JARQ 35(3), 149 – 154 (2001) http://www.jircas.affrc.go.jp

Characteristics of HPLC Columns and Mass Spectra of LC-MS for Phytohormone Analysis

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Abstract

The basic characteristics of 5 columns for high performance liquid chromatography (HPLC) and the mass spectra of phytohormones obtained by HPLC-mass spectrometry (LC-MS) were analyzed to use HPLC efficiently and to determine the optimum conditions for phytohormone analysis. In the case of HPLC, when the MeOH concentration (±20%) of the eluent (1 mL/min) was altered, the retention time became stable after 10 min. For 10 authentic standards of phytohormones, the retention time from 30 to 80% MeOH of an eluent was examined in 5 columns. In the octadecylsilane (ODS) type column, the sequence was zeatin (Z) / zeatin-riboside (ZR) → indole-acetic acid (IAA) → trans-abscisic acid (t-ABA) / indole-propionic acid (IPA) → ABA, and $GA_3 \rightarrow GA_7 \rightarrow GA_4$. In LC-MS, authentic standards and labeled ones of both IAA and ABA types showed almost the same changes in the mass spectrum patterns when the drift voltage increased, indicating the points where the fragmentation occurred. Optimum drift voltages for the highest intensity were 60 V for the IAA and ABA types, 40 V for GA_3 and 80 V for GA_4. Values of drift voltages at which molecular ions could be detected were up to 40 V for IAA, 80 V for ABA, 120 V for GA_3 and 140 V for GA_4.

Discipline: Crop production / Horticulture
Additional key words: abscisic acid, cytokinin, gibberellin, indole-3-acetic acid, ODS

Introduction

In all the crops, phytohormones mainly play important roles in the transmission of information within a plant. Thus as a basis for the cultivation and breeding of crops, the elucidation of the physiological effects of phytohormones is essential. To achieve this objective, it is necessary to analyze the endogenous levels of phytohormones. As endogenous phytohormones usually occur at very low levels, only a small amount can be extracted from plant materials. Thus, it is very difficult to analyze the endogenous levels of these substances.

In the purification process of the endogenous phytohormones, high performance liquid chromatography (HPLC) has been used[4)]. HPLC exhibits a higher performance in terms of separation, quantitative properties and reproducibility. Thus it enables to remove impurities efficiently by fractionating the substances for study. Especially, it is important to extract multiple phytohormones from the same materials[7,8)] Additionally by connecting the fluorescence detector to HPLC, a simple method to quantify indole-3-acetic acid (IAA) using indole propionic acid (IPA) as an internal standard has been developed[1,6)].

In the qualitative and quantitative analysis of phytohormones, gas chromatography-mass spectrometry (GC-MS) in which a highly sensitive gas chromatography apparatus is connected to a mass spectrometer which shows a high identification ability, has been used as the most reliable method[4, 9–12)]. Recently HPLC (LC)-MS in which a high performance liquid chromatography apparatus is connected to a mass spectrometer as a detector has been applied. LC-MS does not require the preparation of a derivative. Therefore, for the quantification of a chemical compound without the use of an internal standard, the accuracy of the measurement can be improved, especially in phytohormone conjugates for which it is difficult to make a derivative for application to GC.

The basic characteristics of the columns for HPLC and the mass spectra of phytohormones obtained by LC-MS were analyzed to use HPLC efficiently and to determine the optimum conditions for phytohormone analysis.

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Received 18 January 2001, accepted 22 February 2001.

Characteristics of HPLC columns for phytohormone analysis

Basic characteristics of the columns and retention times for 10 authentic standards were examined in 5 columns to use HPLC efficiently for phytohormone analysis[13].

