フラボノイド *O*-グリコシドの構造解明のための液体クロマトグラフィー / 質量分析法を用いたプロトコル

隅谷 栄伸，大木 伽耶子，笹井 実佐

Protocol Based on Liquid Chromatography/ Mass Spectrometry for Use in the Structural Elucidation of Flavonoid *O*-Glycosides

Hidenobu Sumitani, Kayako Ogi and Misa Sasai

Numerous glycosides exhibit beneficial functions, but several are toxic, and thus, elucidating the structures of unknown glycosides is critical. Complete elucidation is typically attained using nuclear magnetic resonance spectroscopy, which requires pure, highly concentrated samples, with an extended time generally required to collect a sample. Therefore, we developed a simple, rapid protocol to determine the structures of flavonoid *O*-glycosides. Samples were subjected to acid hydrolysis and the aglycons and sugars were then analyzed separately. Aglycon analysis was conducted via liquid chromatography/electrospray ionization-mass spectrometry. Sugar analysis was conducted via liquid chromatography/atmospheric pressure chemical ionization-mass spectrometry. Using onion peel extract as a model, a glycoside was discovered via product ion scanning. After isolation using preparative liquid chromatography, acid hydrolysis and determination of the sugar and aglycon led to identification of the *O*-glycoside. In this protocol, micrograms of sample are required, and isolation is relatively rapid, indicating the utility of this procedure.

Keywords: flavonoid *O*-glycoside, LC/MS, APCI, acid hydrolysis, sugar analysis

Various functional glycosides are used in the food, cosmetic, and drug industries [1–3]. However, several glycosides are highly toxic, and the correct elucidation of their structures is critical [4]. Complete elucidation often requires the use of nuclear magnetic resonance spectroscopy, which requires pure, highly concentrated samples, with an extended time generally required to collect a sample. The structures of several glycosides have been reported, including *O*-, *S*-, and *C*-glycosides [1,5,6]. The flavonoid *O*-glycosides are the most common and may be hydrolyzed to component sugars and aglycons via treatment with acid. The screening of unknown glycosides should be simplified if the sugars and aglycons can be identified.

Analyses of sugars are conducted using liquid chromatography/mass spectrometry (LC/MS). Atmospheric pressure chemical ionization (APCI) is an ionization method that ionizes compounds via heat and corona discharge [7] and is suitable in the detection of low-polarity molecules.

In this study, we designed a rapid, simple protocol to elucidate the structures of flavonoid *O*-glycosides. First, we used acid hydrolysis to hydrolyze the glycosides to sugars and aglycons, which were then identified separately. To detect low concentrations of sugars, chloride ion adducts were produced using a post-column solvent, and an APCI ion source was used. In this study, we propose a useful protocol for application in glycoside determination.

Material and methods

Materials.

Quercetin and rutinose were purchased from Tokyo Chemical Industry (Tokyo, Japan); quercetin-4'-*O*-glucoside and quercetin-3,4'-diglucoside (QDG) were purchased from Funakoshi (Tokyo, Japan), and rutin, naringin, α-L(+)-rhamnose, D(+)-glucose, L(+)-arabinose, D(−)-fructose, D(+)-mannose, D(+)-galactose, sucrose, lactose, and maltose were purchased from FUJIFILM Wako

Pure Chemical (Osaka, Japan). Hesperidin, cyanidin-3,5-diglucoside (CDG), and neohesperidose were obtained from Sigma-Aldrich (St. Louis, MO, USA), ChromaDex (Los Angeles, CA, USA), and Santa Cruz Biotechnology (Dallas, TX, USA), respectively.

Product ion scans of glycoside standards.

The glycoside standards, rutin (molecular weight: 610 g/mol), hesperidin (610 g/mol), QDG (626 g/mol), CDG (611 g/mol), and naringin (580 g/mol) were dissolved in dimethyl sulfoxide (DMSO) and diluted with ultrapure water to final concentrations of 10, 100, or 1000 ppm. Each sample was filtered using a membrane filter (0.45 µm) and injected into an LC/MS system comprising 1260 Infinity binary LC and 6430 triple-quadrupole LC/MS systems (Agilent Technologies, Santa Clara, CA, USA). Analysis was conducted in accordance with the method described by Kammerer *et al*. [8], with slight modifications. The parameters were: column, Synergi Hydro-RP 100A (100 × 3 mm, ϕ2.5 µm, Phenomenex, Torrance, CA, USA); column oven temperature, 40℃; mobile phase, (A) 2% acetic acid and (B) 0.5% acetic acid/acetonitrile (1:1, v/v); flow rate, 0.4 mL/min; injection volume, 5 µL; ion source, electrospray ionization (ESI); dry gas, nitrogen (350℃, 12 L/min); nebulizing gas, nitrogen (60 psi); capillary voltage, ± 2500 V; fragmentor voltage, 100 V; and mass range, *m/z* 100–1000. Gradient elution was performed as follows: 0–8 min, 10–24% B; 8–16 min, 24–30% B; 16–24 min, 30–55% B; 24–30 min, 55–100% B; 30–33.2 min, 100% B; 33.2–34 min, 100–10% B; 34–36 min, 10% B. Measurements were performed in product ion scan mode, using the precursor ions: *m/z* 611, 609, 627 (positive mode, for rutin, hesperidin, QDG, and CDG), and 579 (negative mode, for naringin), with a collision energy of 10 eV.

