Effect of *Agave americana* and *A. salmiana* Ripeness on Saponin Content from Aguamiel (Agave Sap).

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MATERIALS AND METHODS

- **Saponins Extract Obtention**

Aguamiel from immature *Agave salmiana* (3.5 L) was mixed with 3.5 L of *n*-butanol in a 20 L capacity stirred tank (Chemglass Inc., Vineland, NJ) at 100 rpm for 60 min. The upper phase was recovered after centrifugation (3000 g, 10 min and 25 °C) and mixed with an equal volume of distilled water (100 rpm for 30 min). After centrifugation, the upper phase was washed again following the same conditions. Finally the solvent was eliminated under vacuum at 40 ºC (Rocket, Genevac, Ltd in Ipswich, UK). A total of 840 mg were obtained.

- **Fractionation by Solid Phase Extraction**

Saponins extract (120 mg) was dissolved in 20 mL HPLC grade water and passed through a previously conditioned SPE cartridge Strata C18 (8B-S001-LEG, Phenomenex, Torrance, CA). The cartridge was washed twice with 20 mL HPLC grade water, twice with HPLC grade methanol:water (60:40) solution, and finally the compounds were eluted twice with 20 mL methanol. The compounds of interest were found in the 100% methanol fraction (SPEMeOH) and it was vacuum concentrated (EZ-2-Plus, Genevac, Suffolk, UK) at 50 °C to a volume of 4 mL. The concentrated fraction was stored at -20 °C until use.

- **Purification by Semipreparative HPLC**

A 1 mL aliquot of SPEMeOH was filtered through an Accrodisc GHP 0.45 µm membrane filter (Pall Life Sciences, Port Washington, NY). Fractionation was carried out on a Zorbax SB-C18 semipreparative column, 9.4x250 mm, 5µm (Agilent Technologies, Santa Clara, CA), using water (0.1% formic acid)/methanol 25/75 at 2 mL/min flow rate. Fractions were reprocessed up

to three times to improve sample purity. Only kammogenin glycosides were obtained without contamination of saponins from different aglycones.

- **Fraction Acid Hydrolysis**

In order to determine the glycoside fragment patterns, a fraction that contained compounds **5** and **6** was hydrolyzed in 1 mL HCl (2 N) for 1 h in a water bath at 80 °C. To partially hydrolyze and detect tetraglycosidic saponins derived from compounds **5** and **6,** a different aliquot was hydrolyzed for 15 min. The hydrolyzed fractions were neutralized with NaOH (2 N). Sapogenin and saponin fragments were recovered by adding 2 mL ethyl acetate, mixing and collecting the upper phase, repeated three consecutive times.

- **Separation and Identification by Liquid Chromatography−Electrospray Ionization−Tandem Mass Spectrometry (LC−ESI−MS2) of Penta- and Tetraglycosides of Kammogenin, Manogenin and Gentrogenin.**

Saponin fractions were analyzed by liquid chromatography-electrospray-tandem mass spectrometry (LC-ESI-MS^2) on an 1100 series HPLC instrument equipped with an ion trap mass detector with ESI source (Agilent Technologies, Santa Clara, CA). Ionization conditions were adjusted to 300 °C and 4 kV capillary temperature and voltage, respectively. The full scan covered the mass range from *m/z* 150 to 2000. The nebulizer pressure was 50 psi and flow-rate of nitrogen 10.0 L/min. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas. Data were acquired in positive mode.

Comparison of kammogenin saponins (compound **5** and **6**) was conducted by the isolation of mass peaks *m/z* 1247 and *m/z* 1217 (**Figure S1-A**). Comparison of manogenin saponins (compound **7** and **8**) was conducted by the isolation and fragmentation of mass peaks *m/z* 1249,

1219, 1117 and 1087. Comparison of gentrogenin saponins (compound **9** and **10**) was conducted by the isolation and fragmentation of mass peaks *m/z* 1179, 1047, 915 and 885.