1) HPLC column

The HPLC column which is widely used to analyze phytohormones is based on the separation mode of a reversed phase, and is packed with silica-gel base bonding octadecylsilane (ODS). In the reversed phase column, the retention times for gibberellins (GAs)[5,15] and cytokinins[3] have been examined. In the GAs, a column of the packing material based on the separation mode of a normal phase type and bonds $(CH_3)_2$ has been used[19]. Although this column enables to separate various GAs adequately, the shorter life of the column is a major constraint.

2) Effects of MeOH volume injected and eluent alteration on retention time[13]

In the case of column I, samples from 20 to 180 μL MeOH (10^{-5} M IAA) were injected for HPLC and the retention time and the peak area were examined (eluent, 40% MeOH in 20 mM acetic acid ; flow rate, 1 mL/min (data not shown)). The peak resulting from the injection of 40 μL MeOH showed an ideal shape, while the peak in the case of 60 μL had a shoulder and the peak in the case of 160 μL was divided into 2 parts. Retention times of both the sample and MeOH remained nearly constant up

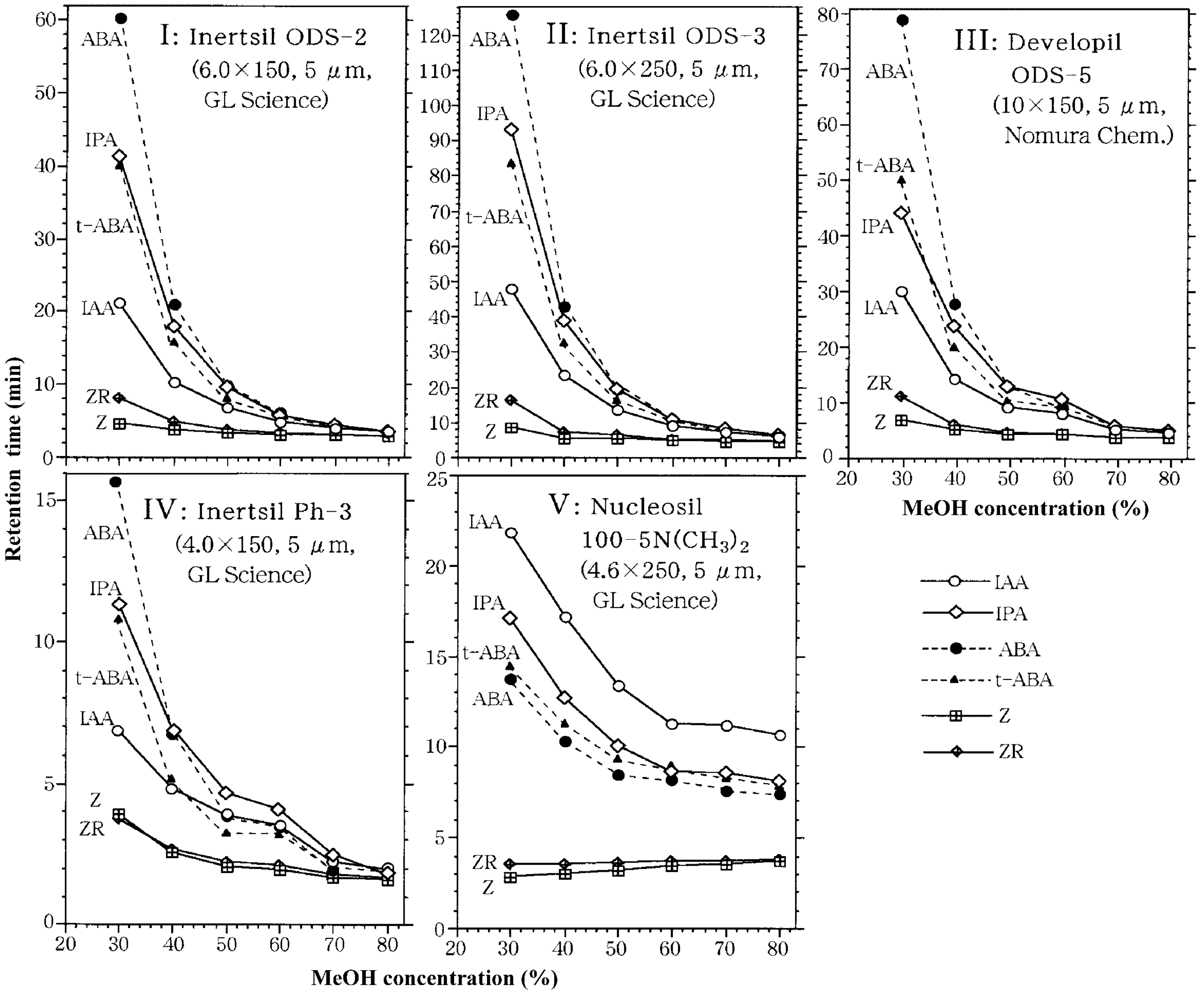

Fig. 1. Relationship between methanol concentration in eluent and retention time of 6 standards in 5 columns
Flow rate: Columns I, II, IV, V, 1 mL/min; Column III, 2 mL/min. Column oven: 40°C.
Eluent constituent: MeOH+H_2O in 20 mM acetic acid (Data from report[13]).

