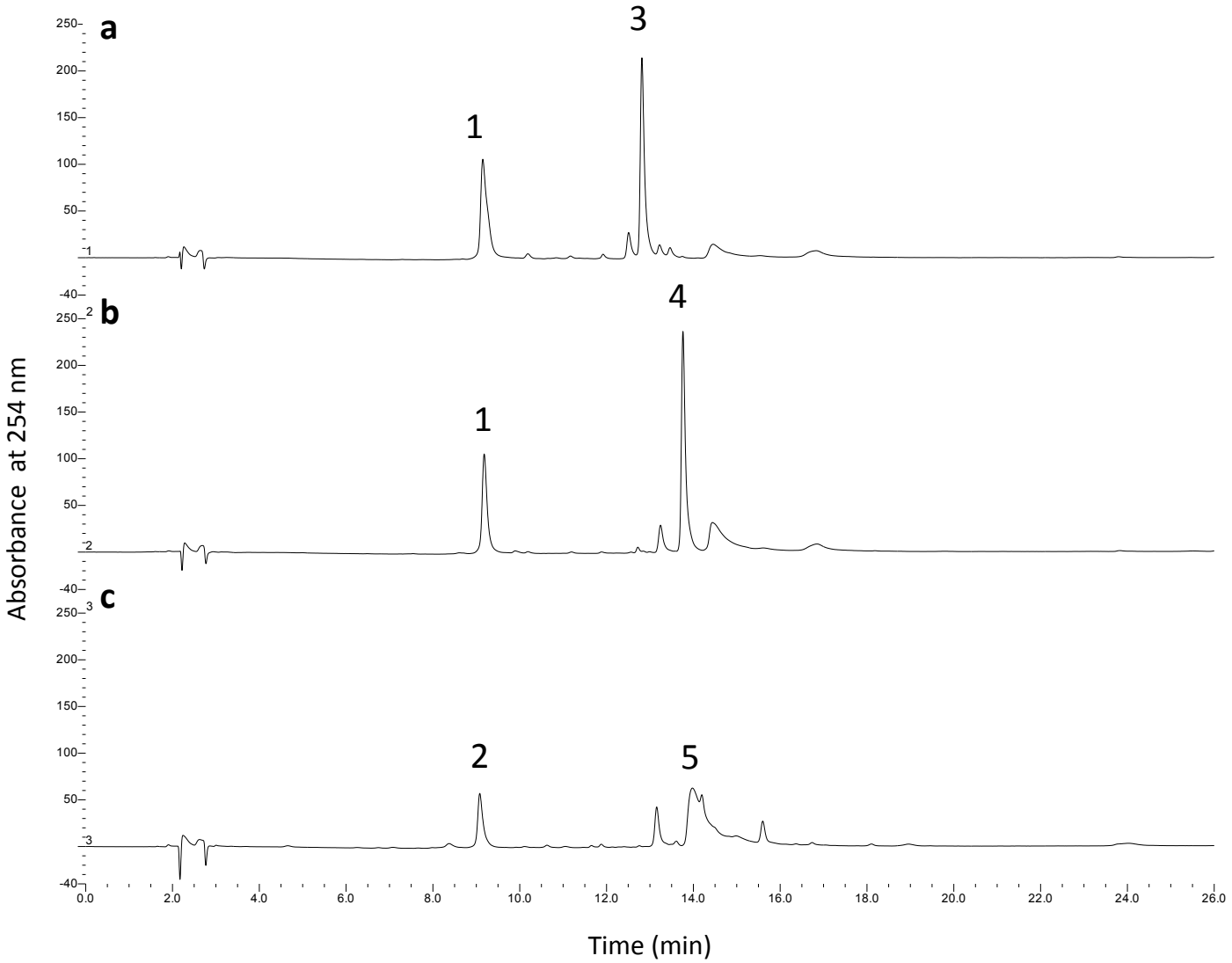
# Naringenin chalcone 20 ng



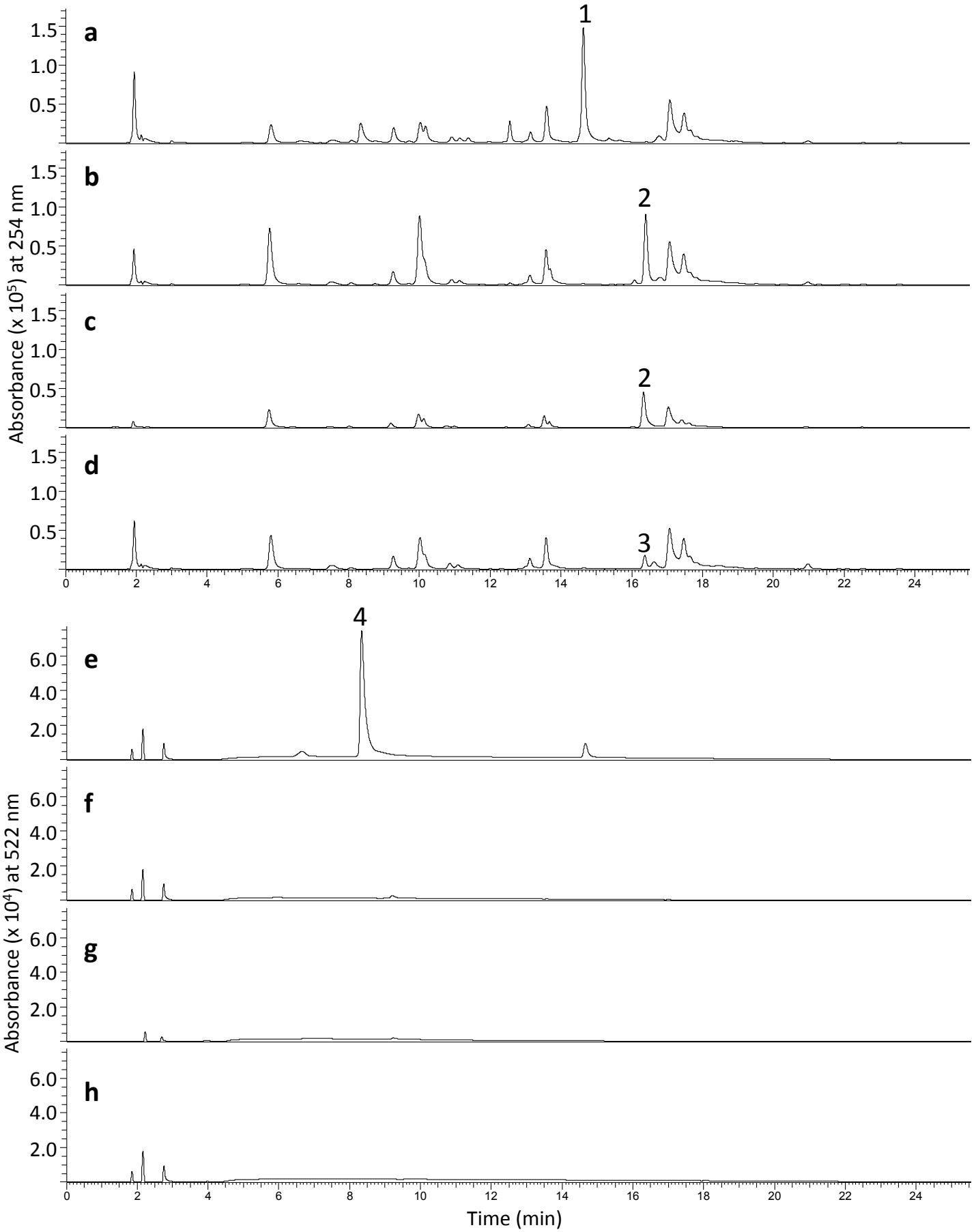
# *Nco* extract


r
Relative absorbance
366.00
245.00
UV spectrum
at RT 19.03
wavelength (nm)


s
Relative absorbance
371.00
245.00
UV spectrum
at RT 22.41
wavelength (nm)




**Supplementary Figure S2.** Chromatograms of non-hydrolyzed lettuce extracts from 18-week old plants. Shown are representative chromatograms of *cv.* Firecracker (**a**), *kfoA* (**b**) and *nco* (**c**) at 254 nm. Labeled peaks are: co-eluted chlorogenic acid and cyanidin 3-O-malonylglucoside (1), chlorogenic acid (2), quercetin-3-O-malonylglucoside (3), kaempferol 3-O-malonylglucoside (4) mix of naringenin chalcone glycosides (5). Note that not all flavonoid peaks are visible on these chromatograms at the scale shown. An additional run using different solvent gradients was used to separate chlorogenic acid and cyanidin 3-O-malonylglucoside (not shown).




**Supplementary Figure S3.** Chromatograms of acid hydrolyzed lettuce extracts from 18-week old plants. Shown are representative chromatograms of *cv.* Firecracker (a, e), *kfoA* (b, f) , *kfoB* (c, g) and *nco* (d, h) at 254 nm (a-d) and 522 nm (e-h). Labeled peaks are: quercetin (1), kaempferol (2), naringenin (3), cyanidin (4). Note that not all flavonoid peaks are visible on these chromatograms at the scale shown.

**Supplementary Table S1.** Phenotype segregation ratios in individual *kfoA, kfoB* and *nco* lines. M, mutant allele; +, wild type allele.