RESULTS AND DISCUSSION

The difference between kammogenin glycosides (compounds **5** and **6)** was the substitution of a xylose for a hexose. Magueyoside A (compound **5)** has two xyloses at the end of the ramifications of the glycosidic chain, when it was fragmented at the ion trap an ion with *m/z* 1085.51 was observed. The compound **5** (kammogenin glycoside) exhibited characteristic ions at *m/z* 1247.3 ($[M+ Na]^+$) and *m/z* 1225.3 ($[M+ H]^+$) **(Figure S1-B)**. The compound **6** (kammogenin glycoside) exhibited the presence of ions at *m/z* 1217.3 ($[M+ Na]^+$) and *m/z* 1195.3 ($[M+ H]^+$) **(Figure S1-C).** Compound **5** differed from compound **6** only by *m/z* 30, which can be attributed to the substitution of a hexose for a pentose. The ions *m/z* 1093.5 ($[M+H-132]^+$) and *m/z* 1063.5 ($[M+H-132]^+$) were obtained as a result of the loss of one pentose from the compound **5** and **6**, respectively (**Figure S2-B** and **S2-C**). The triglycoside fragment was also observed with a characteristic ion 931.4 *m/z* (**Figure S2-D**). This ion was formed from the loss of a second sugar unit, a hexose for the compound **5** ($[M+H-294]^+$) and a pentose in the case of the compound **6** ($[M+H-264]^+$). The ions *m/z* 769.4 ($[M+H-456]^+$), *m/z* 607.4 ($[M+H-618]^+$) and *m/z* 445.3 ($[M+H-780]^+$) correspond to the di, mono and aglycone of compound **5** and **6 (Figure S2-E to S2-G)**. These three ions, along with the triglycoside, were confirmed in the sample hydrolyzed during 60 minutes (**Figure S3**). Compound **5** was similar to compound **6** reported as Magueyoside A by Pérez et al.,[1,2] with the difference of the substitution of a hexose (compound **6**) instead a pentose (compound **5**).

