9 chapter

Mass Spectrometry with High-Performance Liquid Chromatography

Baraem P. Ismail

Department of Food Science and Nutrition, University of Minnesota, St. Paul, MN, USA
e-mail: bismailm@umn.edu

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9.1 INTRODUCTION

9.1.1 Background

Mass spectrometry (MS) is an analytical technique that provides information about the molecular weight and chemical characteristics of a compound. This technique is commonly linked to high-performance liquid chromatography (LC–MS), gas chromatography (GC–MS), inductively coupled plasma (ICP–MS), and other methods.

A mass spectrometer has three essential functions: sample ionization, ion separation, and ion detection. Components in a sample can be ionized by various ionization techniques; regardless of the source, the charged particles that are generated are separated based on their mass-to-charge ratio. Separation occurs in the mass analyzer which sorts the different ion masses by applying electric and magnetic fields. These ions are then detected which provide data on the abundance of each ion fragment present.

For LC–MS analysis, the compounds are first separated on a LC system. After detection in the LC system (by ultraviolet–visible spectroscopy (UV–VIS)), the eluent enters the ion source of a mass spectrometer where the compounds get ionized and subsequently separated based on "mass-to-charge" (m/z) ratio in the analyzer. The separated ions are then detected using an electron multiplier. Ionization using electrospray ionization (ESI) often produces precursor ions with some fragmentation. The ion which undergoes fragmentation is the precursor ion, and the ions that are produced upon fragmentation of the precursor ion are called product ions. To obtain further structural information about the compound, tandem mass spectrometry (MS/MS) can be employed. After ionization, the ions of interest are separated and fragmented by collision-induced dissociation (CID) by using an inert gas (helium, argon, etc.) as the collision gas. The energy given to the collision gas is varied depending on the desired extent of fragmentation, which will provide valuable structural information. The mass spectrum obtained by tandem MS contains only the product ions. A case study is presented to better understand the advantages of MS in tandem.

9.1.2 Reading Assignment

Reuhs, B.L. 2017. High-performance liquid chromatography. Ch. 13 in *Food Analysis*, 5th ed. S.S. Nielsen (Ed.), Springer, New York.

Smith, J.S., and Thakur, R.A. 2017. Mass spectrometry. Ch. 11 in *Food Analysis*, 5th ed. S.S. Nielsen (Ed.), Springer, New York.

9.1.3 Objectives

Determine and identify various phytochemicals (mainly isoflavones, Table 9.1 and Fig. 9.1) in soy flour, and understand how to analyze a mass spectrum to obtain mass and structural information of a compound.

9.1.4 Chemicals

	CAS No.	*Hazards*
HPLC grade acetonitrile	1334547-72-6	Highly flammable, acute toxicity, irritant
HPLC grade methanol	67-56-1	Highly flammable, acute toxicity, serious health hazard

9.1.5 Hazards, Precautions, and Waste Disposal

Adhere to normal laboratory safety procedures. Wear safety glasses at all times. Methanol and acetonitrile waste must be handled as hazardous waste. Other wastes likely may be put down the drain using a water rinse, but follow good laboratory practices outlined by environmental health and safety protocols at your institution.