Acid hydrolyses of glycoside standards.

Glycoside standards were dissolved in DMSO / ultrapure water (1:1, v/v) to final concentrations of 100 ppm. Each solution was mixed with an equal volume of 2 M HCl and heated at 90 ℃ for 5, 10, or 20 min using a dry block bath (THB-1, AS ONE, Osaka, Japan). After filtration through 0.45-µm membrane filters, the samples were used in sugar analysis.

LC/APCI-MS analyses of sugars.

Sugar analysis was conducted according to the method described by Imtakt (Kyoto, Japan) [i], with slight modifications. The LC/MS system comprised 1260 InfinityⅡ binary LC and 6125 single quadrupole LC/MS systems (Agilent Technologies), with two flow paths. The main flow led from the binary pump to the autosampler, thermostatted column compartment, and mass selective detector (MSD). To produce the chloride ion adducts, a make-up path, which was supplied by the isocratic pump to the MSD, was added after column separation. The conditions were: column, Unison UK-Amino (150 × 3 mm, ø 3.0 µm, Imtakt); column oven temperature, 60 ℃ ; mobile phase, acetonitrile (A) and water (B); mobile phase flow rate, 0.4 mL/min; injection volume, 1 µL; ion source, atmospheric pressure ionization (negative mode); make-up flow solution, chloroform/acetonitrile (1:1, v/v, 0.35 mL/min); drying gas, nitrogen (250℃, 12 L/min); nebulizing gas, nitrogen (0.24 MPa); capillary voltage, 3000 V; fragmentor voltage, 70 V; mass range, *m/z* 100–1000; and measurement mode, selected ion monitoring (Cl^- adducts, Table 1). Gradient elution was performed as follows: 0–7 min, 9% B; 7–30 min, 9–21% B; 30–30.01 min, 21–9% B; 30.01–35 min, 9% B.

Screening of glycosides in onion peel extract.

Onion peel was freeze-dried, ground into a fine powder, and extracted using water at 80 ℃ for 30 min (10 mg/mL). LC/MS scanning was conducted using the same conditions as those of the product ion scans, with the scan mode reset. Product ion scans were conducted for the two largest peaks (compounds X and Y) in the chromatogram using the same conditions as those described above, with the precursor ions reset as *m/z* 303 and 465 (positive mode).

Structural determination and isolation of compound X.

Of the two peaks examined, peak X represented a glycoside, which was used in further processes. Accurate mass analysis was conducted using an LC / quadrupole time-of-flight mass spectrometry (qTOFMS) system comprising an LC-20A (Shimadzu, Kyoto, Japan) and a micrOTOF-Q Ⅱ (Bruker, Billerica, MA, USA). The LC parameters were the same as those described above, and the MS parameters were: detection mode, AutoMSMS; mass range, *m/z* 50–1000; dry gas, nitrogen (200 ℃ , 8 L/min); nebulizing gas, nitrogen (1.6 bar); capillary voltage, − 4500 V for positive ions; hexapole RF, 200 V; quadrupole ion energy, 5 eV; collision gas, nitrogen (1.6 bar); collision energy, 10 eV; collision RF, 200 V; and end plate offset, 500 V. Data analysis was performed using the Data Analysis software (Bruker).

Compound X was isolated using a preparative LC/

MS system comprising 1260 InfinityⅡ binary LC and 6120 single quadrupole LC/MS systems (Agilent Technologies), with two flow paths. The main flow led from the binary pump to the autosampler, thermostatted column compartment, and active splitter before reaching the fraction collector. Because the MSD is a destructive detector, and the main flow cannot be directly routed into the electrospray source, a make-up flow is supplied by the isocratic pump. This make-up flow led from the isocratic pump to the active splitter before reaching the MSD. To facilitate mass detection, the active splitter transported an aliquot of the main flow into the make-up flow, which then carried it into the MSD. The conditions were as follows: column, Discovery® HS F5-5 (250 × 10 mm, ø 5 μm, SUPELCO, Sigma-Aldrich); mobile phase, water (A) and acetonitrile (B); mobile phase flow rate, 4.0 mL/min; injection volume, 100 μL; ion source, ESI (positive and negative modes); make-up flow solution, 0.5% formic acid in water/methanol (1:1, v/v , 0.80 mL/min); drying gas, nitrogen (250 ℃ , 12 L/min); nebulizing gas, nitrogen (0.24 MPa); capillary voltage, ± 3000 V ; fragmentor voltage, 70 V; mass range, *m/z* 100–1000; and measurement mode, scan (positive and negative). Gradient elution was performed as follows: 0–25 min, 0–100% B; 25–25.01 min, 100–0% B; 25.01–30 min, 0% B, and the fraction eluted from 12.9–13.5 min was collected. To isolate the sample, a high concentration of onion extract (75 mg/mL) was prepared. Isolation was conducted twice and the collected fractions were dried using an evaporator and dissolved in 200 μL of ultrapure water.

Acid hydrolysis and analysis of compound X.