to 180 μL. Peak areas from both the sample and MeOH nearly corresponded to the MeOH volume injected. Thus in 1 mL/min in the column (6 mm I.D.), the MeOH volume within 40 μL was suitable for the determination of the retention time and peak area. After the MeOH concentration in an eluent was altered (40 → 60%, 60 → 40%), the retention time became stable after 6 minutes in columns II and III, and after 9 minutes in columns I, IV and V (data not shown).

3) MeOH concentration in eluent and retention time[13]

For 10 authentic standards of phytohormones, the relationship between the MeOH concentration in an eluent and the retention time was examined in 5 columns. In column I of the ODS type, the retention times between zeatin (Z) and zeatin-riboside (ZR) and between trans-abscisic acid (t-ABA) and IPA were close (not separate), and the sequence was Z / ZR → IAA → t-ABA / IPA → abscisic acid (ABA) (Fig. 1-I). Columns II and III of the ODS type showed a similar tendency (Fig. 1-II, III), confirming an early report on the ODS type column[18].

In column IV of the phenyl (Ph) type in the reversed phase mode, the sequence showed the same tendency as that of the ODS type, but generally the retention times were shorter (Fig. 1-IV). In column V of the normal phase mode, the pattern was different from that of the reversed phase mode (Fig. 1-V). The retention times among ABA, t-ABA and IPA were close, and the retention time of IAA was especially long.

GAs were examined by using authentic standards available commercially. In columns I, II, III and IV, the sequence of the retention time was GA_3 → GA_{13} → GA_7 → GA_4 (Fig. 2-I, II, III, IV). In column V in the normal phase, the sequence of the retention time which was GA_4