9.1.6 Reagents

- Deionized distilled water
- HPLC grade water
- Methanol/water mixture, 80/20

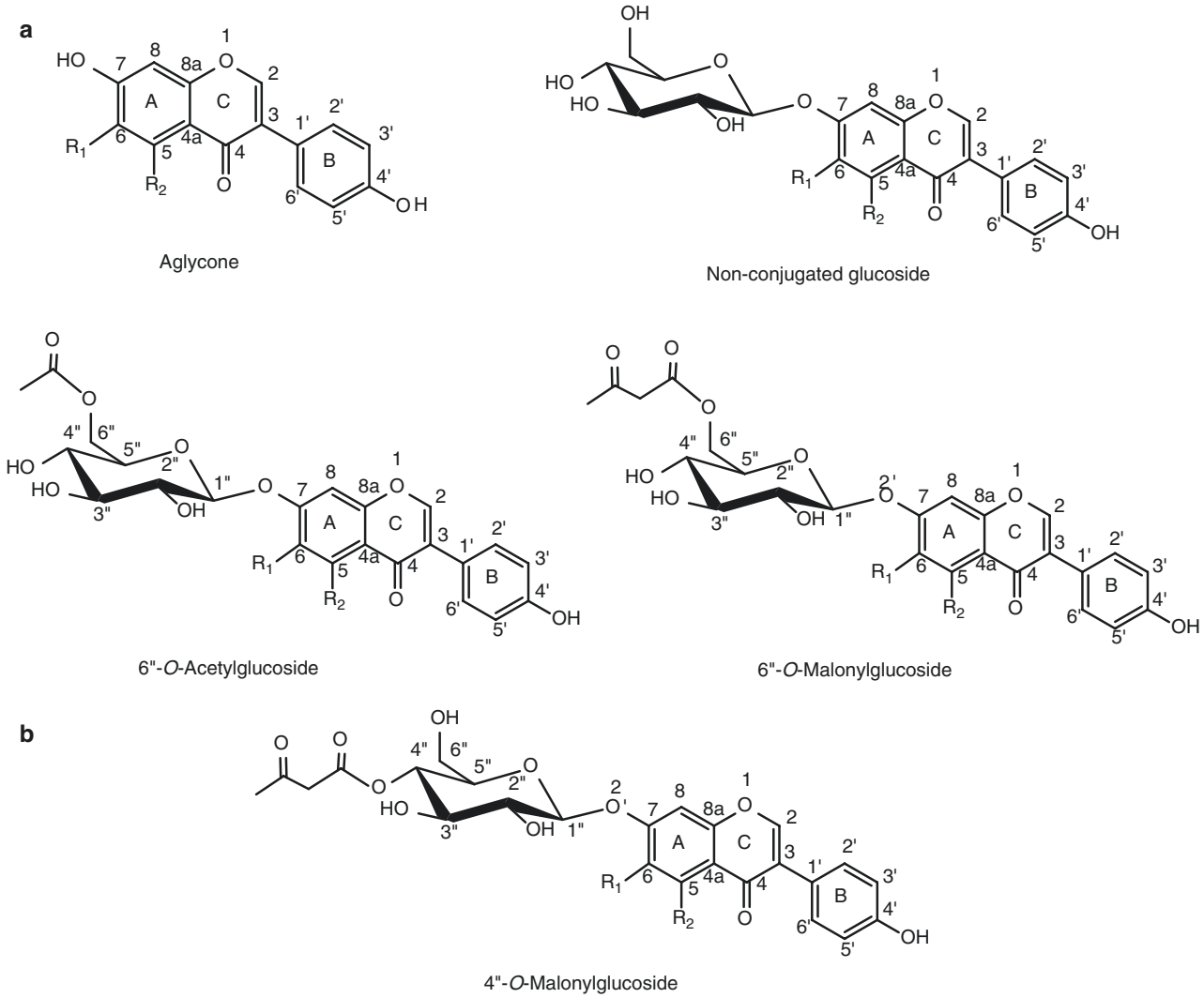
9.1.7 Supplies

- Aluminum foil
- Automatic pipettors, 1000 μL, 200 μL, 100 μL, and 10 μL
- Autosampler vials, 1.5 mL, with caps
- 3 Beakers, 125 mL, each containing either 80/20 methanol, deionized distilled water, or acetonitrile
- 1 Beaker, 125 mL, for balancing the centrifuge tubes
- Buchner funnel
- 2 Centrifuge tubes, 50 mL centrifuge tubes
- Defatted soy flour
- Erlenmeyer flask, 25 mL (rinsed prior with 80/20 methanol)
- Erlenmeyer flask, 10 mL (rinsed prior with 80/20 methanol)
- 3 Graduated cylinders, 10 mL
- Filter paper, Whatman 42, 90 mm diameter
- Glass rods
- Markers
- Parafilm
- Rotary evaporator (rotovap) collection flasks (125 mL or smaller) (rinsed prior with 80/20 methanol)
- Sidearm flask, 125 mL, with a nozzle to plug the vacuum pump (rinsed prior with 80/20 methanol)
- Stir bars and stirrer
- Syringes, 3 mL
- Syringe filters, non-sterile, nylon; pore size 0.45 μm; diameter 25 mm