The isolated compound X (200 μL) was mixed with an equal volume of 2 M HCl and heated at 90 ℃ for 10 min, and the sugars and aglycons in the hydrolyzed samples were analyzed. The same conditions as those of the LC/APCI-MS analyses of sugars were used in sugar analysis. The same conditions as those of product ion scanning were used in aglycon analysis, with the product ion scan mode reset to scan mode.

Results and discussion

Product ion scanning of glycoside standards.

A product ion scan should be performed to confirm whether an unidentified target component is a glycoside. The *m/z* values of the dehydrated sugars detected in the product ion scans of flavonoid glycosides are shown in **Table 1**. The product ion scan spectra of several typical disaccharide glycosides are shown in **Figure 1**, and **Table 2** shows the *m/z* values of the product ions detected using these disaccharide glycosides.

Rutin is a glycoside comprised of quercetin and the disaccharide rutinose (6-*O*-α-L-rhamnosyl-D-glucose). Protonated molecules (*m/z* 611) and two product ions (*m/z* 303 and 465) are detected in the positive-ESI spectrum of rutin. The differences in *m/z* are 308, 162, and 146, which are consistent with the *m/z* values of dehydrated rutinose, glucose, and rhamnose, respectively. Hesperidin is a glycoside comprised of hesperetin and the disaccharide rutinose. Deprotonated molecules (*m/z* 609) and product ions (*m/z* 301) are detected in the negative-ESI spectrum of hesperidin. The difference in *m/z* is 308, representing dehydrated rutinose. However, product ions consistent with the loss of dehydrated glucose, and rhamnose are not detected. QDG is the 3,4'-*O*-diglucoside of quercetin. Protonated molecules (*m/z* 627) and two product ions (*m/z* 303 and 465) are detected in the positive-ESI spectrum of QDG. The differences in *m/z* are 162, representing dehydrated glucose. CDG, which is an anthocyanin, is the 3,5-*O*-diglucoside of cyanidin. Charged molecules (*m/z* 611) and two product ions (*m/z* 287 and 449) are detected in the positive-ESI spectrum of CDG. The differences in *m/z* are also 162, representing dehydrated glucose. Naringin is a glycoside comprised of naringenin and the disaccharide neohesperidose (2-*O*-α-L-rhamnosyl-D-glucose). Deprotonated molecules (*m/z* 579) and two product ions (*m/z* 271 and 459) are detected in the negative-ESI spectrum of naringin. The differences in *m/z* are 308, 188, and 120, which are consistent with the *m/z* values of dehydrated neohesperidose. However, product ions consistent with the loss of dehydrated glucose and rhamnose are not detected.

Although the structure may not be determined using only product ion scanning, whether the target component is a mono- or disaccharide glycoside may be indicated by referring to the data shown in **Tables 1** and **2**.

Table 1. *m/z* values of sugar-Cl⁻ adducts and dehydrated sugars in the LC/APCI-MS spectra and LC/ESI-MS product ion scans, respectively

Classification	Sugars	Molecular weight (compositional formula, g/mol)	Selected ion monitored using LC/APCI-MS	Detected ion at LC/ESI-MS product ion scan
			m/z of [M + Cl]⁻	*m/z* of dehydrated sugar
Pentoses	Arabinose Xylose	150 ($C_5H_{10}O_5$)	185	132
Hexoses	Fructose Mannose Galactose Glucose	180 ($C_6H_{12}O_6$)	215	162
Two hexoses	Sucrose Lactose Maltose	342 ($C_{12}H_{22}O_{11}$)	377	342
Others	Rhamnose	164 ($C_6H_{12}O_5$)	199	146
	Neohesperidose Rutinose	326 ($C_{12}H_{22}O_{10}$)	361	308

Table 2. Molecular weights (MWs) and compositional formulae (CFs) of the aglycons and glycosides of typical flavonoids

Flavonoid		Aglycon		Glycoside		Composition of disaccharide glycoside			
Name	MW (CF)	Name	MW (CF)	Name	MW (CF)	Saccharide	MW (CF)	Other saccharide	MW (CF)
Rutin	610 ($C_{27}H_{30}O_{16}$)	Quercetin	302 ($C_{15}H_{10}O_7$)	Rutinose	326 ($C_{12}H_{22}O_{10}$)	Glucose	180 ($C_6H_{12}O_6$)	Rhamnose	164 ($C_6H_{12}O_5$)
				dehydrated (− H_2O)	308	dehydrated (− H_2O)	162	dehydrated (− H_2O)	146
Hesperidin	610 ($C_{28}H_{34}O_{15}$)	Herperetin	302 ($C_{16}H_{14}O_6$)	Rutinose	326 ($C_{12}H_{22}O_{10}$)	Glucose	180 ($C_6H_{12}O_6$)	Rhamnose	164 ($C_6H_{12}O_5$)
				dehydrated (− H_2O)	308	dehydrated (− H_2O)	162	dehydrated (− H_2O)	146
Quercetin-3,4'-diglucoside (QDG)	626 ($C_{27}H_{30}O_{17}$)	Quercetin	302 ($C_{15}H_{10}O_7$)	Glucose (x2)	180 ($C_6H_{12}O_6$)				
				dehydrated (− H_2O)	162				
Cyanidin-3,5-diglucoside (CDG)	611 ($C_{27}H_{31}O_{16}^+$)	Cyanidin	287 ($C_{15}H_{11}O_6^+$)	Glucose (x2)	180 ($C_6H_{12}O_6$)				
				dehydrated (− H_2O)	162				
Naringin	580 ($C_{27}H_{32}O_{14}$)	Naringenin	272 ($C_{15}H_{12}O_5$)	Neohesperidose	326 ($C_{12}H_{22}O_{10}$)	Glucose	180 ($C_6H_{12}O_6$)	Rhamnose	164 ($C_6H_{12}O_5$)
				dehydrated (− H_2O)	308	dehydrated (− H_2O)	162	dehydrated (− H_2O)	146