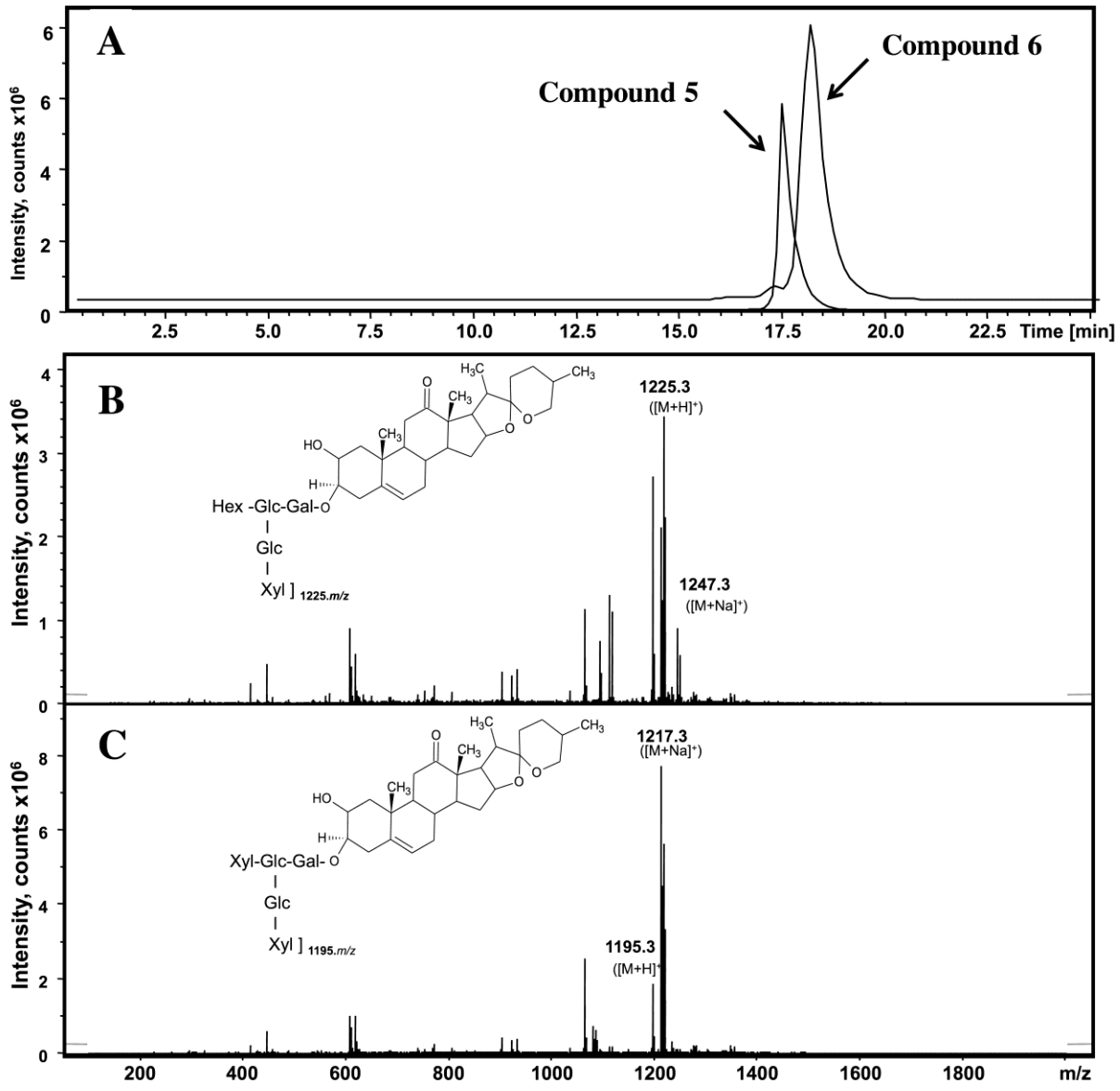

Figure S1 A. Chromatogram of extracted ions for compounds **5** and **6**. **B.** Mass spectrum and tentative structure for compound **5**, kammogenin pentaglycoside. **C.** Mass spectrum and reported structure for compound **6**, magueyoside A.[1,2]