9.1 table

Twelve known isoflavones found in soybean and their molecular weight (MW)

Isoflavone	*MW*	*Isoflavone*	*MW*	*Isoflavone*	*MW*
Daidzein	254	Genistein	270	Glycitein	284
Daidzin	416	Genistin	432	Glycitin	446
Acetyldaidzin	458	Acetylgenistin	474	Acetylglycitin	488
Malonyldaidzin	502	Malonylgenistin	518	Malonylglycitin	532



9.1 figure

(**a**) Structures and numbering of the 12 known isoflavones categorized as aglycone, nonconjugated glucoside, acetylglucoside, and malonylglucoside. R1 can be -H in the case of daidzin and genistin or -OCH_3 in the case of glycitin, while R2 can be -H in the case of daidzin and glycitin or -OH in the case of genistin. (**b**) Structures and numbering system of 4″-*O*-malonylglucosides (malonylglucoside isomers)

9.2 table Example combinations of ionization sources and mass analyzers coupled with chromatographic separation

Name	*Ionization source*	*Mass analyzer*	*Tandem MS*	*Separation mode*
LTQ Orbitrap	Electrospray ionization (ESI)	Linear ion trap and orbital trap	Yes	Liquid chromatography
5500 QTRAP®	ESI	Triple quadrupole with Q3 linear trap	Yes	Liquid chromatography
4000 QTRAP®	ESI	Triple quadrupole with Q3 linear trap	Yes	Liquid chromatography
LTQ	ESI	Linear ion trap	Yes	Liquid chromatography
LCT Premier	ESI	Time of flight (TOF)	No	Liquid chromatography
4800 AB Sciex	Matrix-associated laser desorption ionization (MALDI)	TOF–TOF	Yes	Liquid chromatography
Pegasus 4D	Electron impact (EI)	TOF	No	Gas chromatography
Agilent 6130	Dual ESI/atmospheric pressure chemical ionization (APCI)	Quadrupole	Yes	Liquid chromatography
LCQ (2)	ESI	Ion trap	Yes	Liquid chromatography
LCQ(1),	Dual ESI/APCI	Ion trap	Yes	Liquid chromatography
Biflex III	MALDI	TOF	No	Liquid chromatography
QSTAR XL	ESI	Quadrupole TOF	Yes	Liquid chromatography
Saturn 3	EI/chemical ionization (CI)	Ion trap	Yes	Gas chromatography

- Tape
- Vacuum pump
- Vortex

9.1.8 Equipment

- Analytical balance
- Centrifuge, for 15 mL tubes
- High-performance liquid chromatography system
- Mass-selective detector (quadrupole), equipped with electrospray ionization source
- Rotary evaporator

Various combinations of ionization sources and mass analyzers can be used for LC–MS and GC–MS analysis (see Table 9.2). This laboratory exercise as describes utilizes a LC–MS system coupled with electrospray ionization (ESI) and a quadrupole mass analyzer.

