Problem Session

2022/04/23 Kensuke Miura

Please read the description below and answer the questions

Question 1.

Crude extract from *Corydalis yanhusuo* was subjected to LC-MS and LC-MS/MS experiments and the following data were obtained (**Figure 1** and **Table 1**)。

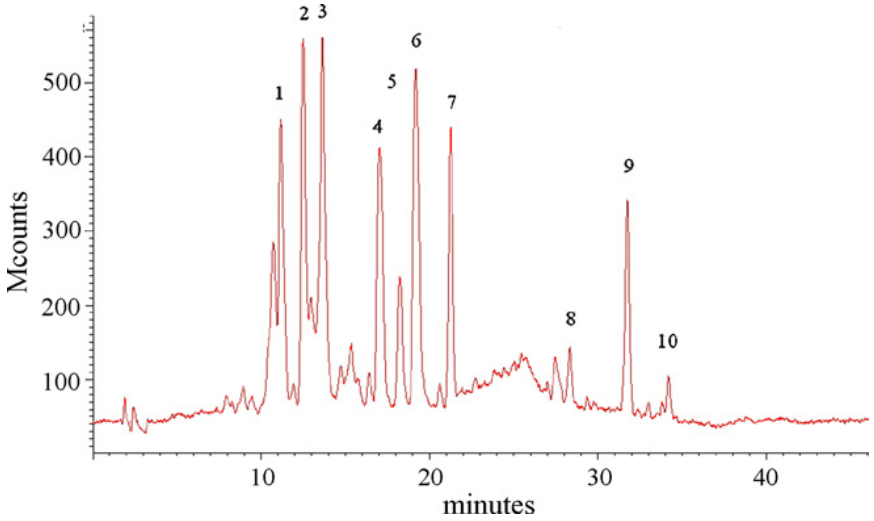


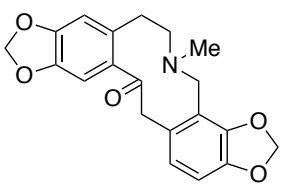
Figure 1. HPLC-MS total ion chromatogram of the extract of *Corydalis yanhusuo.* Column: Diamonsil C_{18}-column 4.6 × 200 mm, eluent A: aceonitrile, eluent B: H_2O + 2% AcOH adjusted with Et_3N to pH 5, A/B = 20/80 (0-15 min), 20/80 to 80/20 (15-35 min), 20/80 (35- min), flow rate: 1.0 mL/min

Table 1. Retention times, MS data, and MS/MS data of main alkaloids in *Corydalis yanhusuo.*

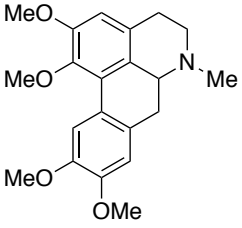
peak number (analytes)	retention time (min)	$[M+H]^+$ (*m/z*)	$[M]^+$ (*m/z*)	MS/MS (*m/z*)
1	11.13	354	-	206, 188, 149
2	12.46	342	-	178, 165, 151
3	13.51	356	-	324, 294, 279
4	17.05	-	352	337, 336, 322, 308
5	18.23	-	336	321, 306, 292, 278
6	19.28	-	366	351, 350, 334, 322
7	21.17	356	-	192, 165, 151
8	28.28	340	-	176, 165, 149
9	31.67	370	-	192, 165, 151
10	34.18	324	-	176, 149

condition of MSMS:

<u>Please identify the compounds in each peak from MS/MS fragmentation.</u> The 10 alkaloids extracted from *Corydalis yanhusuo* are shown below.



1



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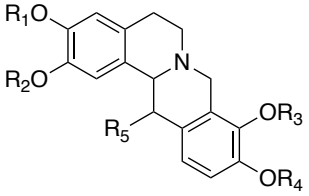
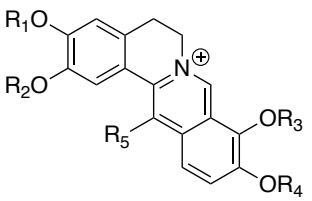


3: R_1=R_2=R_3=R_4=R_5=Me
4: R_1=R_2=R_3=R_4=Me, R_5=H
5: R_1,R_2=R_3,R_4=-CH_2-, R_5=H
6: R_1=R_3=R_4=Me, R_2=R_5=H
7: R_1,R_2=-CH_2-, R_3=R_4=Me, R_5=H



8: R_1=R_2=R_3=R_4=Me, R_5=H
9: R_1,R_2=-CH_2-, R_3=R_4=Me, R_5=H
10: R_1=R_2=R_3=R_4=R_5=Me

Question 2.

A stock solution containing 10 analytes was prepared by dissolving the reference compounds in methanol and then diluted with methanol to appropriate concentrations for establishing calibration curves. Solutions containing different concentrations of the 10 analytes were injected in triplicate (see **Figure 2**). Calibration curves for estimating the amount of alkaloids were obtained from the experiments (**Table 2**).

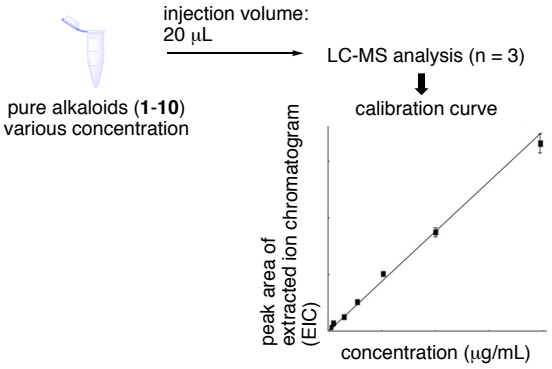


Figure 2. Scheme of establishing calibration curve

Table 2. Linear regression of 10 analytes in *Corydalis yanhusuo*

Analytes	Linear regression		
	Calibration curves	Correlation coefficient	Linear range (μg/ml)
1	$y = 69.49x + 266.97$	0.9981	20–500
2	$y = 56.63x + 185.00$	0.9977	20–500
3	$y = 65.66x + 160.50$	0.9997	50–1000
4	$y = 56.03x + 201.75$	0.9994	20–500
5	$y = 68.21x + 76.50$	0.9980	20–500
6	$y = 62.95x + 42.99$	0.9975	20–500
7	$y = 56.03x + 201.75$	0.9997	20–500
8	$y = 46.07x + 105.30$	0.9996	10–100
9	$y = 57.66x + 202.48$	0.9988	20–500
10	$y = 55.39x + 261.50$	0.9977	10–100

y and x stand for the peak area and the concentration (μg/ml) of the analytes, respectively.

