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Liquid Chromatography MS/MS Responses on Lentinan for Structure Characterization of Mushroom Polysaccharide β -D-Glucan

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Abstract

Lentinan, a complex polysaccharides β-D-glucan found in Shiitake Mushroom has been known to have medicinal effect such as anticancer and immune-modulatory properties. Analysis on lentinan through application of high-end biotechnology instrument such as mass spectrometry is still limited. In this study, Liquid Chromatography Mass Spectrometry-Quadropole Time of Flight (LC/MS Q-TOF) has been used to optimize the MS/MS response in elucidating the lentinan characteristics. Analysis was done by using lentinan standard at different concentrations through different combinations of four types of columns and four types of buffers. Four levels of MS/MS collision energies or known as Collision Induced Dissociation (CID) were applied. Data presented shown that the optimum MS/MS CID that generated all six product ion of lentinan at the most combinations of column and buffer was at 20.0 V. Combination of Altima Hydrophilic Interaction Liquid Chromatography (HILIC) column without any buffer in mobile phase (AH; NB) and Hypercarb column with the present of 0.1% acetic acid in mobile phase (TH; 0.1% AA) respectively were found to be the best combinations among other combinations as these combinations were able to generate product ions of lentinan at lentinan concentration as low as 0.156 mg/mL. This suggested that these column and buffer combinations have given good sensitivity to enhance peak efficiency of lentinan at low concentration which was required to meet MS/MS abundance threshold. As the conclusion, suitable combinations of column and buffer are crucial in order to enhance peak efficiency of lentinan at optimum ionization intensity to allow optimum MS/MS responses for elucidation of their structure characteristics. Determination of appropriate collision energy also plays an important role to generate MS/MS spectra of all product ions for the lentinan.

Keywords: Mushroom β-D-glucan; Lentinan; LC/MS Q-TOF; MS/MS spectra; Structure characterization

Introduction

Lentinan, a polysaccharide β-D-glucan, is widely used as an alternative medicine and also as dietary supplement nowadays as it claimed to have a lot of health benefits such as antimicrobial, antitumor, hypocholesterolemic actions and antioxidants. It was detected for years in a well-known edible mushroom namely Shiitake Mushroom (*Lentinus edodes*) [1- 4]. This compound has been known as Biology Response Modifier (BRM) as it is actively mediates anticancer activity through activation of human immune system [1,5-7]. Lentinan has been reported to have ability in treating many types of cancers including liver, stomach, lung, ovarian and colon cancers through regression of tumour formation [2,6,8]. Daily consume of lentinan on rats has also shown the ability of the compound on weight gains and increased of white blood cells such as monocytes [5].

Lentinan is a type of 1,3/1,6-β-D-glucan with molecular mass of 400000 Dalton [1,3,4]. It is comprised of repeated units of glucose with seven glucose molecules in each repeating unit (Figure 1) that gives the molecular formula of C42H72O36 and molecular mass of 1152 Dalton [1,3]. The solubility of lentinan reported was varies. Chihara et al. has reported that lentinan is mostly soluble in alkali solution and formic acid, slightly soluble in hot water and not soluble in cold water, acid solution and organic solvents such as alcohol, ether, chloroform, pyridine and hexamethylphosphoramide [9]. However, latter, this compound has been reported as water soluble β-D-glucan which is readily soluble in water [4,7] but not soluble in 50% (v/v) ethanol [4]. There was a study shown that no peak able to be detected by LC/MS/MS Q-TOF from lentinan extracted by alkaline solution as compared to lentinan from hot water extract [10].

Several methods have been applied by researchers to determine the presence of lentinan in mushroom such as GC/MS (for detection of monosaccharide), methylation analysis (for determination of the positions of glycosidic linkages) and NMR spectroscopy (for determination of degree of branching and the degree of polymerization) [7]. Nowadays, Liquid Chromatography-Mass Spectrometry (LC-MS) also has been used on determination of lentinan [10]. LC/MS/MS Quadropole-Time of Flight is one of very powerful instruments that can provides exact mass information (molecular weight) and fragmentation patterns (product ions) for elucidation on the compound structure [11].

The use of this high end instrument in determining the optimum MS/MS response for Lentinan is still not reported. Selection of right columns, types of buffers and level of MS/MS collision energy need to be emphasized in order to obtain better response [12]. Therefore objective of this study was to gain better LC/MS/MS response in elucidating lentinan characteristics through application of different types of columns, mobile phase's buffers and level of collision energies.

Materials and Methods

Chemicals

All solvents were LC/MS grade. Water was Milli-Q grade with the

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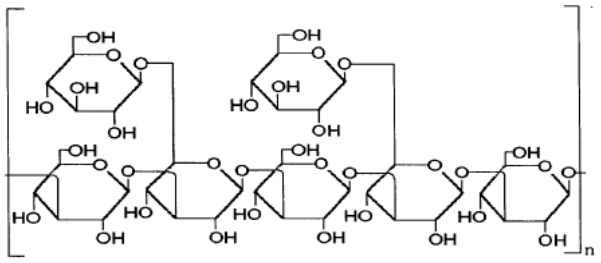


Figure 1: Structure of one unit of lentinan with seven glucose molecules

total organic compound less than 3 ppb.