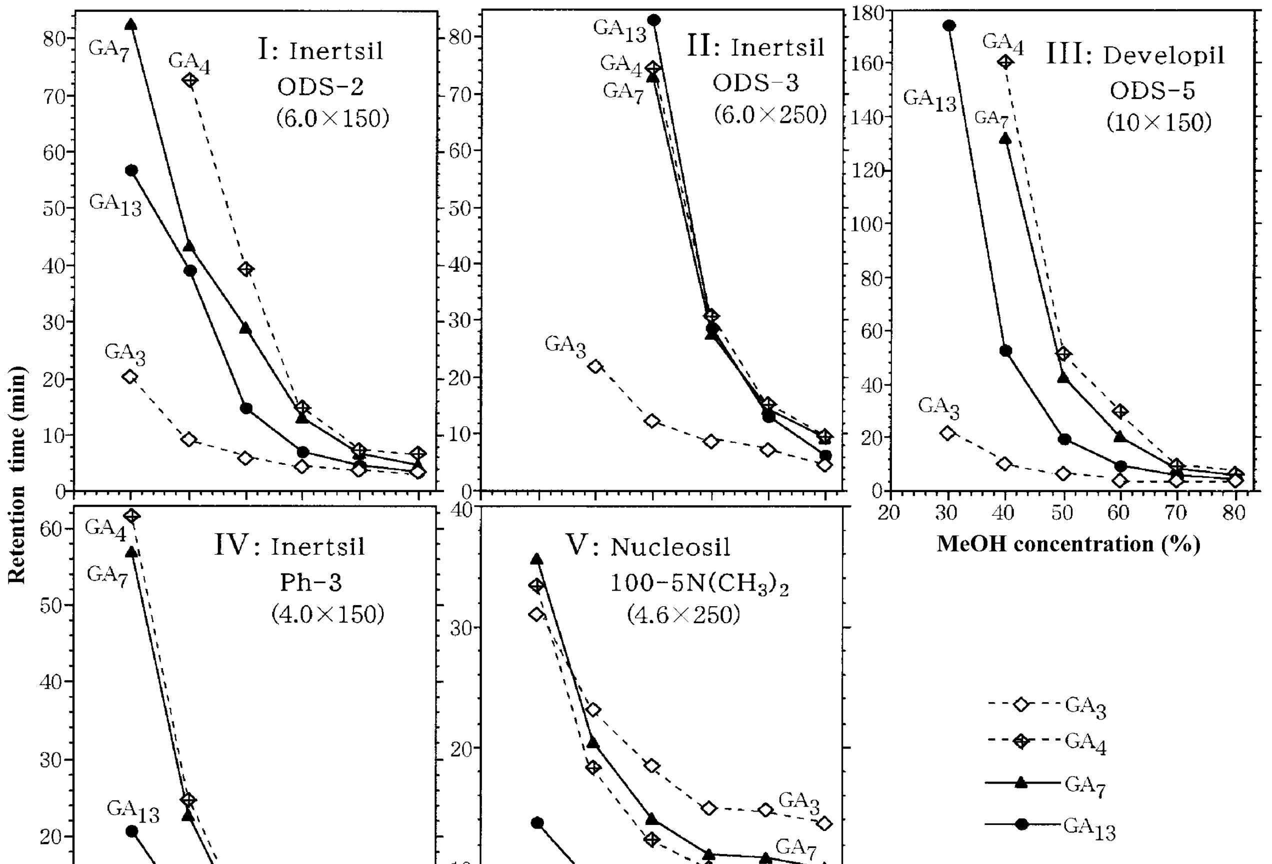

Fig. 2. Relationship between methanol concentration in eluent and retention time of 4 GAs in 5 columns
Same conditions as in Fig. 1.

→ GA_7 → GA_3 (Fig. 2-V), was in the opposite order to the sequence in the reversed phase except for GA_{13}, confirming the tendency observed in the early report[19]. However, in the same column V, the retention time slightly changed and the reproducibility was lower although the sequence was the same.

Mass spectra of phytohormone standards

Mass spectra (pattern and intensity) of each standard were measured when the drift voltage which controls the degree of fragmentation of the standard changed, to obtain the highest sensitivity conditions for LC-MS for IAA, ABA and GA analysis[14].

1) MS

There are 2 measurement modes in LC-MS; 1) the scan mode which monitors repeated scan within a certain mass range, 2) the selected ion monitoring (SIM) mode which monitors only selected ions which are characteristic of the chemical compound studied. The SIM mode shows a higher sensitivity (intensity of a fragment ion) than the scan mode. When the drift voltage increases, the sample is fragmented into smaller parts, and the sensitivity may also change.

As the negative mode was used, the molecular ion weight (m/z) of a standard was 1 atomic mass unit (equivalent to one hydrogen atom) less than the molecular weight (Table 1). $^{13}C_6$-IAA[2] and $^{2}H_6$-ABA[17] have been used as internal standards in phytohormone analysis by GC-MS.