Please calculate the amounts of the alkaloids per crude *Corydalis yanhusuo*, respectively ($mg_{alkaloid}/g_{crude}$). Please use the value of peak areas (**Table 3**), and concentration of crude as shown.

*Concentration of crude and peak areas were virtually set, independent of the original paper.

concentration of crude: 100 mg in 300 mL methanol, injection volume: 20 μL

peak number	peak area of EIC
1	11686
2	8019
3	29773
4	9970
5	4237
6	24090
7	15273
8	1257
9	4450
10	1572

Question 3.

In the case of question 2, the LC-MS quantification was performed without problems. However, since crudes contain many compounds other than analyte, LC-MS quantification is potentially affected by **matrix effects**.

Matrix effects：the effect on an analytical assay caused by all other components of the sample except the analyte. Matrix effects are observed either as a loss in response, resulting in an underestimation of the amount of analyte, or as an increase in response, producing an overestimated result.

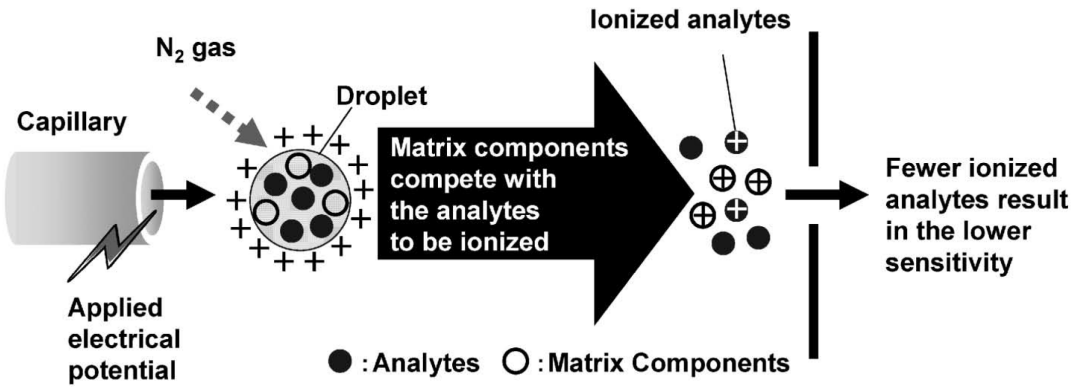


Figure 3. Conceptual scheme of electrospray ionization with ion suppression

Please provide a method to avoid the effects.

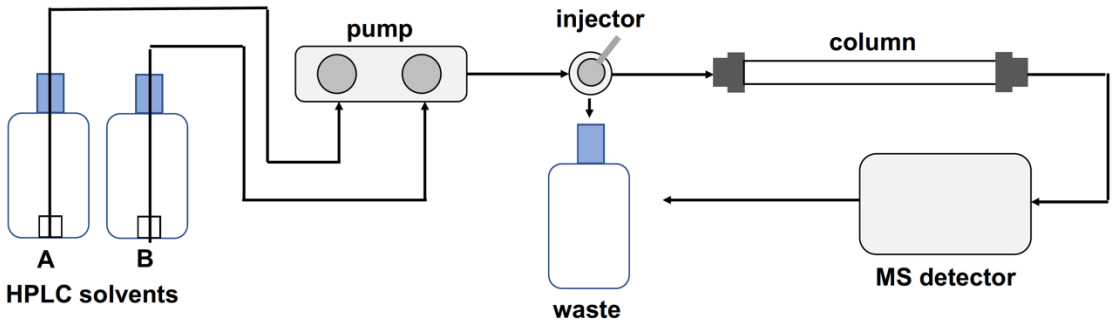
Problem Session Answer

2022/04/23 Kensuke Miura

Topic. Determination and quantification of compounds using LC-MS, LC-MS^n

0. Introduction

0-1. LC-MS analysis



analytical techniques combining liquid chromatography (LC) with the mass spectrometry (MS).

High sensivity, selectivity of mass-based analysis→detecting microgram or even nanogram quantities

0-1. Liquid chromatography

examples of separation mode:

adsorption chromatography, ion-exchange chromatography, size-exclusion chromatography, and affinity chromatography. reverse-phase chromatography

→ selection the separation mode that separates the analytes.

0-2. inonization technics

ESI (Electrospray Ionization) [1)] ionization process: capillary with high voltage

analytes: moderately polar molecules (e.g., metabolites, xenobiotics, and peptides).

APCI (Atmospheric Pressure Chemical Ionization)[2)] ionization process: corona discharge

analytes: small, neutral, relatively non-polar, and thermally stable molecules (e.g., steroids, lipids)

APPI (Atmospheric Pressure Photoionization)[3)] ionization process: photons coming from a discharge lamp.

analytes: molecules of lower polarity

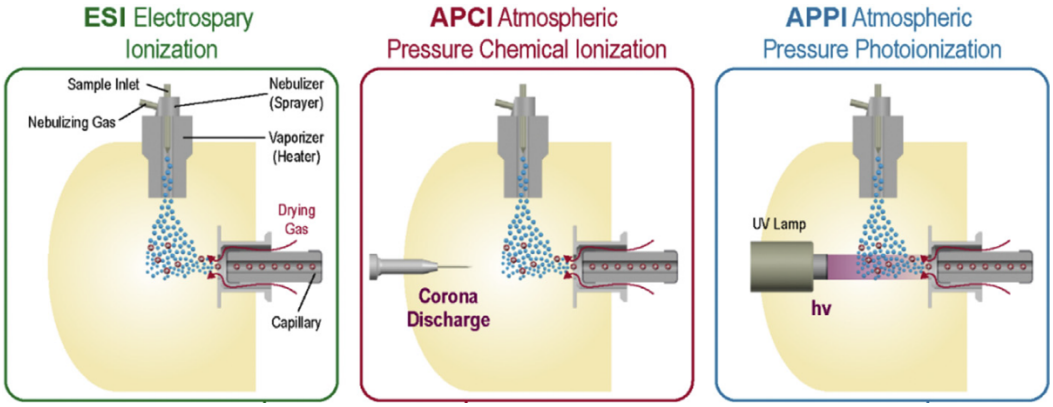


Figure 1. Different ionization techniques

0-3. application of LC-MS, LC-MSMS quantification

1. food analysis[4)]

determination of pesticide residues and toxic substances

2. pharmaceuticals analysis[5)]

measurement of drug blood concentration

3. Determination of known compounds (in this problem)[6)7)]

In order to discovering novel compounds from natural source, dereplication strategy was developed. Dereplication refers to the rapid identification of known compounds. The identification of known compounds in the crudes is important for the efficient investigation of new compounds, and LC-MS is used for this purpose because it allows analysis without the need to isolate samples. In addition, MSMS can be used to identify substructures, and known compounds are identified by searching the database for information such as molecular weight, UV absorption, and substructures.