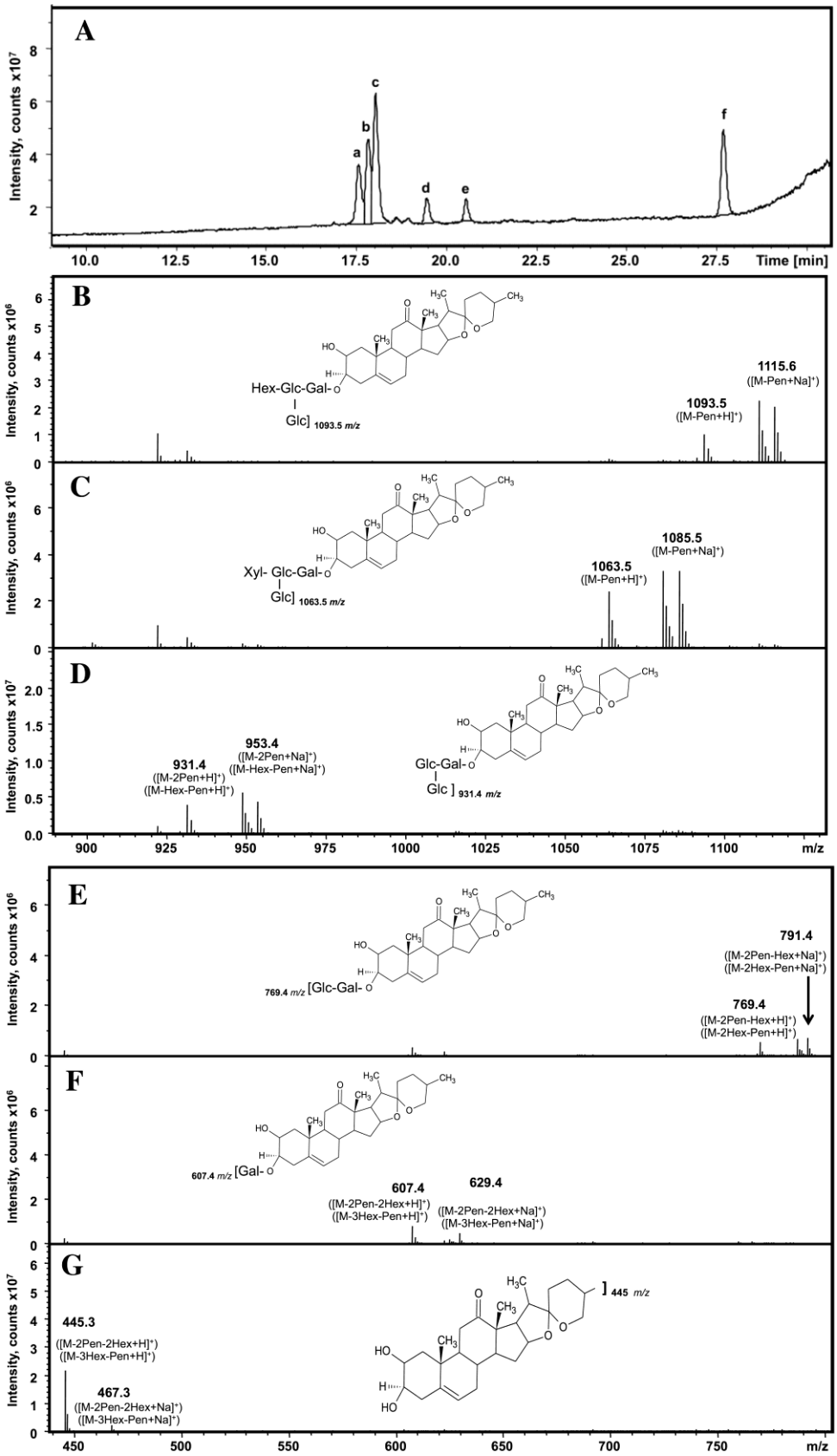
A
Intensity, counts x10^7
a
b
c
d
e
f
Time [min]
B
Intensity, counts x10^6
Hex-Glc-Gal-
Glc] 1093.5 m/z
1115.6
([M-Pen+Na]+)
1093.5
([M-Pen+H]+)
C
Intensity, counts x10^6
Xyl- Glc-Gal-
Glc] 1063.5 m/z
1085.5
([M-Pen+Na]+)
1063.5
([M-Pen+H]+)
D
Intensity, counts x10^7
931.4
([M-2Pen+H]+)
([M-Hex-Pen+H]+)
953.4
([M-2Pen+Na]+)
([M-Hex-Pen+Na]+)
Glc-Gal-
Glc] 931.4 m/z
m/z
E
Intensity, counts x10^6
769.4 m/z [Glc-Gal-
791.4
([M-2Pen-Hex+Na]+)
([M-2Hex-Pen+Na]+)
769.4
([M-2Pen-Hex+H]+)
([M-2Hex-Pen+H]+)
F
Intensity, counts x10^6
607.4 m/z [Gal-
607.4
([M-2Pen-2Hex+H]+)
([M-3Hex-Pen+H]+)
629.4
([M-2Pen-2Hex+Na]+)
([M-3Hex-Pen+Na]+)
G
Intensity, counts x10^7
445.3
([M-2Pen-2Hex+H]+)
([M-3Hex-Pen+H]+)
467.3
([M-2Pen-2Hex+Na]+)
([M-3Hex-Pen+Na]+)
] 445 m/z
m/z

Figure S2 A. Total ion chromatogram for 15 minute hydrolyzed kammogenin glycoside fraction, a – tetraglycoside from compound **5**, b – tetraglycoside from compound **6**, c –triglycoside fragment, d – diglycoside fragment, e – monoglycoside fragment, f – aglycone. **B.** Mass spectrum and tentative structure for tetraglycoside derived from compound **5**. **C.** Mass spectrum and tentative structure of tetraglycoside derived from compound **6**. **D.** Mass spectrum and tentative structure triglycoside derived from compounds **5** and **6**. **E.** Mass spectrum and tentative structure of diglycoside derived from compounds **5** and **6. F.** Mass spectrum and tentative structure of monoglycoside derived from compounds **5** and **6**. **G.** Mass spectrum and aglycone structure.