9.2 PROCEDURE

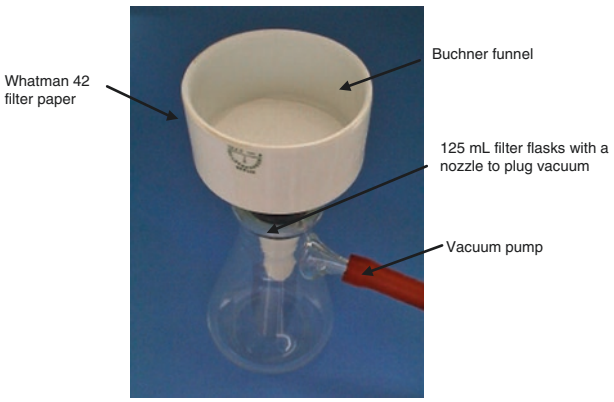
9.2.1 Sample Preparation

1. Weigh 0.05 gm of defatted soy into a labeled 25 mL Erlenmeyer flask.
2. Add 10 mL of HPLC grade acetonitrile to the sample and put in a stir bar.
3. Stir on a magnetic stir plate until the sample is thoroughly mixed (stirrer speed = 7, time = 5 min).
4. Add 9 mL DDW and stir for 2 h (stirrer speed = 7). Occasionally, scrape the surface of the plastic bottle with a glass rod to make the sample sticking to the sides go into the solution. (There will be a sample on the stirrer prepared by the TA and ready for you to take to the next step.)
5. Quantitatively transfer the sample solution from the Erlenmeyer flask to a labeled centrifuge tube. Rinse the flask three times with 1 mL acetonitrile and add the rinse to the centrifuge tube. Use the scale to balance the tube with a tube containing water.
6. Centrifuge the solution in a centrifuge. Example conditions with a Marathon 3200 centrifuge are:

   Speed = 4000 rpm
   Time = 15 min
   Temperature = 15 °C

7. Take the centrifuge tube carefully out of the rotor so that the precipitate does not get disturbed.
8. Filter the permeate using a Buchner funnel, labeled 125 mL filter flask with a nozzle to plug the vacuum pump, Whatman 42 filter paper, and vacuum pump. The setup should look like in Fig. 9.2.
9. Quantitatively transfer the filtered solution into a labeled rotary evaporator (rotovap) collection flask. Again rinse three times with 1 mL acetonitrile and add the rinse to the rotovap collection flask.
10. Evaporate to dryness using a rotovap. Example conditions with a Buchi R-II Rotavapor are:

    Cooler temperature = 4 °C.
    Water bath temperature = 38 °C.
    Rotovap RPM = 120.
    Time = 15 min (may take up to 30 min or so).



9.2 figure Setup to filter sample permeate

Pressure: since it is a two-step evaporation, evaporating of acetonitrile will be done at 115 mbar, and evaporation of water will be done at 30 mbar.
Note: If the available pump is not adequate enough to use for evaporating water from the sample, shell freezing is used to freeze the sample so it can be dried using a lyophilizer (freeze dryer).

11. There will be a sample already dried out for you and ready for the next step. Add 10 mL of 80/20 methanol/water and quantitatively transfer the solution into a labeled 25 or 10 mL Erlenmeyer flask. This time do not rinse; just transfer using a pipette.
12. Vortex for 2 min (Speed = 5).
13. Attach a 0.45 µm filter onto the end of a 3 mL syringe.
14. Pour some of the sample into the filter assembly.
15. Push sample through the filter into an autosampler vial.
16. Label the sample vial (include group name, date, sample name).
17. Seal the flask with parafilm, wrap with silver foil, and label adequately.

9.2.2 LC–MS Procedure

A Shimadzu LC-10 AD HPLC equipped with two solvent pumps and a CT-10A column oven will be used. The chromatographic column is a YMC AM-303 (ODS, 250 mm × 4.6 mm i.d.) comprising C18 reversed-phase packing (5 µm average pore size), equipped with a C18 guard column (4 mm × 20 mm i.d.).

The chosen flow rate is 1 mL/min and oven temp of 45 °C. A linear HPLC gradient will be used: Solvent A will be 0.1 % (v/v) formic acid, and solvent B will be acetonitrile. The gradient time program is described in Table 9.3.

9.3 table HPLC gradient time program

Solvent B concentration (5 %)	*Time (min)*
11	0 (Initial)
14	30
14	35
30	40
30	50
11	52
11	60

9.2.3 MS Conditions

Mass spectrometry is performed on a Waters ZQ quadrupole instrument. Positive ionization will be employed. Masses are scanned from 200 to 600 m/z over 60 min, at a scan rate of one scan/s.