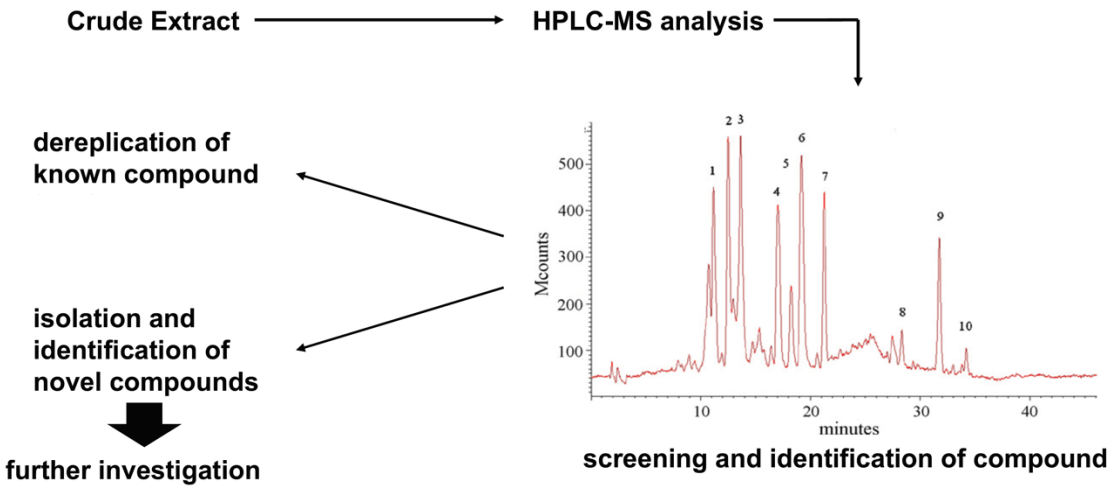


Figure 2. Conceptual scheme of dereplication

Question 1. Determination of compounds in each peak

1-0. Introduction

Corydalis yanhusuo[6)]

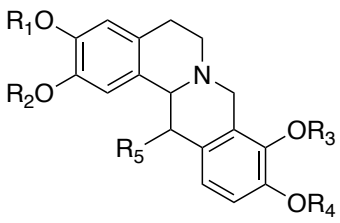
important kind of traditional Chinese medicine]

Tertiary alkaloids Quaternary alkaloids

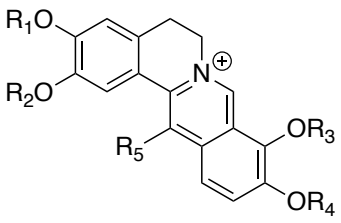
from the tuber of *Corydalis yanhusuo*, main bioactive compounds

Structure of tertiary alkaloids and quaternary alkaloids[6)7)8)9)]

tertiary alkaloids

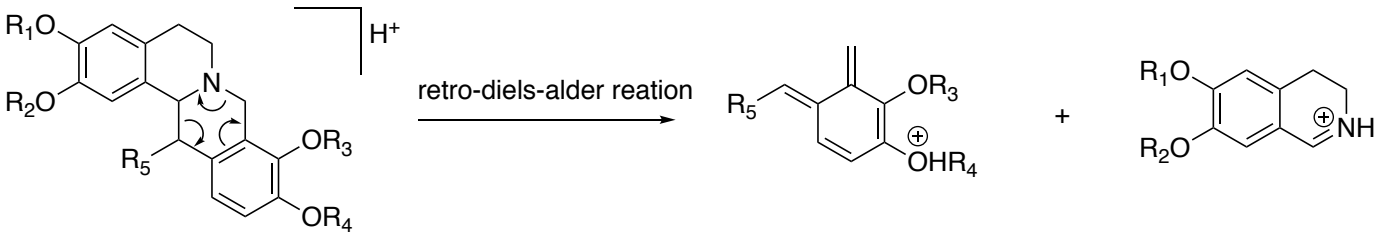


quatenary alkaloids



bioactivity[6)]: antibacterial, antiviral, and anticancer activities

retro-diels-alder reaction in fragmentation of tertiary alkaloids[10)11)]



1-1. Solution of question 1.

Table 1. Retention times, MS data, and MS/MS data of main alkaloids in *Corydalis yanhusuo.*

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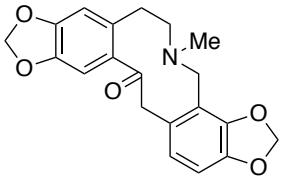
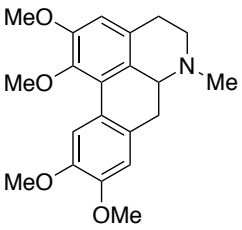
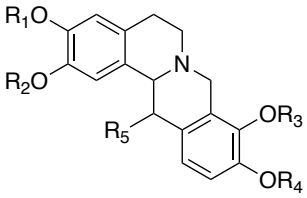