Table 1. Properties of IAA and ABA types for LC-MS

Type Chemical	IAA $^{12}C_6$-	 $^{13}C_6$-	ABA $^{1}H_6$-	 $^{2}H_6$-
Mol wt	175	181	264	270
Optimum voltage[a]	60	60	60	60
Base peak (m/z)[b]	144	150	263	269

a): Drift voltage for the highest intensity of base peak.
b): Optimum drift voltage.

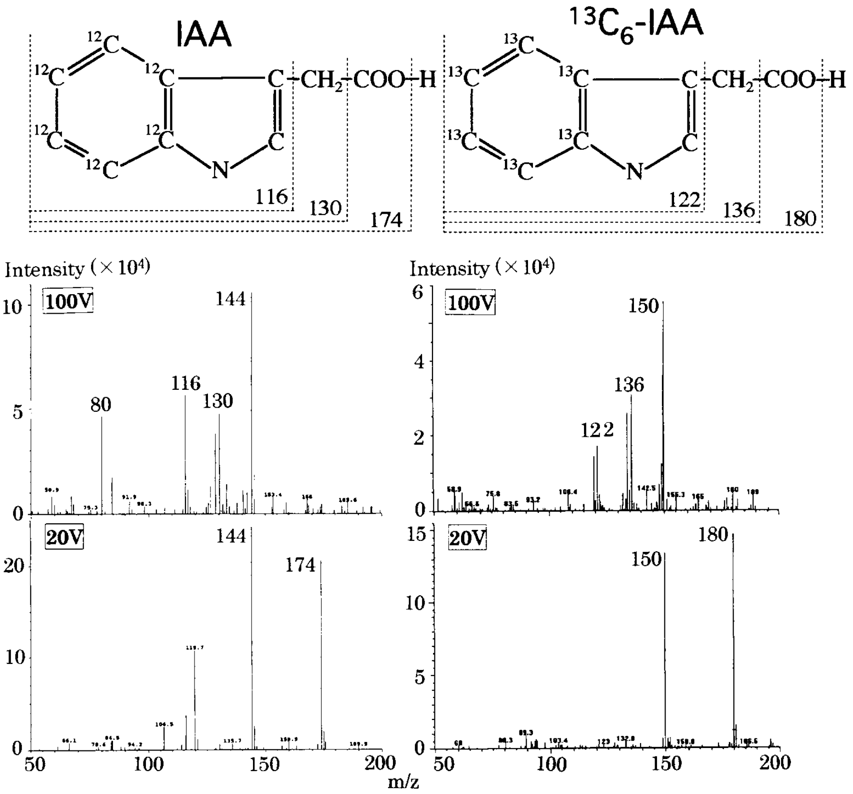

Fig. 3. Chemical structure and mass spectra of IAA and $^{13}C_6$-IAA at drift voltages of 20 and 100 V
LC-MS: M-1200H Hitachi, Ionization: Atmospheric pressure chemical ionization (APCI), Polarity: Negative mode, Dwell count: 1,000, Eluent: MeOH+H_2O in 20 mM acetic acid, Flow rate: 1 mL/min, Sample: 1,000 pmol (Data from report[14]).

2) IAA and $^{13}C_6$-IAA

Substitution of 6 stable ^{13}C isotopes in $^{13}C_6$-IAA for six ^{12}C isotopes of an indole ring in IAA (Fig. 3) was performed. For a drift voltage of 20 V, the molecular ion peak of IAA was observed at 174 m/z, and that of $^{13}C_6$-IAA at 180 m/z (Fig. 3). For 100 V, the fragment ion peaks, 116 and 130 from IAA corresponded to the 122 and 136 peaks from $^{13}C_6$-IAA, respectively, of which the chemical structure and fragmented points were presented. However the fragmented point of the fragment ions, 144 and 150 of base peaks could not be determined. Continuous injection of the same amount of IAA after changing the drift voltage from 20 to 100 V indicated that the optimum drift voltage for the highest intensity was 60 V[14] (Table 1).