Eight selected ions of the following m/z are monitored, $[M+H^+]$: 255, 271, 417, 433, 459, 475, 503, and 519.

The injection volume is 20 µL; the split ratio is 1:3 (so ¼ of the flow goes into the mass spectrometer, ¾ flow through the UV detector). The source temperature is 150 °C; the desolvation temperature is 450 °C. The desolvation gas flow is 600 L/h; the cone gas flow is 75 L/h. The cone voltage is 30 eV; the capillary voltage is 3 kV.

1. Turn on the gas flow by pressing the button "API gas" on the tune page.
2. Click the button "Press to Operate" on the tune page.
3. Turn on the oven and the degasser.
4. Load the HPLC conditions on the inlet page and turn on the flow.
5. Wait until the HPLC column is conditioned and the oven has reached the desired temperature.
6. Enter the file name, MS file, inlet file, and MS tune file name, and save the data set.
7. Before you inject sample, make sure that the mass spectrometer is in the "Load" mode.
8. Press the "Start" button, inject the sample, click on "Start" again, and press the "Inject" button on the mass spectrometer.

9.3 DATA AND CALCULATIONS

Analyze the total ion chromatogram and mass spectra of the selected isoflavones provided to you at the end of the lab exercise. To help you understand how to analyze a mass spectrum to obtain mass and structural information of a compound, see the Case Study, Sect. 9.5. Use the resource materials provided to answer the general questions below, and analyze the ion chromatogram and mass spectra of the isoflavones.

9.4 QUESTIONS

9.4.1 General Questions

1. What is a molecular ion? Does electron impact (EI) ionization produce a single precursor ion? How about ESI? Answer by explaining the difference between soft ionization to hard ionization.
2. List the ionization interfaces that are capable of delivering soft ionization.
3. Which ion travels farthest in unit time (low m/z or high m/z) in time-of-flight mass analyzer? How does the length of time-of-flight mass analyzer affect the resolution?
4. Explain the different between a quadrupole and an ion trap.
5. What more information can MS/MS (i.e., MS in tandem) provide versus just one MS?

9.4.2 Questions Specific to Isoflavone Analysis

1. Discuss the peaks and how you identify them.
2. Indicate if there were any co-elutions and how one can determine that.
3. Discuss the presence of isomers, if any, and how useful the MS was in identifying their presence, pointing to advantages and limitations.
4. Were you able to see precursor ions? Were you able to see product ions? Use a table format (Table 9.4) to list the precursor ions identified and their product ions as in the example table below.

9.4 table Identified isoflavones, precursor, and product ions m/z values

Retention Time	*Isoflavone*	*Precursor ion m/z*	*List of product ions (name and m/z)*

9.5 CASE STUDY

Malonylgenistin is a type of isoflavone abundantly found in soybeans. Epidemiological studies have shown the association of isoflavones with many health benefits such as reducing the risk of cancer, alleviation of postmenopausal symptoms, etc. Processing of soybeans into various soy products results in the conversion of malonylgenistin to various compounds. Monitoring the conversions of malonylgenistin to different compounds is important to determine if the resultant compounds have any biological significance.