Sample material

Lentinan Standard isolated from *Lentinus edodes* was purchased from CarboMer Incorporation (San Diego, USA).

Sample preparation

Lentinan standard was prepared at 2.5 mg/mL with clean Milli-Q water. The standard was dissolved in Milli-Q water and mixed well. The solution was heated at 80°C in sonicator water-bath for complete mixing. Serial dilution of the standard was carried out up to 0.078 mg/mL.

LC/MS Q-TOF analysis

Analysis was performed by using an Agilent 1290 RRLC (Rapid Resolution Liquid Chromatography) series equipped with 6550 iFunnel Q-TOF LC/MS System. Lentinan standard was run from the lowest concentration to the highest concentration. Clean Milli-Q water was used as blank. Four types of columns were used for the analysis (Table 1). Mobile phases used were 100% water (A) and 100% acetonitrile (B) with addition of different types of buffers; i) No buffer; ii) 0.1% formic acid; iii) 0.1% acetic acid; and iv) 10 mM ammonium formate respectively. Columns parameters were set as per Table 1.

Gradient for normal phase was 90%-75% B (0-5 min); 75%-50% B (5-10 min); 50%-5% B (10 –12 min); 5% B (12-13 min) and 5%-90% B (13-15 min). Reverse phase gradient was set at 5%-60% B (0-7 min); 60%-90% B (7-11 min); 90%B (11-12 min) and 90%-5% B (12-15 min). The total run time was 15 minutes for each sample.

Analysis was performed in MS/MS run mode at negative ion polarity with the following settings:- Gas Temperature: 250°C; Gas Flow: 13 L/min; Nebulizer pressure (N_2): 35 psig; Sheath gas: 11 L/min at 350°C; Capillary voltage: 3500 V; Nozzle voltage: 1000 V; Fragmentor voltage: 175 V; and Drying gas: 5 L/min at 350°C. MS/MS collision energies were set at 0.0 V, 10.0 V, 20.0 V and 40.0 V.

The profile patterns of total ion chromatogram (TIC), retention time, generation of product ions (fragmentation patterns) and MS/MS response sensitivity on lentinan were analysed.