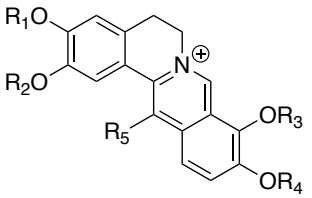
1



2

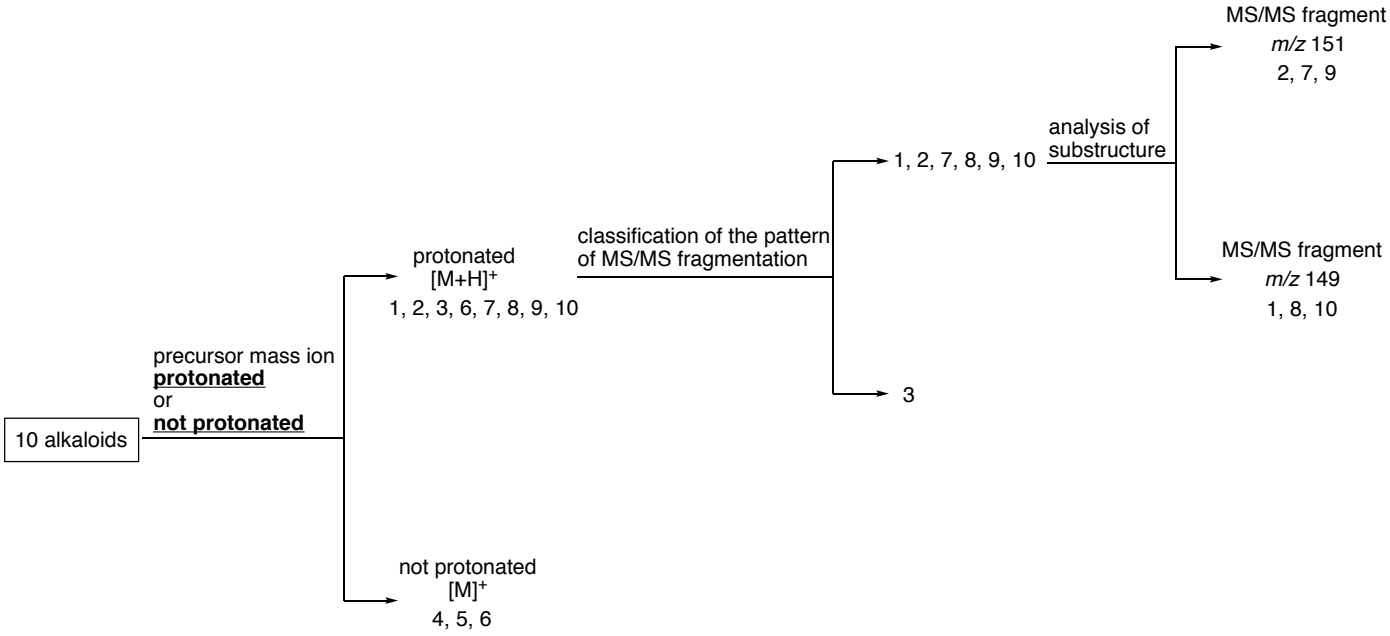


3: $R_1=R_2=R_3=R_4=R_5$=Me
4: $R_1=R_2=R_3=R_4$=Me, R_5=H
5: $R_1,R_2=R_3,R_4$=-CH_2-, R_5=H
6: $R_1=R_3=R_4$=Me, $R_2=R_5$=H
7: R_1,R_2=-CH_2-, $R_3=R_4$=Me, R_5=H