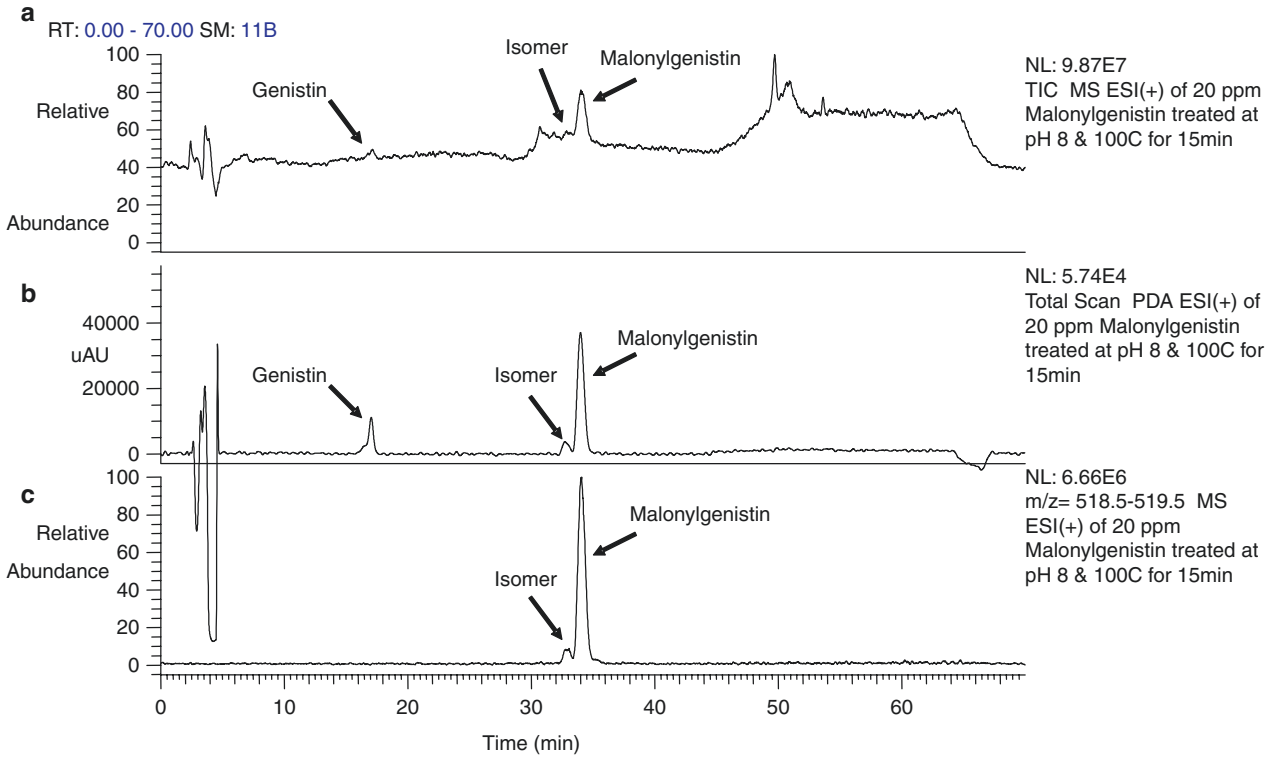
Recent studies have shown the formation of a new derivative upon heat treatment of malonylgenistin. The wavescans obtained using a photodiode array detector (PDA) of the new derivative and malonylgenistin were similar. Hence, LC/UV failed to provide any information about the structural differences between the two compounds. MS analysis of the two compounds revealed that the compounds had same mass (m/z = 519 Dalton) indicating that the new derivative is an isomer of malonylgenistin (Fig. 9.3). Tandem mass spectrometry was employed to probe structural differences between the two isomers. There were two noticeable differences in the product ion spectra (Fig. 9.4).

1. Isomer fragmented more at a lower collision level as compared to malonylgenistin.
2. Malonylgenistin product ion spectra had an ion with m/z = 433, which was absent in the isomer product ion spectra.

These differences highlight the structural differences between the two compounds. Nuclear magnetic resonance has to be employed to obtain further structural details.