| Parent line | Parent genotype | Number of mutant offspring | Number of wild type offspring | Total number of offspring | Percentage of mutant offspring | Expected number of mutant | Expected number of wild type | Chi square value ( p=0.05, Chi |
|---|---|---|---|---|---|---|---|---|
| *kfoA*-3-3 | M/M | 45 | 0 | 45 | 100.0% | 45 | 0 | |
| *kfoA*- 6-1 | M/M | 68 | 0 | 68 | 100.0% | 68 | 0 | |
| *kfoA*-6-101 | M/M | 92 | 0 | 92 | 100.0% | 92 | 0 | |
| *kfoA-6-1-88* | M/M | 42 | 0 | 42 | 100.00% | 42 | 0 | |
| *kfoA-3-3-37* | M/M | 38 | 0 | 38 | 100.00% | 38 | 0 | |
| *kfoA-6-1-25* | M/M | 45 | 0 | 45 | 100.00% | 45 | 0 | |
| *kfoA-6-1-58* | M/M | 55 | 0 | 55 | 100.00% | 4.5 | 13.5 | 3.630 |
| *kfoA*-3 | +/M | 13 | 20 | 33 | 39.4% | 8.25 | 24.75 | 3.646 |
| *kfoA*-6 | +/M | 11 | 23 | 34 | 32.4% | 8.5 | 25.5 | 0.980 |
| **Sum *kfoA*** | **M/M** | **385** | **0** | **385** | **100.0%** | **385** | **0** | |
| **Sum *kfoA*** | **+/M** | **32** | **53** | **85** | **37.6%** | **21.25** | **63.75** | **7.251** |
| **Sum *kfoA*** | **+/+** | **n/a** | **n/a** | **n/a** | **n/a** | | | |
| | | | | | | | | |
| *kfoB*-1 | M/M | 19 | 0 | 19 | 100.0% | 19 | 0 | |
| *kfoB-2-2* | M/M | 15 | 0 | 15 | 100.00% | 15 | 0 | |
| *kfoB-2-3* | M/M | 33 | 0 | 33 | 100.00% | 33 | 0 | |
| *kfoB*-2 | +/M | 3 | 17 | 20 | 15.0% | 5 | 15 | 1.067 |
| *kfoB*-3 | +/M | 6 | 14 | 20 | 30.0% | 5 | 15 | 0.267 |
| *kfoB*-7 | +/M | 5 | 15 | 20 | 25.0% | 5 | 15 | 0.000 |
| *kfoB*-4 | +/+ | 0 | 18 | 18 | 0.0% | 0 | 18 | |
| *kfoB*-5 | +/+ | 0 | 19 | 19 | 0.0% | 0 | 19 | |
| *kfoB*-6 | +/+ | 0 | 1 | 1 | 0.0% | 0 | 1 | |
| **Sum *kfoB*** | **M/M** | **67** | **0** | **67** | **100.0%** | **67** | **0** | |
| **Sum *kfoB*** | **+/M** | **14** | **46** | **60** | **19.4%** | **18** | **54** | **2.074** |
| **Sum *kfoB*** | **+/+** | **0** | **38** | **38** | **0.0%** | **0** | **38** | |
| | | | | | | | | |
| *nco*-1 | M/M | 49 | 0 | 49 | 100.0% | 49 | 0 | |
| *nco*-1-3 | M/M | 25 | 0 | 25 | 100.0% | 25 | 0 | |
| *nco-2-203* | M/M | 28 | 0 | 28 | 100.00% | 28 | 0 | |
| *nco 1-413* | M/M | 10 | 0 | 10 | 100.00% | 10 | 0 | |
| *nco*-2 | +/M | 12 | 28 | 40 | 30.0% | 10 | 30 | 0.533 |
| *nco*-4 | +/M | 6 | 18 | 24 | 25.0% | 6 | 18 | 0.000 |
| *nco*-3 | +/+ | 0 | 11 | 11 | 0.0% | 0 | 11 | |
| *nco*-6 | +/+ | 0 | 16 | 16 | 0.0% | 0 | 16 | |
| **Sum *nco*** | **M/M** | **112** | **0** | **112** | **100.0%** | **112** | **0** | |
| **Sum *nco*** | **+/M** | **18** | **46** | **64** | **28.1%** | **16** | **48** | **0.333** |
| **Sum *nco*** | **+/+** | **0** | **27** | **27** | **0.0%** | **0** | **27** | |

**Supplementary Table S2.** Primers for PCR amplification and sequencing of *CHI, F3H* and *F3'H* in lettuce.

| Amplified gene | Primer name | Primer sequence | Product size |
|---|---|---|---|
| *CHI* | CHI_16F | AAGAGCTAAACCATCAGTCAAACAC | 658 |
| | CHI_673R | CCATACCTTCCGATCACCGAC | |
| | CHI_86F | AGTCTCCAGATCGAATCCGTCG | 783 |
| | CHI_868R | TGAGCTTTATTTTAAGATCACATGCGTAG | |
| *F3H* | F3H_4F | CACCGTCTTCTTCCTCCCTTG | 1186 |
| | F3H_1189R | TCATTACATATGATTGGTGGTAGATGC | |
| *F3'H* | F3'H_61F | CCGGTACTCACCATTCAAACGC | 1835 |
| | F3'H_1895R | TCCATTGCTATCACTAGAATGTGGG | |
| | F3'H_155F | TCCCACCGCTCATTTTACCAAC | 1815 |
| | F3'H_1969R | TGTTGTTTGATTATTGACGCACAC | |
| | F3'H_249F | ACTTTTTGCAGTGAGCTCCCATTT | for sequencing |
| | F3'H_1345R | CAACATCGGTGACTTCATCCCG | |
| | F3'H_1524F | AAGATGATGCCGATGGAGAGGG | |