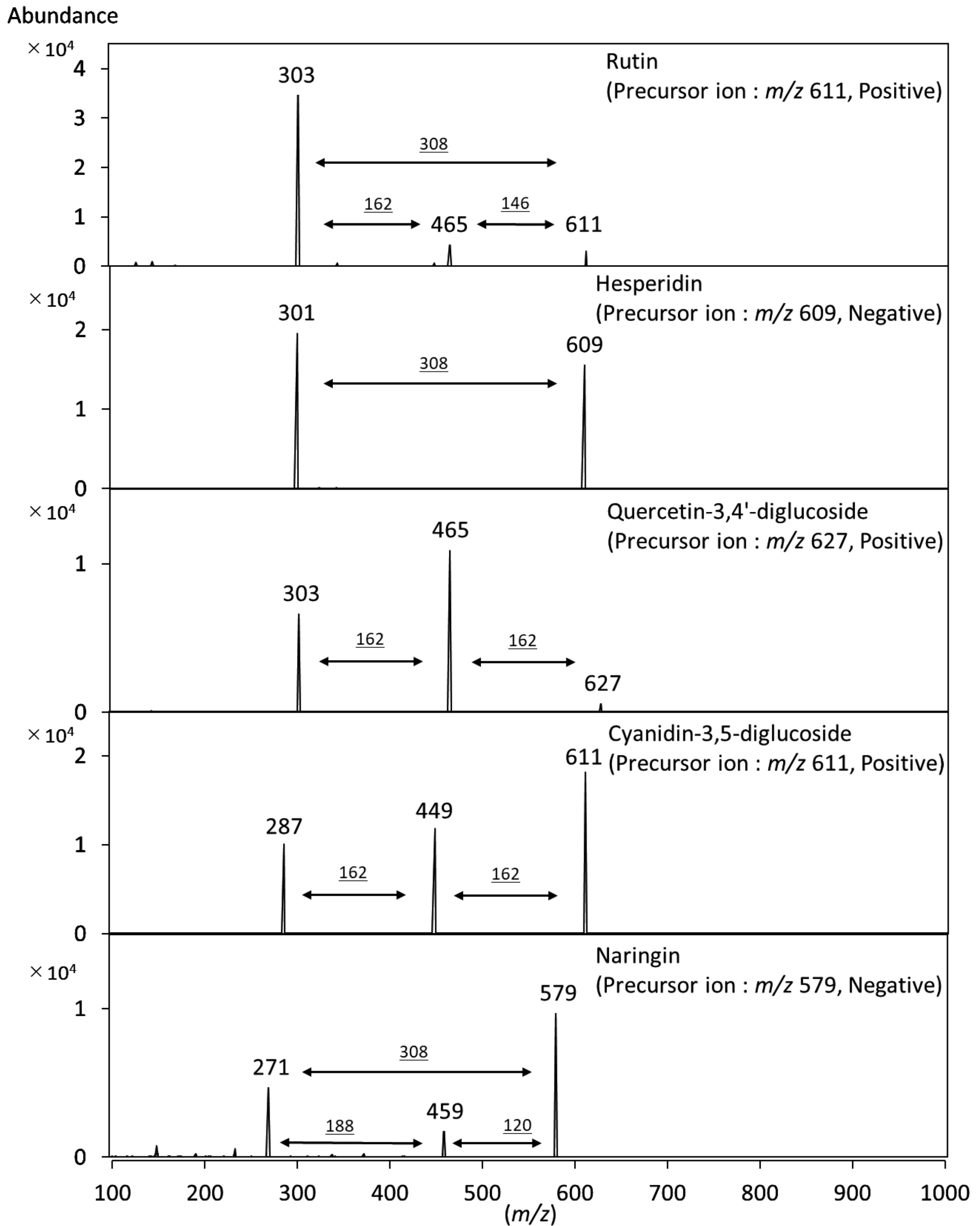

Figure 1. Product ion scan spectra of rutin (10 ppm), hesperidin (100 ppm), quercetin-3,4'-diglucoside (10 ppm), cyanidin-3,5-diglucoside (1000 ppm), and naringin (100 ppm). In each spectrum, the differences between detected ions are indicated with double-headed arrows.

LC/APCI-MS analyses of sugars and acid hydrolyses of the glycoside standards.