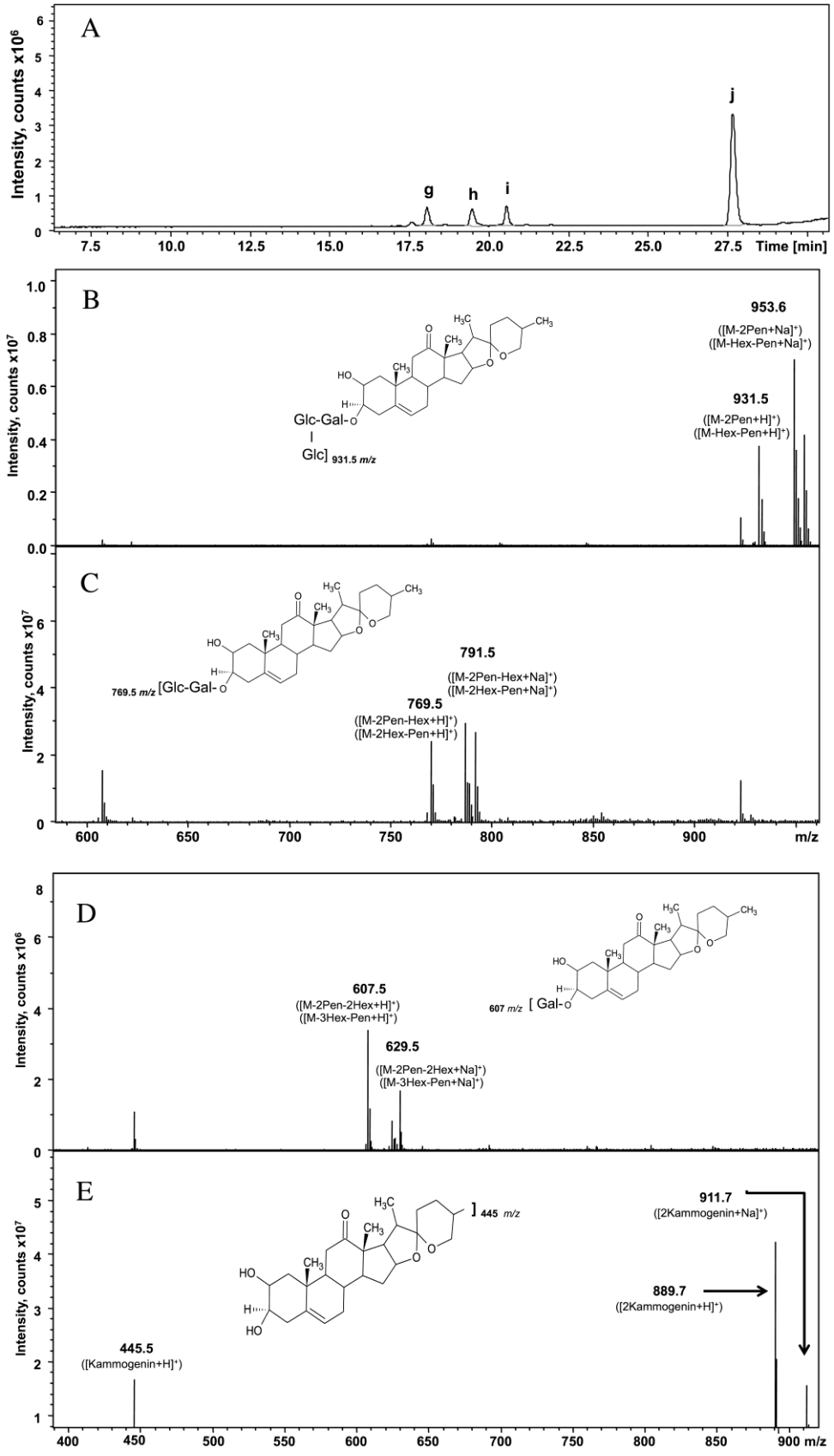
A
Intensity, counts x10^6
g
h
i
j
Time [min]
B
Intensity, counts x10^7
953.6
([M-2Pen+Na]+)
([M-Hex-Pen+Na]+)
931.5
([M-2Pen+H]+)
([M-Hex-Pen+H]+)
Glc-Gal-O
Glc] 931.5 m/z
C
Intensity, counts x10^7
791.5
([M-2Pen-Hex+Na]+)
([M-2Hex-Pen+Na]+)
769.5
([M-2Pen-Hex+H]+)
([M-2Hex-Pen+H]+)
769.5 m/z [Glc-Gal-O
m/z
D
Intensity, counts x10^6
607.5
([M-2Pen-2Hex+H]+)
([M-3Hex-Pen+H]+)
629.5
([M-2Pen-2Hex+Na]+)
([M-3Hex-Pen+Na]+)
607 m/z [Gal-O
E
Intensity, counts x10^7
] 445 m/z
911.7
([2Kammogenin+Na]+)
889.7
([2Kammogenin+H]+)
445.5
([Kammogenin+H]+)
m/z