8: $R_1=R_2=R_3=R_4$=Me, R_5=H
9: R_1,R_2=-CH_2-, $R_3=R_4$=Me, R_5=H
10: $R_1=R_2=R_3=R_4=R_5$=Me

overview of classification of alkaloids



MSMS fragmentation path

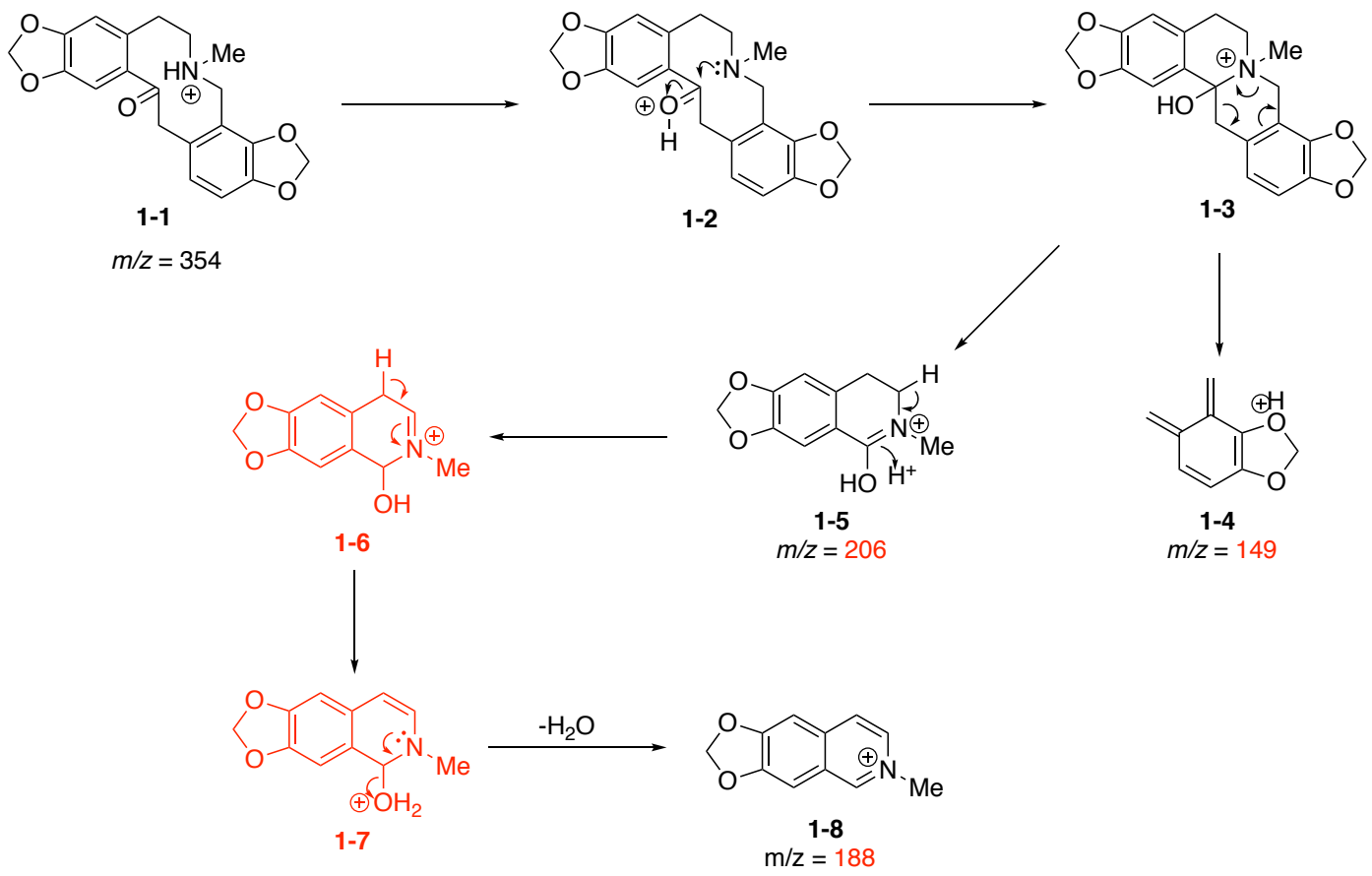
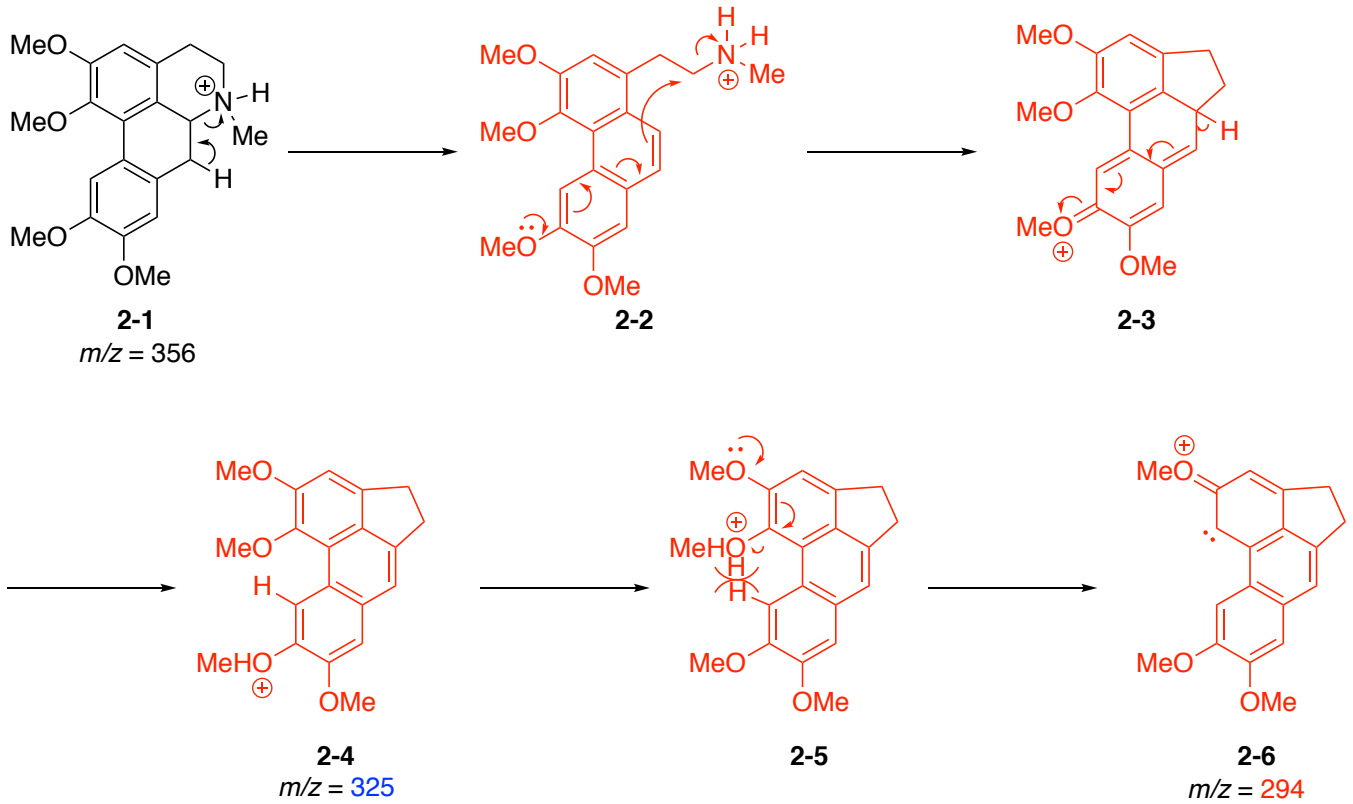