3) ABA and $^{2}H_6$-ABA

ABA and $^{2}H_6$-ABA showed almost the same changes in the mass spectrum patterns (Fig. 4). The fragment ion peaks, 153 and 219 from ABA corresponded to the 159 and 225 peaks from $^{2}H_6$-ABA, respectively, confirming the previous report in which the determination was performed only for ABA[16]. The fragment ion peaks, 204 from ABA and 207 from $^{2}H_6$-ABA showed a fragmented point in ring-CH_3, correcting what was previously reported[16]. The optimum drift voltage for the highest intensity was also 60 V when the drift voltage changed from 20 to 140 V[14] (Table 1) .

4) GA_3 and GA_4[14]

For GA_3, the molecular ion peak, 345 was observed up to 140 V (data not shown). From 100 V, characteristic fragment ion peaks, 143 and 221 appeared. The highest intensity corresponded to 40 V when the drift voltage had been changed from 20 to 180 V. For GA_4, a molecular ion, 331 was only observed from 20 to 80 V, and above 100 V, fragment ion peaks, 213 and 225 appeared. The highest intensity corresponded to 80 V when the drift voltage had been changed from 20 to 180 V[14].

Values of drift voltages at which molecular ions were detected were up to 40 V for the IAA types, 80 V for the ABA types and 140 V for GA_3 and GA_4. This voltage might reflect the stability of the standard molecule.

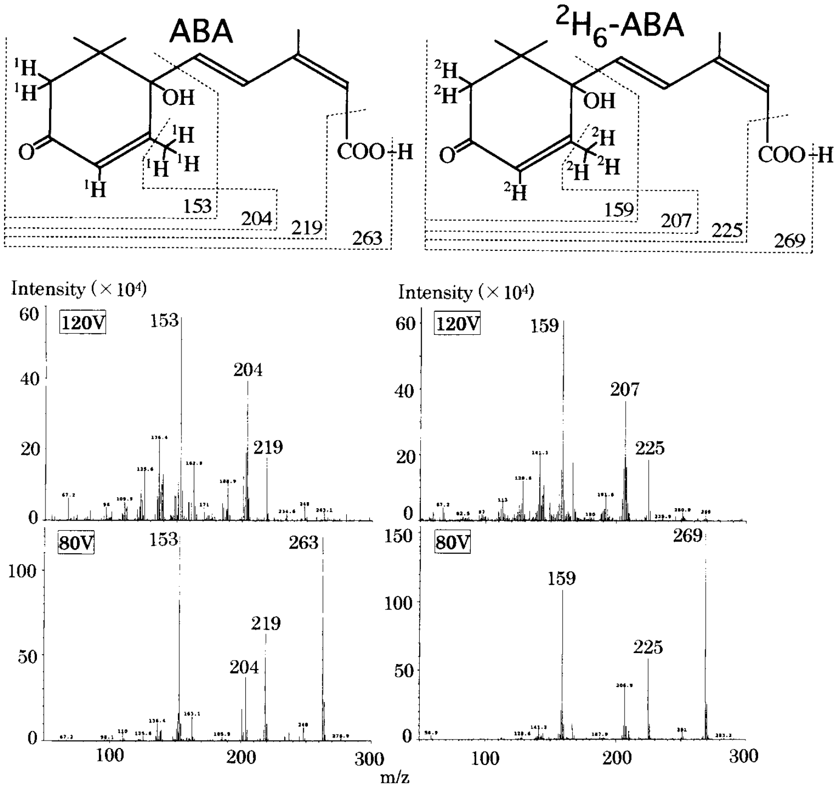

Fig. 4. Chemical structure and mass spectra of ABA and $^{2}H_6$-ABA at drift voltages of 80 and 120 V
Same conditions as in Fig. 3.

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