Figure S3 A. Total ion chromatogram for 60 minute hydrolyzed kammogenin glycoside fraction, g – triglycoside fragment, h – diglycoside fragment, i – monoglycoside fragment, j – aglycone. **B.** Mass spectrum and tentative structure for triglycoside derived from compounds **5** and **6**. **C.** Mass spectrum and tentative structure for diglycoside derived from compounds **5** and **6**. **D.** Mass spectrum and tentative structure for monoglycoside derived from compounds **5** and **6**. **E.** Mass spectrum and kammogenin structure.

The fraction containing compounds **7** and **8** (**Figure S4-A**) was analyzed by tandem mass spectroscopy to compare their fragmentation patterns. For both compounds, the sodium adducts were found at higher intensity than their molecular ions or fragments. Similar to kammogenin glycosides, compound **7** had a hexose instead of the xylose previously reported for magueyoside D or compound **8 (Figure S4)**. Compound **7** presented the parent ion *m/z* 1249.5 ($[M+Na]^+$) (**Figure S4-B**), which is *m/z* 30 greater than parent ion for compound **8** (**Figure S4-C**), with *m/z* 1219.5 ($[M+Na]^+$). Particularly, for compound **7**, two different tetraglycosidic ions patterns were exhibited: *m/z* 1117.5 ($[M+Na-132]^+$) and *m/z* 1087.5 ($[M+Na-132]^+$). In contrast, for compound **8,** only the ion at *m/z* 1087.5 ($[M+Na-132]^+$) was observed, confirming that the two end sugars were xylose as reported for magueyoside D.[2] Different triglycoside fragments were observed. Compound **7** had an ion at *m/z* 955.4 ($[M+Na-294]^+$), when hexose and pentose units were lost **(Figure S4-B)**, whereas compound **8** had one at *m/z* 925.4 ($[M+Na-294]^+$). Contrastingly, compound **7** exhibited the loss of a hexose, whereas compound **8** loss a pentose to come up with the di and monoglycosides with *m/z* 793.4 ($[M+Na-456]^+$) and *m/z* 631.3 ($[M+Na-618]^+$), respectively. This evidence suggests that compound **7** was similar to compound **8**, which was previously reported by Magueyoside D Perez et al.[2] but had a hexose instead of one of the xyloses.