Figure 3. Proposed fragmentation of **1** in MSMS analysis



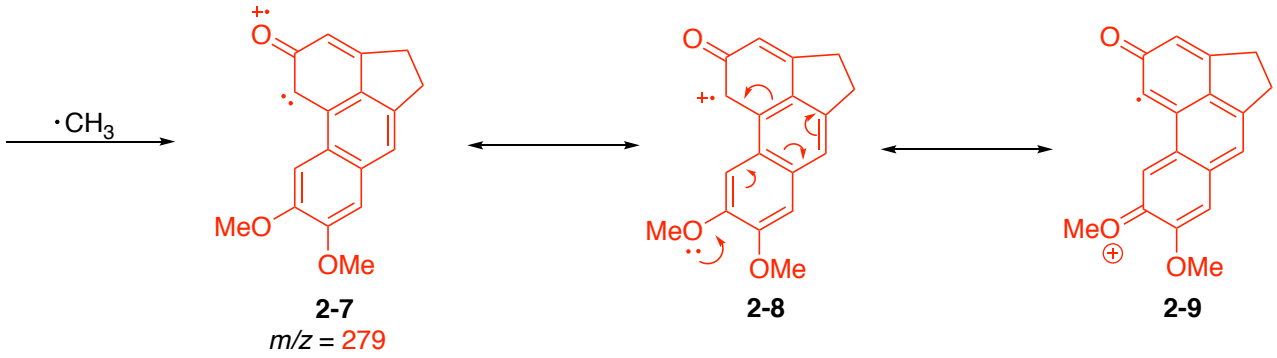


Figure 4. Proposed fragmentation of **2** in MSMS analysis

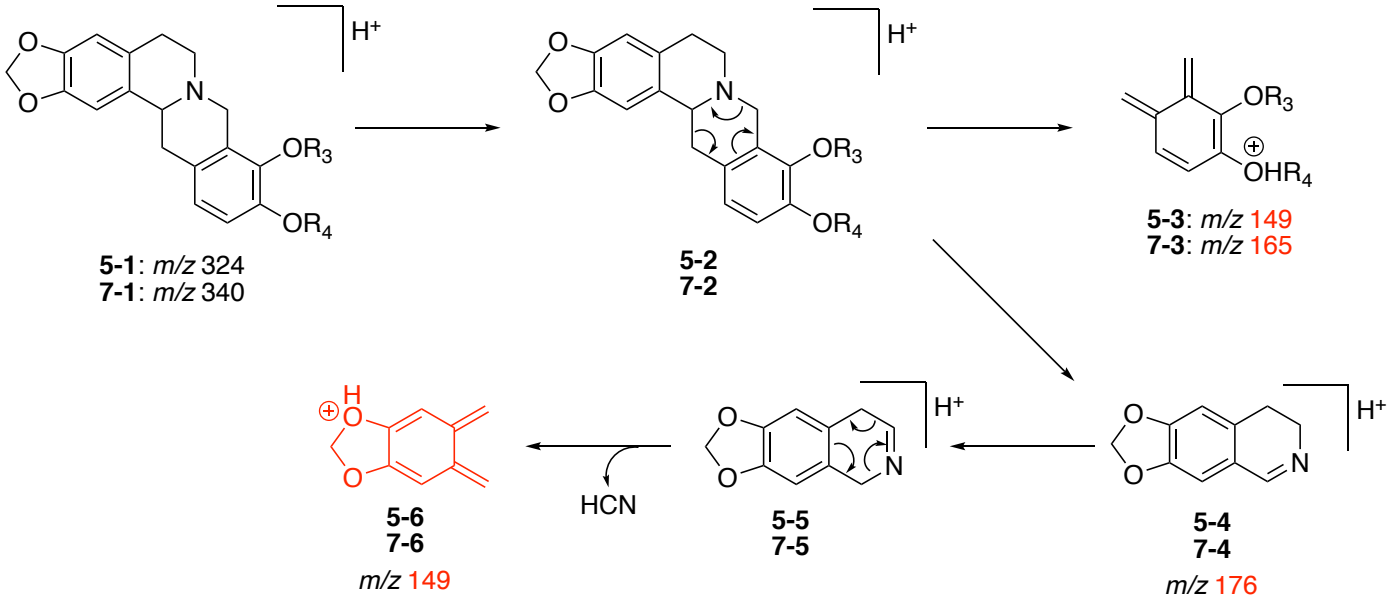
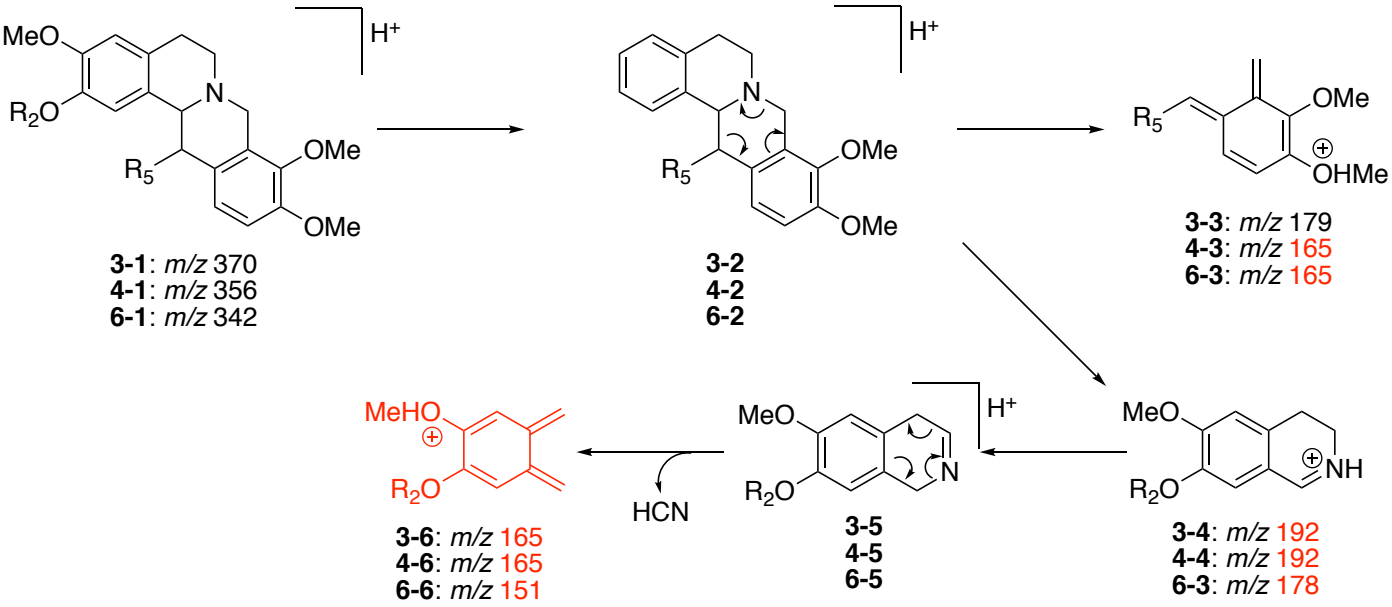