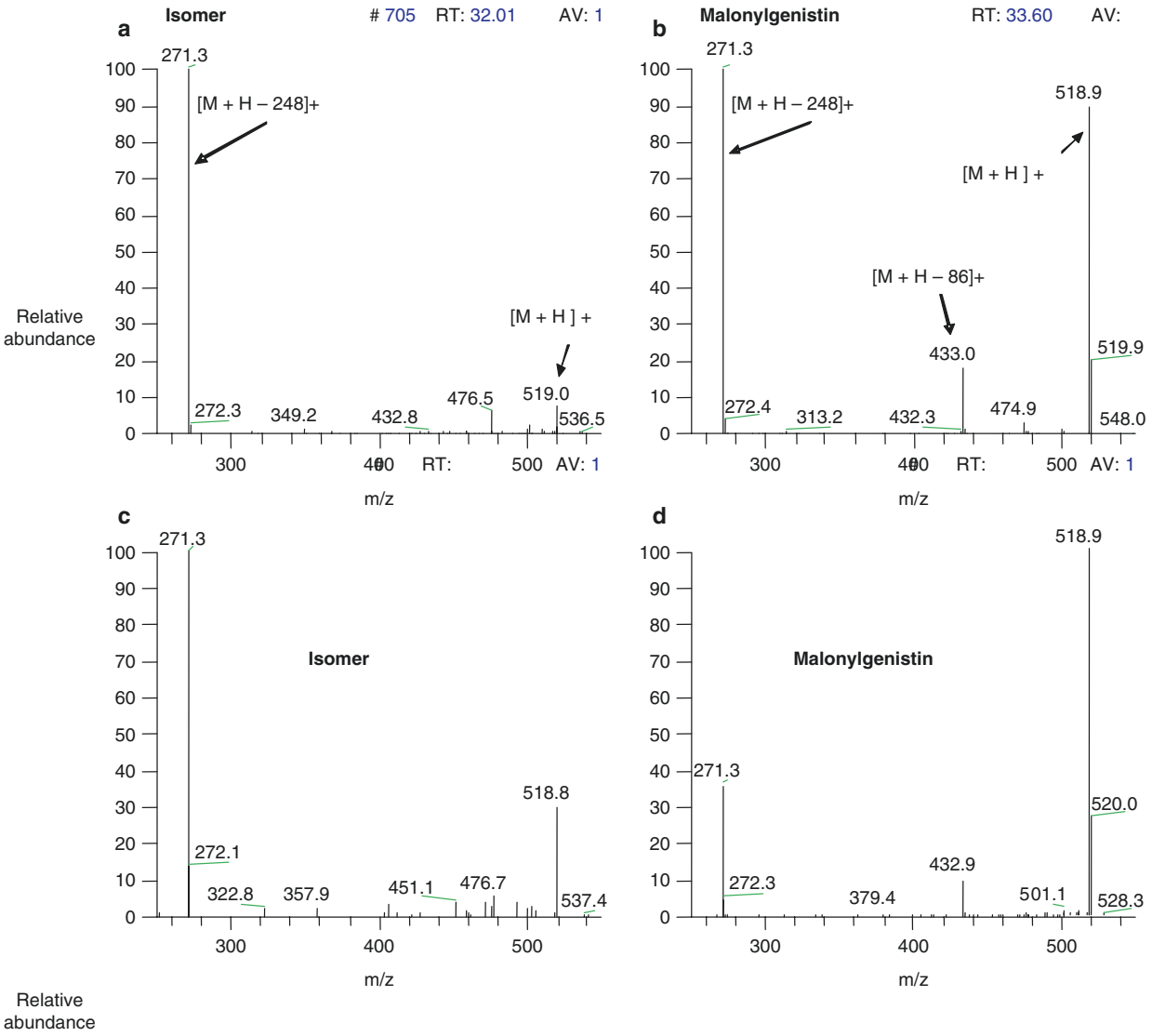
RESOURCE MATERIALS

Smith JS, Thakur RA (2017) Mass spectrometry. Ch. 11. In: Nielsen SS (ed) Food analysis, 5th edn. Springer, New York



9.3 figure

ESI–MS analysis malonylgenistin and its isomer: (**a**) total ion chromatogram, (**b**) PDA view of malonylgenistin solution, and (**c**) reconstructed single ion chromatogram of m/z 519 ion (protonated molecule of a malonylgenistin)



9.4 figure

ESI–MS/MS analysis of the protonated forms of isomer and malonylgenistin at various collision levels: (**a**) isomer at 20%, (**b**) malonylgenistin at 20%, (**c**) isomer at 17%, and (**d**) malonylgenistin at 17% collision