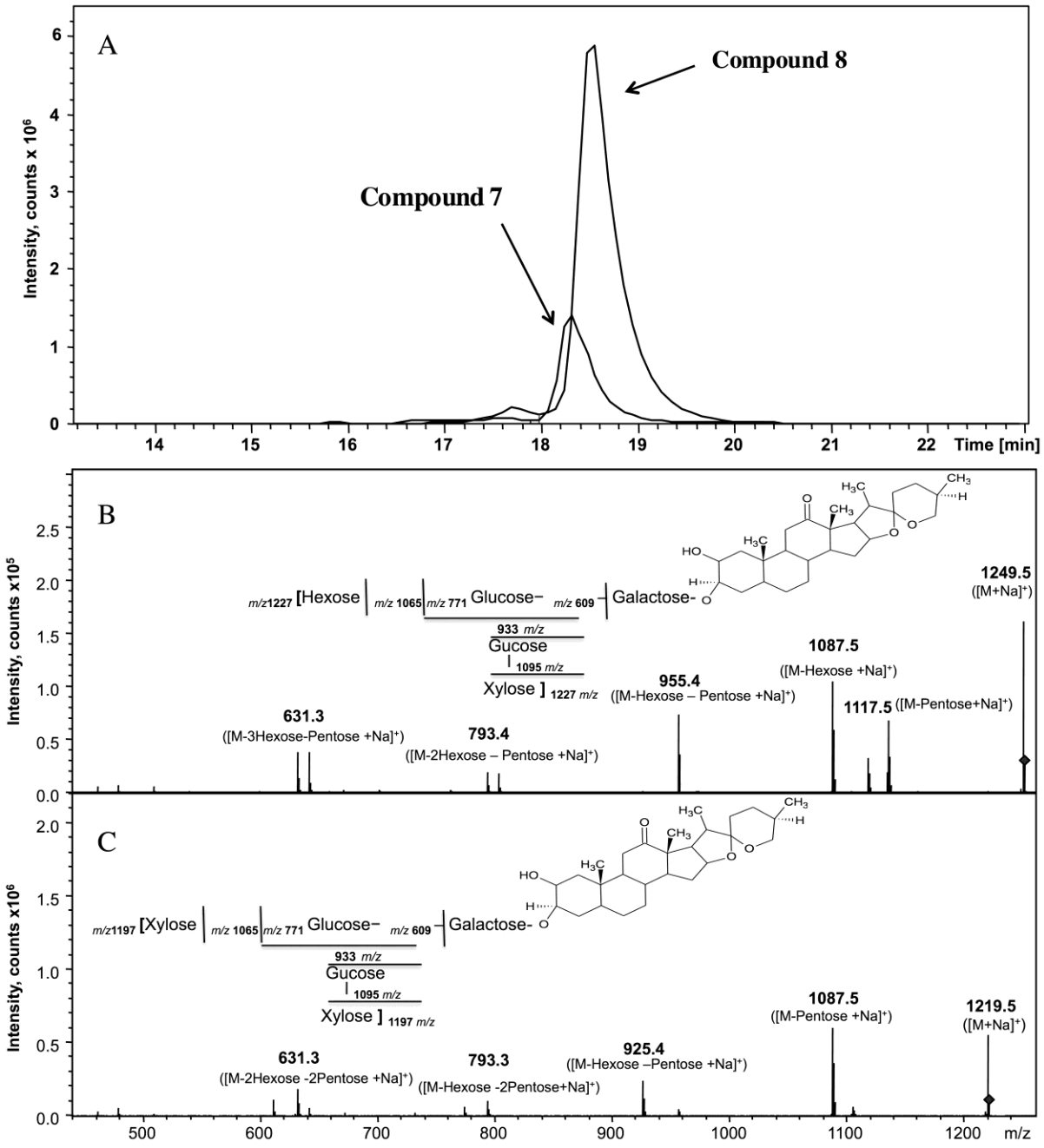

Figure S4 A. Chromatogram of extracted ions for compounds **7** and **8**. **B.** Mass spectrum, tentative structure and fragmentation pattern for compound **7**, manogenin pentaglycoside. **C.** Mass spectrum, reported structure and fragmentation pattern for compound **8**, magueyoside D.[2]

The fraction containing compounds **9** and **10** (**Figure S5-A**) was also analyzed by tandem mass spectrometry. Compound **9** presented the parent ion *m/z* 1179.3 ($[M+H]^+$) (**Figure S5-B**), which corresponds to a gentrogenin pentaglycoside. When losing one sugar unit, compound **9** had only one ion representing the tetraglycoside at *m/z* 1047.3 ($[M+H-132]^+$), indicating that a pentose was lost. Compound **10** showed the parent ion: *m/z* 1047.3 ($[M+H]^+$) (**Figure S4-C**), similar to the tetraglycoside of compound **9**. Further fragmentation of both compounds produced ions that show structural similarities by the triglycoside (*m/z* 885.2), monoglycoside (*m/z* 591.2) and aglycone (*m/z* 429.2) fragments. Similarities in fragmentation patterns demonstrate that compound **10** had a similar glycosylation pattern except for the xylose found at the end of the glycosidic chain of magueyoside H **(Figure S5)**.