As the sugars are relatively insensitive to ESI, APCI was applied. To enhance their sensitivities, various sugars may be detected via their chloride ion adducts produced using a post-column solvent. Using the combination of APCI and post-column analysis, various sugars are detected (**Figure 2**), including rhamnose (detection limit: 0.23 ppm, S/N = 3), arabinose (0.32), xylose (0.20), fructose (0.09), mannose (0.23), galactose (0.48), glucose (0.40), neohesperidose (0.14), rutinose (0.09), sucrose (0.36), lactose (1.31), and maltose (2.59). The acid hydrolyses of rutin and hesperidin should yield rutinose, rhamnose, and glucose, and the concentrations of sugars obtained are shown in **Figures 3a** and **3b**. To determine the different sugar moieties, incomplete hydrolysis of the disaccharides is critical, because if they are completely hydrolyzed into the monosaccharide components, determining whether two monosaccharides or the disaccharide were bound to the aglycon is challenging. All experimental conditions used result in the detection of three sugar moieties (**Figures 3a** and **3b**). The acid hydrolyses of QDG and CDG should produce glucose, and glucose is detected under all conditions used (**Figures 3c** and **3d**). Acid hydrolysis of naringin should produce neohesperidose, rhamnose, and glucose, and three sugars are detected after heating for 5 or 10 min (**Figure 3e**). Because acid hydrolysis for 10 min yields a higher concentration of sugars than that obtained after 5 min, 2 M HCl for 10 min is selected as the most effective condition.

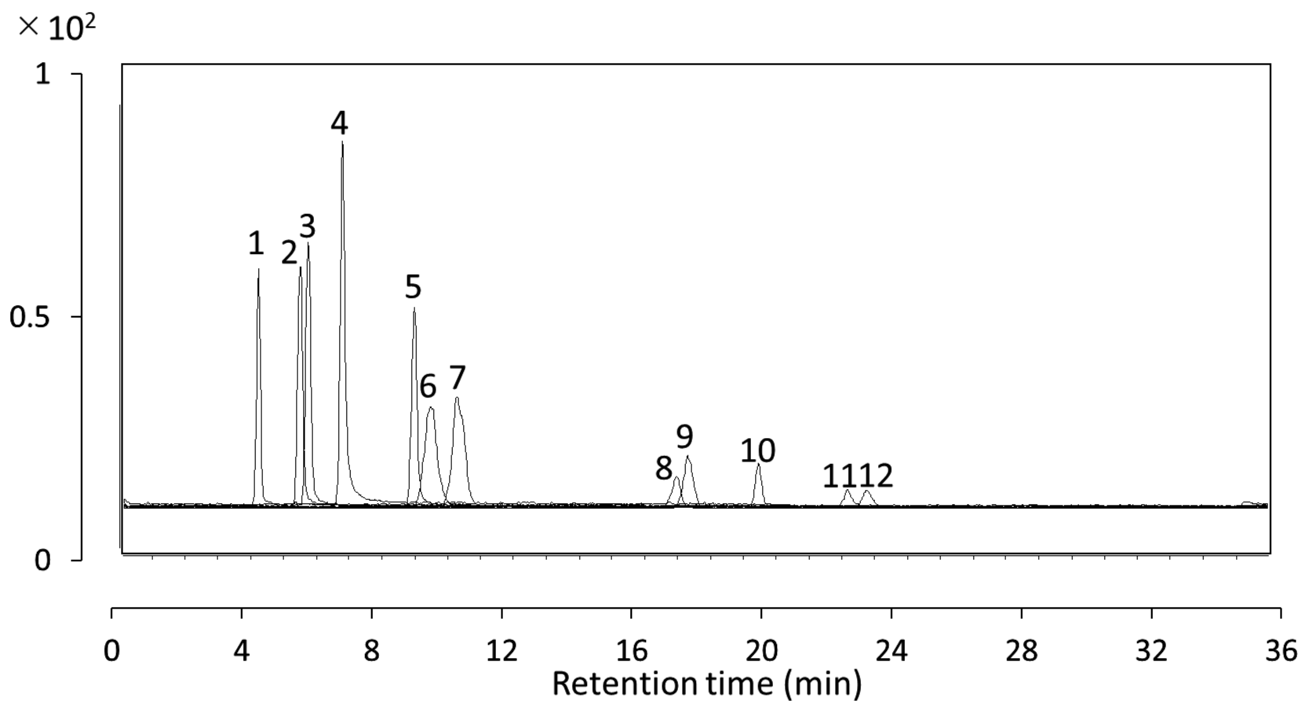

Figure 2. Selected ion chromatograms of sugars (10 ppm)

1: rhamnose, 2: arabinose, 3: xylose, 4: fructose, 5: mannose, 6: galactose, 7: glucose, 8: neohesperidose, 9: rutinose, 10: sucrose, 11: lactose, and 12: maltose.