Figure 5. Proposed fragmentation of **5** and **7** in MSMS analysis



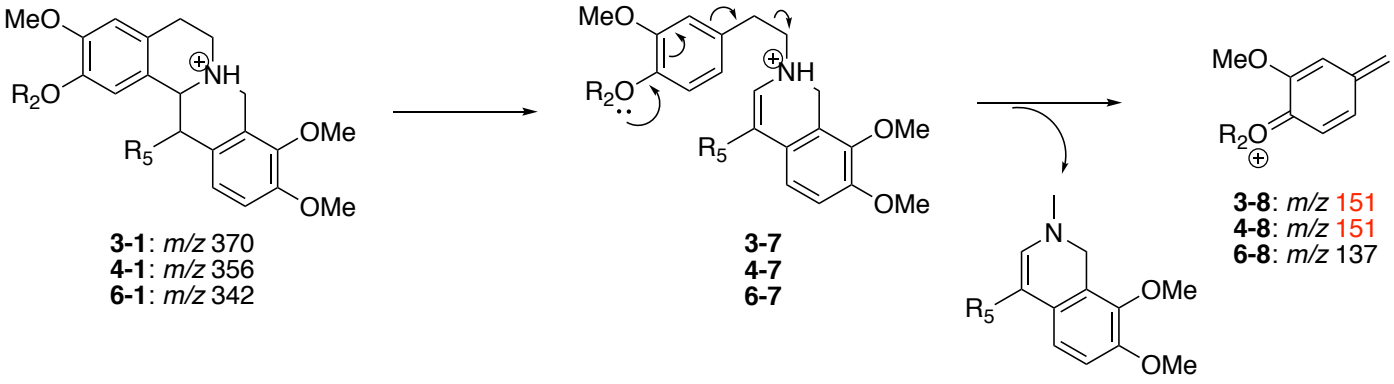


Figure 6. Proposed fragmentation of **3**, **4**, and **6** in MSMS analysis

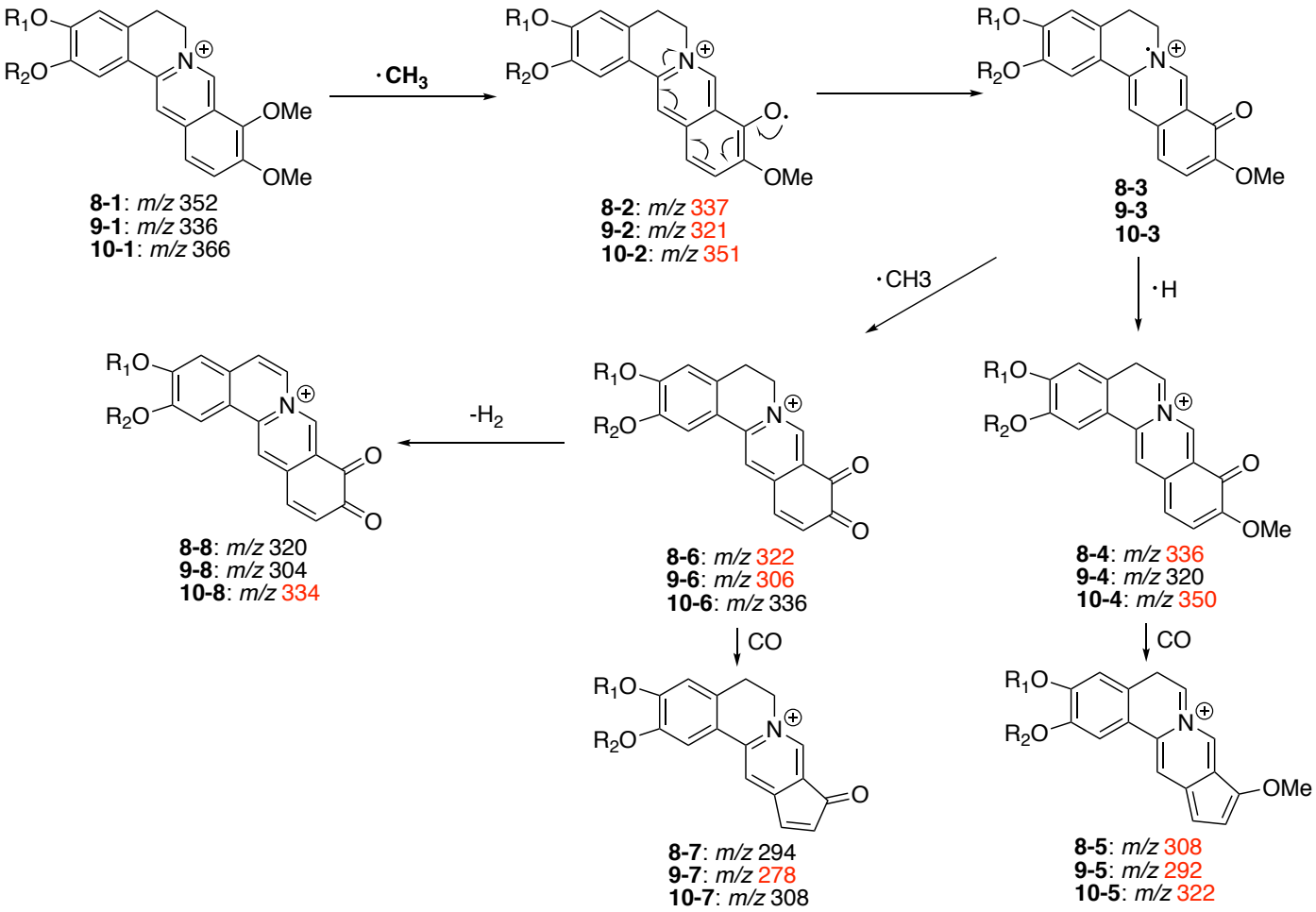
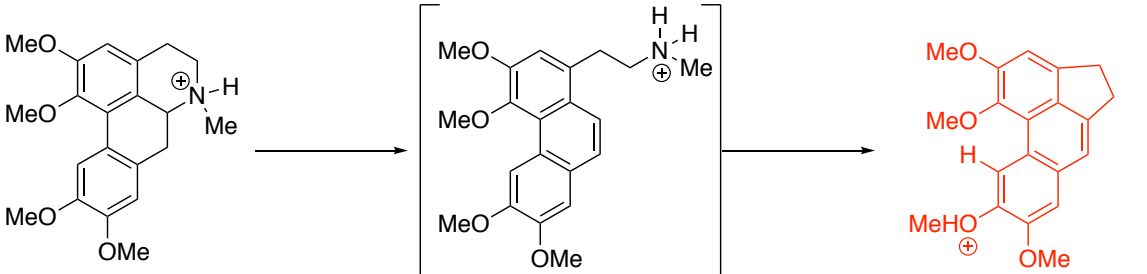


Figure 7. Proposed fragmentation of **8**, **9**, and **10** in MSMS analysis

Answer of Question 1

peak number (analytes)	compound
1	**1** (Protopine)
2	**6** (Tetrahydrocolumbamine)
3	**2** (Glaucine)
4	**8** (Palmatine)
5	**9** (Berberine)
6	**10** (Dehydrocorydaline)
7	**4** (Tetrahydropalmatine)
8	**7** (Canadine)
9	**3** (Corydaline)
10	**5** (Tetrahydrocoptisine)

MS/MS fragmentationin of **2** in other report[11)12)]



2-1
$C_{21}H_{26}NO_4$
[M+H] calcd: 356.1856
found: 356.1846

2-2

2-4
$C_{20}H_{21}NO_4$
[M+H] calcd: 325.1434
found: 325.1441

The fragment ion (*m/z* =324) was not reported in other literature, instead the fragment ion (*m/z* =325) was reported. The mass number of fragmentation was determined in high resolution measurement using calibration mix (error: 2.15 ppm), which suggested that this mass number was correct.

Since it was not reported that the high resolution MSMS analysis was conducted in the main paper, the answer was given after correcting 325 as the correct mass number of the fragment ions.

solution of Question 2

Please calculate the amounts of the alkaloids per crude *Corydalis yanhusuo*, respectively ($mg_{alkaloid}/g_{crude}$).

peak number	calibration curves y = ax + b	peak area y_n
1	y = 69.49x + 266.97	11686
2	y = 56.63x + 185.00	8019
3	y = 65.66x + 160.50	29773
4	y = 56.03x + 201.75	9970
5	y = 68.21x + 76.50	4237
6	y = 62.95x + 42.99	24090
7	y = 56.03x + 201.75	15273
8	y = 46.07x + 105.30	1257
9	y = 57.66x + 202.48	4450
10	y = 55.39x + 261.50	1572

y and x stand for the peak area and the concentration (μg/mL) of the analytes, respectively.

1. concentration of alkaloids injected to LC-MS

$$x_n(\mu g/mL) = \frac{y_n - b_n}{a_n}$$

peak number	concentration (μg/mL) x_n
1	164.3
2	138.3
3	451.0
4	174.3
5	61.0
6	382.0
7	269.0
8	25.0
9	73.7
10	23.7

2. calculate the amounts of the alkaloids per crude using the concentration of crude

$$z_n\left(mg_{alkaloid}/g_{crude}\right) = x_n(\mu g/mL) \times \frac{300}{100} \times 10^{-3}$$

peak number	amount of compound (mg/g) z_n
1	0.493
2	0.415
3	1.35
4	0.523
5	0.183
6	1.15
7	0.807
8	0.750
9	0.221
10	0.0710

3-0. Introduction of matrix effect[15)]

Matrix effect:

quantitative analysis with electrospray ionization (ESI) can be substantially affected by the occurrence of ion-suppression or-enhancement caused by the presence of a matrix or other interferences in the sample.

3-1. mechanistic study

The processes that lead to matrix effect in LC–MS are not fully understood, but they are probably due to the influence of co-eluting compounds on the analyte ionization. There are hypotheses to explain the processes that lead to ion suppression.