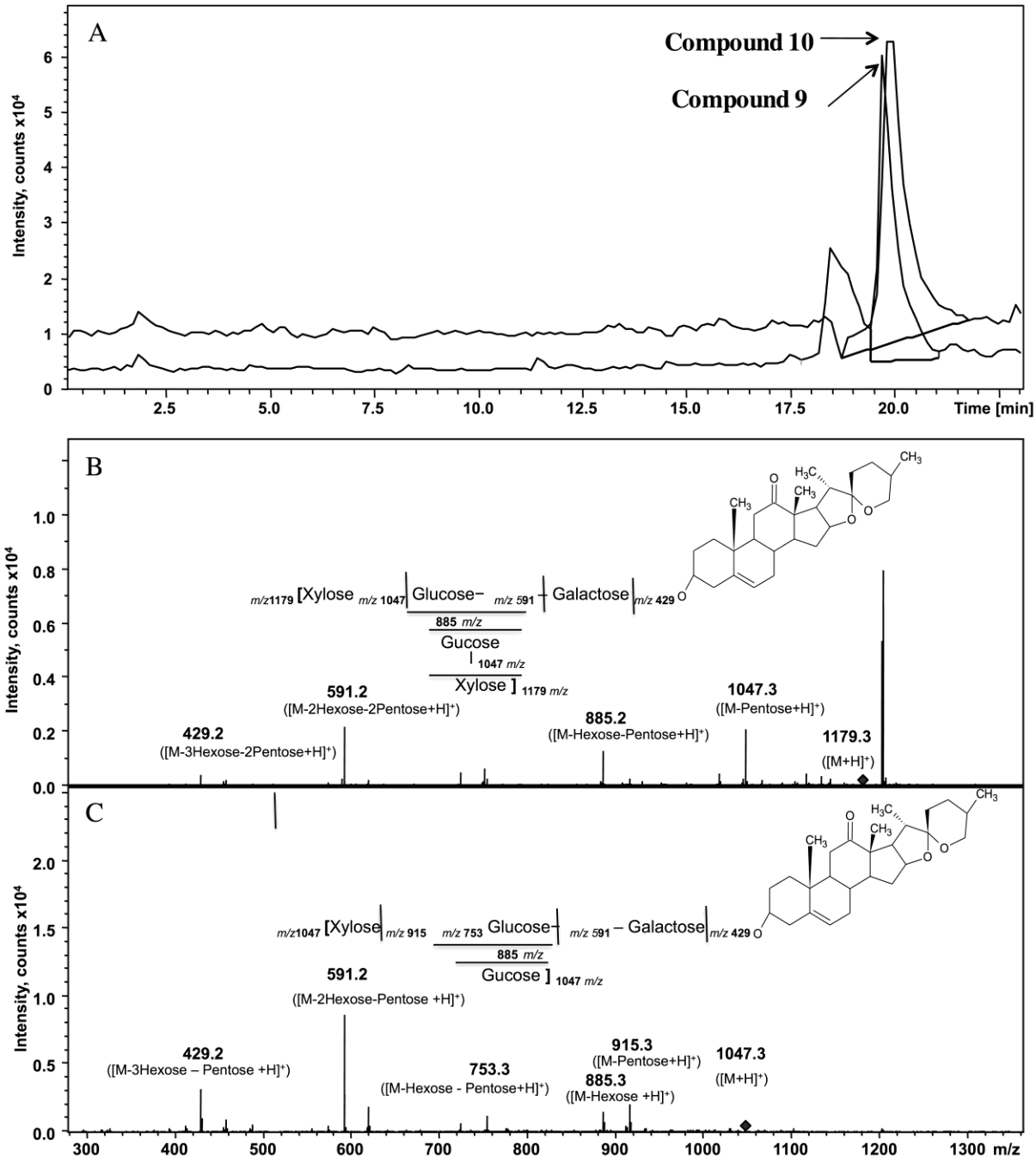

Figure S5 A. Chromatogram of extracted ions for compounds **9** and **10**. **B.** Mass spectrum, tentative structure and fragmentation pattern for compound **9**, magueyoside H.[1] **C.** Mass spectrum, tentative structure and fragmentation pattern for compound **10**, gentrogenin tetraglycoside.

REFERENCES

(1) Pérez, A. J.; Simonet, A. M.; Calle, J. M.; Pecio, Ł.; Guerra, J. O.; Stochmal, A.; Macías, F. A. Phytotoxic Steroidal Saponins from Agave Offoyana Leaves. *Phytochemistry* **2014**, *105*, 92–100.

(2) Pérez, A. J.; Calle, J. M.; Simonet, A. M.; Guerra, J. O.; Stochmal, A.; Macías, F. A. Bioactive Steroidal Saponins from Agave Offoyana Flowers. *Phytochemistry* **2013**, *95*, 298–307.