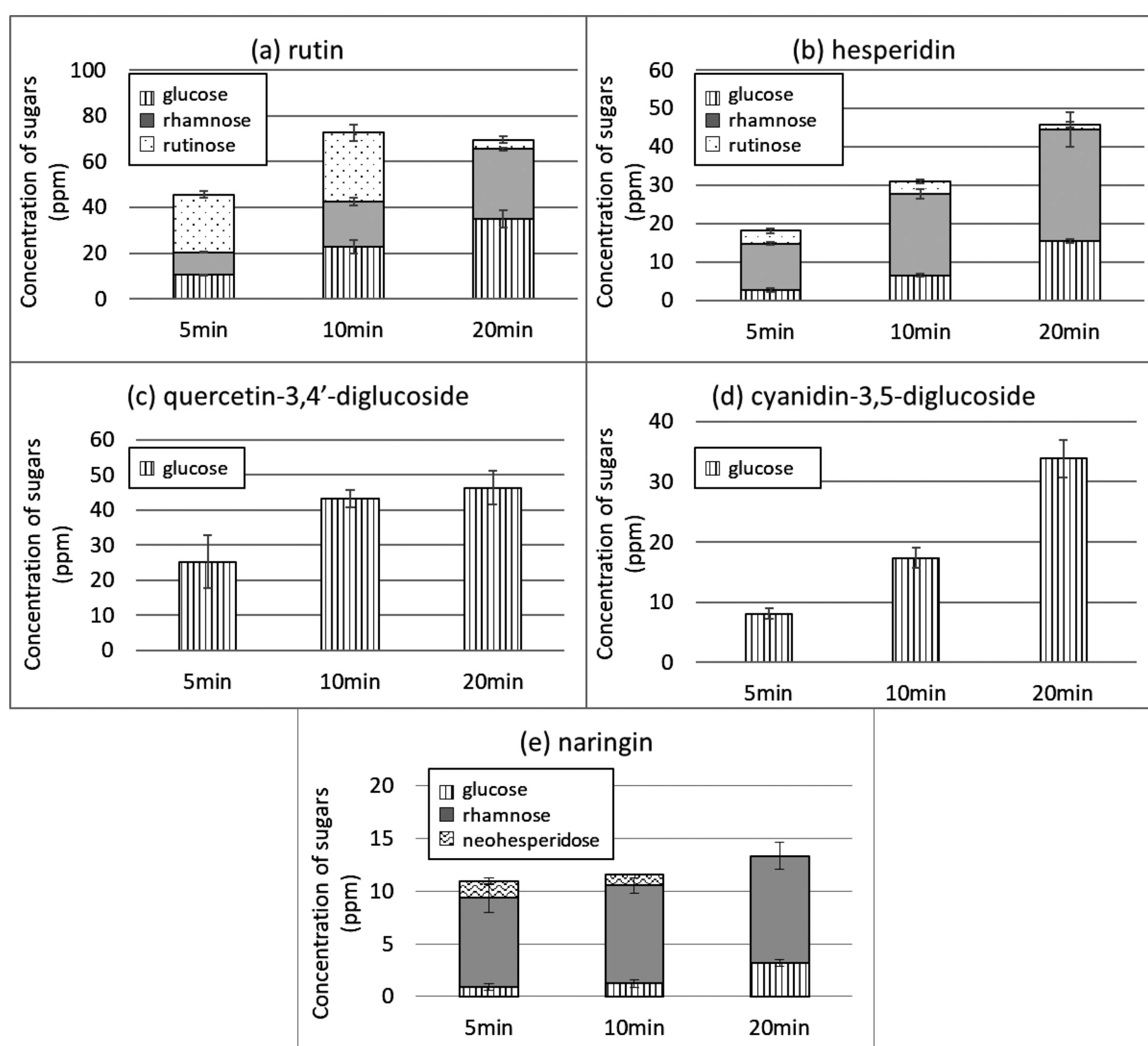

Figure 3. Concentrations of sugars detected in hydrolyzed (a) rutin, (b) hesperidin, (c) quercetin-3,4'-diglucoside, (d) cyanidin-3,5-diglucoside, and (e) naringin, which were hydrolyzed using 2 M HCl for 5/10/20 min.

Screening of glycosides in onion peel extract.

As an example, onion peel extracts were subjected to LC/ESI-MS and the two peaks obtained were then analyzed via product ion scanning (Figure 4). Of the two peaks examined, the peak of compound X may be a glycoside, because the mass spectrum reveals the presence of sugar groups in the structure. The mass spectrum of compound Y exhibits only one peak, *m/z* 303, which suggests that it is not a glycoside. Further analysis revealed that compound Y was quercetin (data not shown). For compound X, two fragment ions are detected, *m/z* 465 and 303. The difference between them is 162, which indicates that compound X is a glycoside comprised of a hexose (Table1) and an aglycon with a molecular weight of 302 g/mol.

Acid hydrolysis and analysis of compound X.

Accurate mass analysis showed that the estimated formula $[M + H]^+$ of compound X is $C_{21}H_{21}O_{12}$, and thus, the formula of compound X is $C_{21}H_{20}O_{12}$. The results of sugar analysis via LC/APCI-MS show that compound X contains a glucose moiety (Figure 5). Additionally, LC/ESI-MS and comparison with a quercetin standard indicates that the aglycon of compound X is quercetin (Figure 6). Therefore, compound X is a glycoside comprised of glucose and quercetin, with the formula $C_{21}H_{20}O_{12}$.