3-1-1. competition for the available charges/ the access to the droplet surface between the analyte and the matrix[14)]

This loss can be due to a limited amount of excess charge available on the ESI droplets, or to saturation of the ESI droplets with analyte at their surfaces, thus to inhibit ejection of an ion trapped inside the droplets.

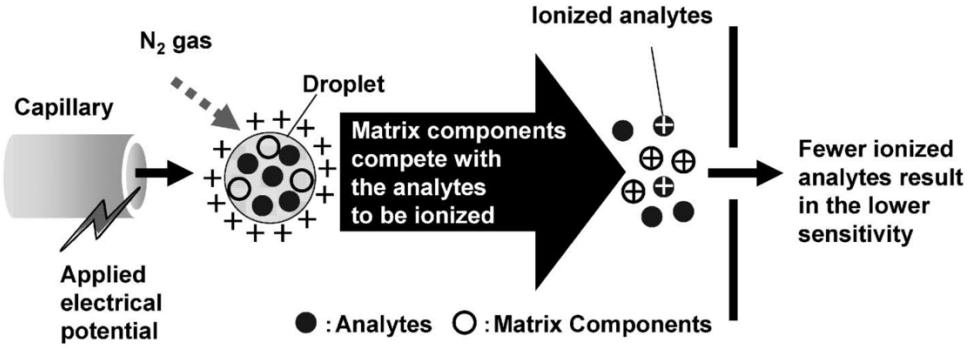


Figure 8. ion suppression by competing other compounds

3-1-2. increasing the viscosity, boiling point and the surface tension of the droplets[15)]

This property of droplet might change the efficiency of their formation and evaporation, which affects the amount of charged ions in the gas phase that ultimately reaches the mass analyzer

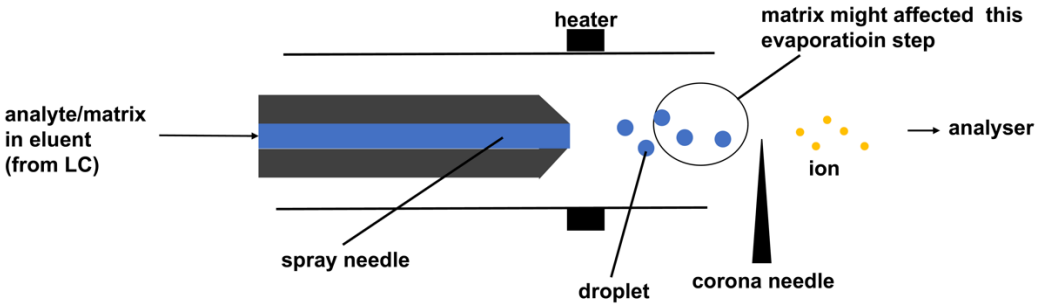


Figure 9. ion suppression by increasing the viscosity, boiling point and the surface tension of the droplets

3-2. solution to the problem caused by matrix effect

A. sample extraction and clean-up

solid-phase extraction, liquid–liquid extraction

B. chromatography

Investigating the column/solvent conditions for separation from impurity

C. calibration methods

C-1. standard-addition method[16)]

The standard-addition method probably represents the most-effective way to compensate for the adverse influence of the matrix on method performances. However, this approach is laborious and time-consuming because spiked samples must be analyzed for each sample.

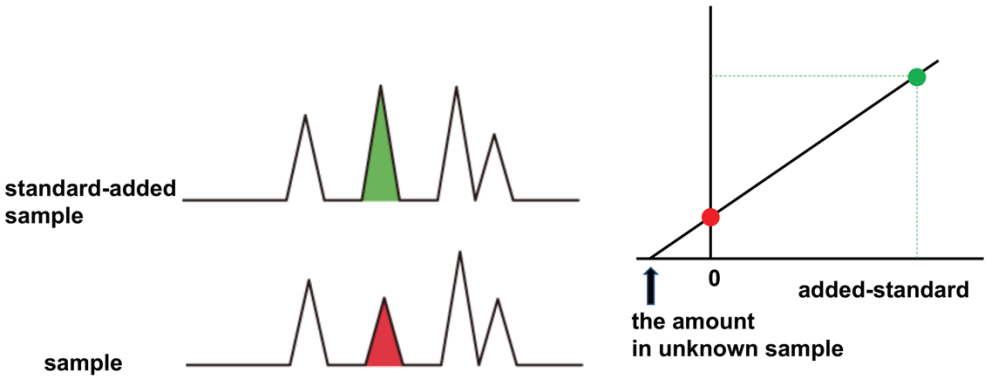


Figure 10. ion suppression by increasing the viscosity, boiling point and the surface tension of the droplets

C-2. stabled isotopic labelled internal standard[17)]

Isotopes are subject to the same matrix effect as the analyte because they have the same retention time. Therefore, the analyte can be quantified by using it as an internal standard and calculating the ratio.

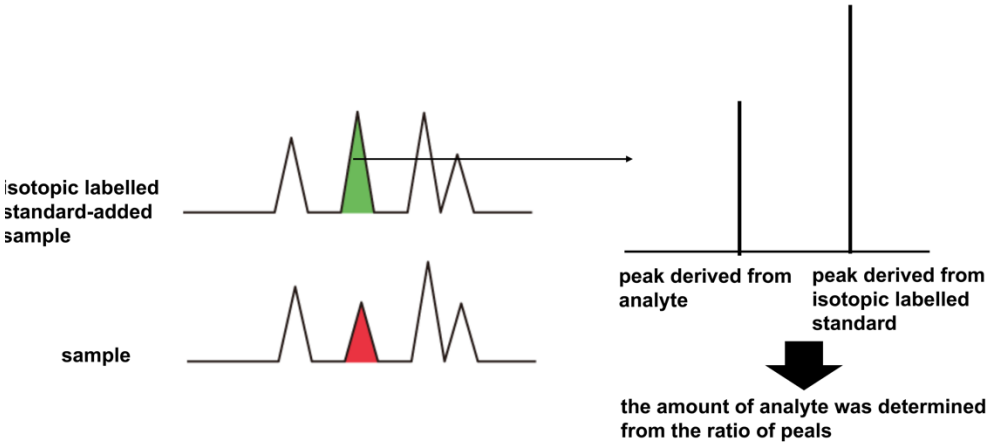


Figure 11. ion suppression by increasing the viscosity, boiling point and the surface tension of the droplets

D. changing ionization techniques

It was reported that APCI[18)190] and APPI[20)] was susceptible to ion suppression than ESI. In the case of ionization suppression by the 3-1 mechanism, the matrix effect can be avoided by changing the ionization mechanism.

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