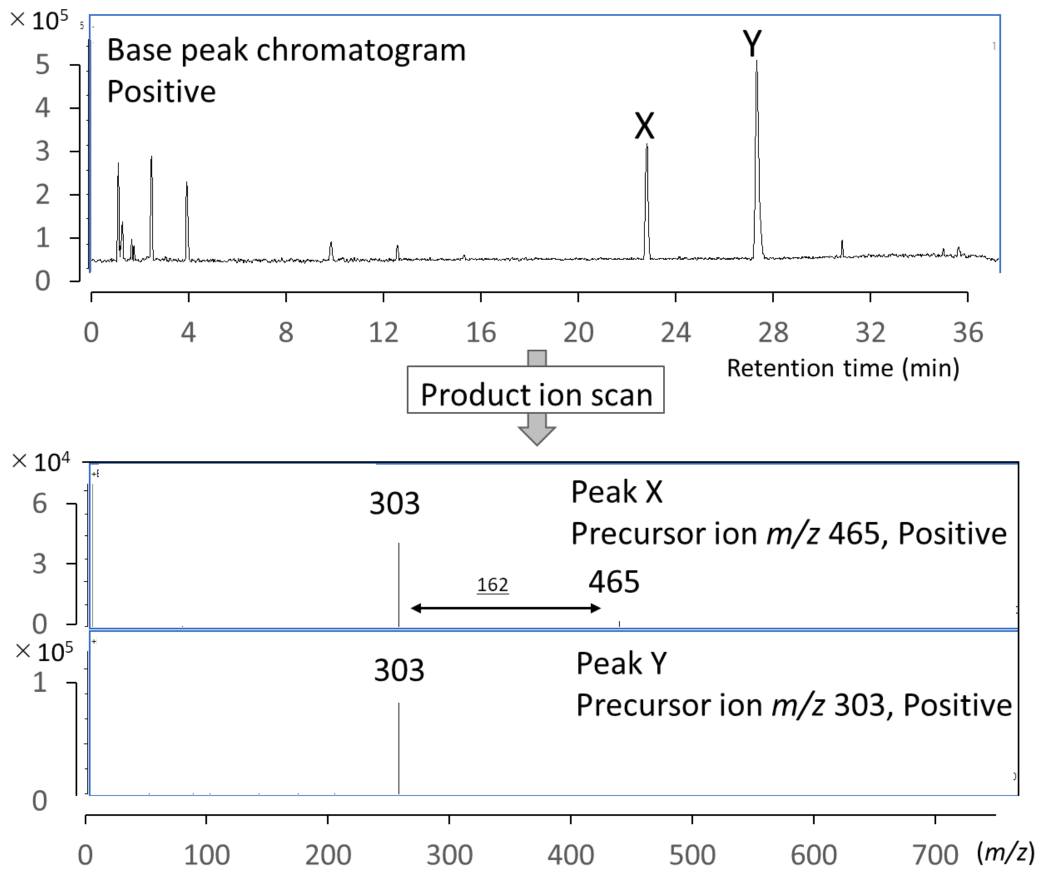

Figure 4. Base peak chromatogram of onion peel extract and the product ion scan spectra of peaks X and Y (precursor ions of X and Y: *m/z* 465 and 303, respectively, positive, ESI).

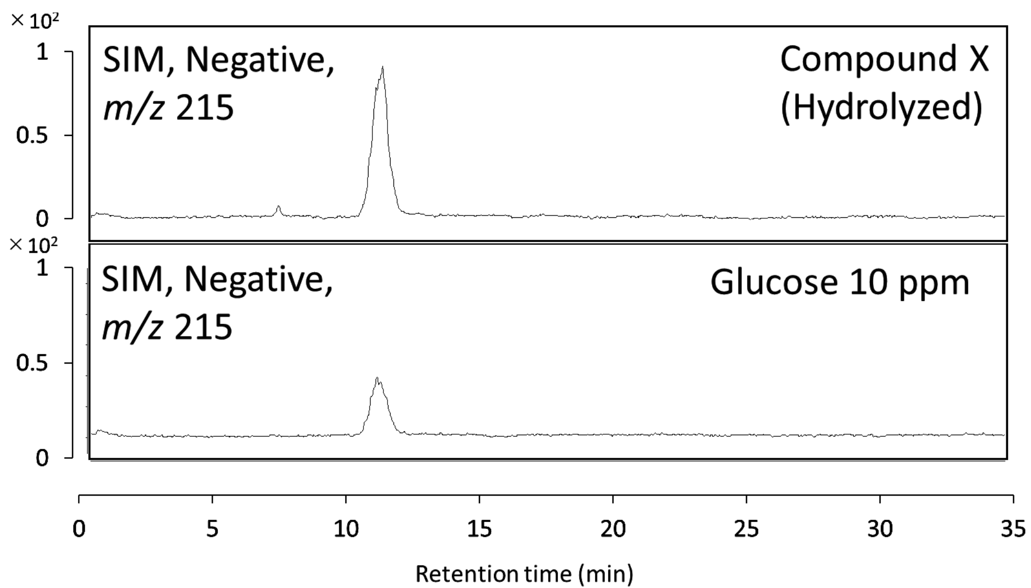

Figure 5. Selected ion chromatograms of hydrolyzed compound X and a 10 ppm glucose solution (negative, *m/z* 215, APCI).

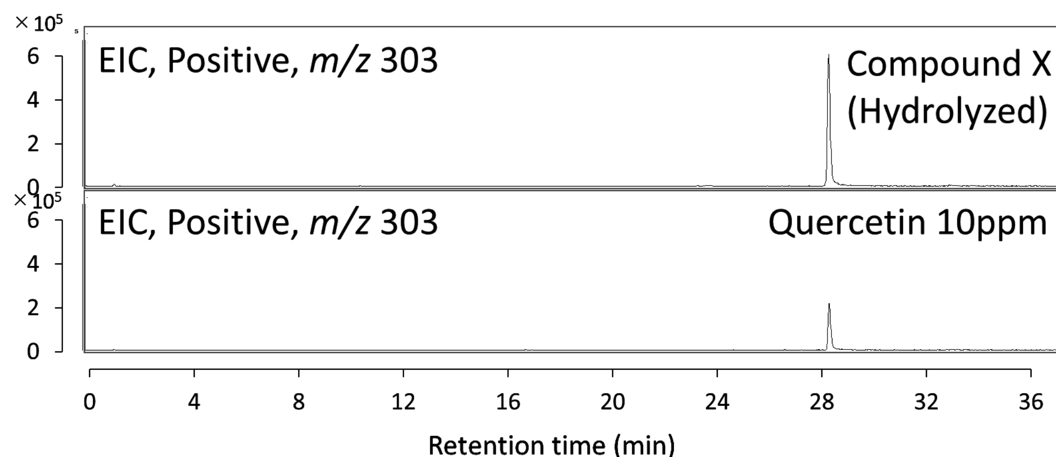

Figure 6. Extracted ion chromatograms of hydrolyzed compound X and quercetin 10 ppm solution (positive, *m/z* 303, ESI).

Conclusion.

Structural identification of glycosides is critical, because several display useful functions, whereas others are toxic. In this study, we propose a protocol to easily identify flavonoid *O*-glycosides (**Figure 7**). If product ion scanning predicts the presence of mono- or disaccharide glycosides, estimating the compositional formula from the accurate mass obtained using techniques such as LC/qTOFMS is considered a better approach. After isolation using preparative liquid chromatography, treatment with 2 M HCl for 10 min hydrolyzed the sample compounds to their sugar and aglycon components. By analyzing the sugars and aglycons separately, we could identify a particular combination of sugars and aglycons, but the binding site of the glycoside remains unclear. Finally, reference materials and cross-checks are required to ensure accuracy of the protocol.

This protocol requires only small amounts (micrograms) of samples; it may be performed easily in numerous fields. Moreover LC/APCI-MS would be useful in detecting a wide variety of sugars, with high sensitivities.

Acknowledgements.

We would like to thank Editage (www.editage.com) for English language editing.

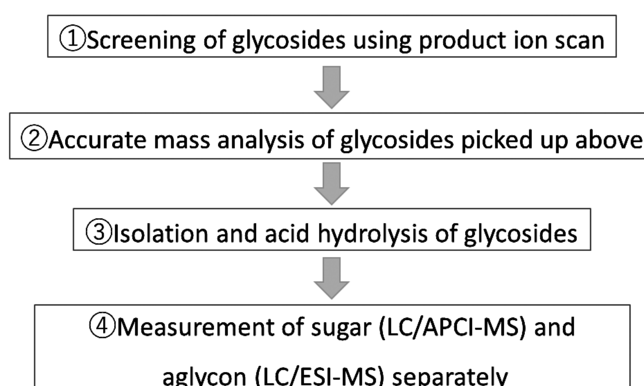

Figure 7. Protocol for use in the structural elucidation of *O*-glycosides.

References

1) Carakostas M.C., Curry L.L., Boileau A.C., and Brusick D.J. (2008). Overview: The history, technical function and safety of rebaudioside A, a naturally occurring steviol glycoside, for use in food and beverages. *Food Chem. Toxicol.*, **46**(7), S1-S10.

2) Tanimoto S., Tominaga H., Okada Y., and Nomura M. (2006). Synthesis and cosmetic whitening effect of glycosides derived from several phenylpropanoids. *Yakugaku Zasshi*, **126**(3), 173-177.

3) Dostanic-Larson, I., Van Huysse, J.W., Lorenz, J.N., and Lingrel, J.B. (2005). The highly conserved cardiac glycoside binding site of Na, K-ATPase plays a role in blood pressure regulation. *Proc. National Acad. Sci.*, **102**(44), 15845-15850.

4) Majak W. (1992). Metabolism and absorption of toxic glycosides by ruminants. *J. Range Manag*., 45, 67-71.

5) Mazumder A., Dwivedi A., and du Plessis J. (2016). Sinigrin and its therapeutic benefits. *Molecules*, **21**(4), 416-427.

6) Obara H., and Onodera J. (1979). Structure of carthamin. *Chem. Lett*., **8**(2), 201-204.

7) Kim Y.H., and Kim S. (2010). Improved abundance sensitivity of molecular ions in positive-ion APCI MS analysis of petroleum in toluene. *J. Am. Soc*. *Mass Spectrom*., **21**(3), 386-392.

8) Kammerer D., Claus A., Carle R., and Schieber A. (2004). Polyphenol screening of pomace from red and white grape varieties (*Vitis vinifera L*.) by HPLC-DAD-MS/MS. *J. Agric. Food Chem*., **52**(14), 4360-4367.

URL cited

i) http://www.imtakt.com/TecInfo/TI823E.pdf (July 1, 2022)