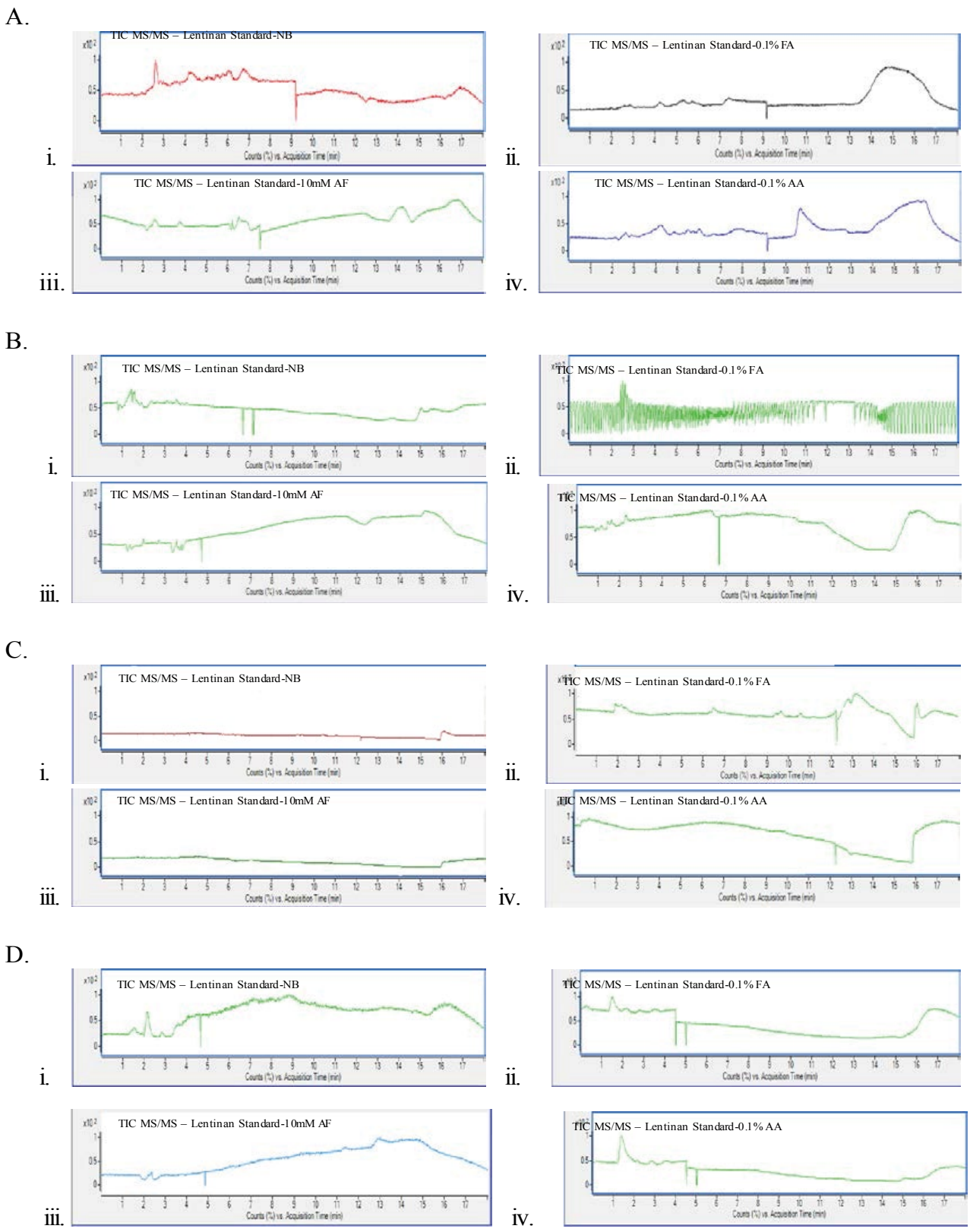
Results and Discussion

The MS/MS chromatogram for total ion chromatogram (TIC) of 2.5 mg/mL lentinan was showed in Figure 2. Lentinan peak was in circled area. Results showed that different combinations of columns and buffers used were led to different TIC patterns. The same type of column with the presence of different types of buffers in mobile phases has produced different TIC patterns of the lentinan standard. The TIC pattern was also totally different with the use of different types of columns. As shown, there was no lentinan peak at the MSMS TIC showed by the Prevail Carbohydrate (PC) column with the presence of 10 mM Ammonium Formate (10 mM AF) in mobile phases. Absence of the MS/MS peak determined the failure generation of MS/MS spectra. This was probably due to insufficient abundance threshold of the lentinan for MS/MS generation. Small changes in pH are able to cause extreme sensitivity of some compounds [13]. Therefore this can suggested that combination PH: 10 mM AF was unable to increase sensitivity of lentinan to be ionized at particular abundance threshold. TIC pattern of lentinan standard that passed through the combination of Poroshell120 HILIC (PH) column with 0.1% Formic Acid (FA) was resulted to a lot of ion suppression. The details of causes of ion suppression are not clear. The use of common buffers also might lead to formation of ion suppression. Proper sample preparation needs to be emphasized in order to minimize or eliminate the ion suppression [14]. This suggested that extra precaution is required when analysing lentinan using this PH: 0.1% FA combination.

Figure 3 showed the Extracted Ion Chromatogram (EIC) for retention time of lentinan. As shown, the retention time was changed with different combination of columns and buffers. However some combination was not affecting the retention time of lentinan. As shown in the figure, lentinan that passed through Altima HILIC (AH) column showed the same retention time at 9.3 min with the use of mobile phases that contained of No Buffer (NB), 0.1% Formic Acid (0.1% FA) and 0.1% Acetic Acid (0.1% AA). The retention time was shifted to 7.5 min with the use of mobile phases that contained 10 mM Ammonium Formate (10mM AF). Retention time of the lentinan was different at all with the presence of these four types of buffers which were 6.7 min (NB), 2.6 min (0.1% FA), 4.7 min (10mM AF) and 6.8 min (0.1% AA) through Poroshell120 HILIC (PH) column. Use of Prevail Carbohydrate (PC) column with mobile phases containing 0.1% FA and 0.1% AA remained the same retention time for lentinan (12.4 min), slightly changed to 12.3 min with the mobile phases without any buffer and fasten to 10.9 min with the use of mobile phases containing 10mM AF. While Hypercarb (TH) column has retained the lentinan at almost the same time with the use of the mobile phases containing NB (4.7 min), 0.1% FA (4.8 min), 10 mM AF (5.0 min) and 0.1% AA (4.8 min). In general, retention time of a compound will be different with the use different types of columns [13]. Therefore different characteristics of columns have affected the time of lentinan to be eluted. However, application of different types of buffer in mobile phases through the same types of column has also led to the changes of retention time of lentinan. Combination of column and buffer could contribute to the modification of the column characteristics. The buffer used has adjusted the pH in mobile phases and caused an effect on the stability of columns. It then affects indirectly the peak efficiency and retention of analytes [15]. Furthermore, the adjustment of pH could also influence the column characteristics which contribute to the polarity of analytes that eluting analytes at different times [16]. Alteration of pH

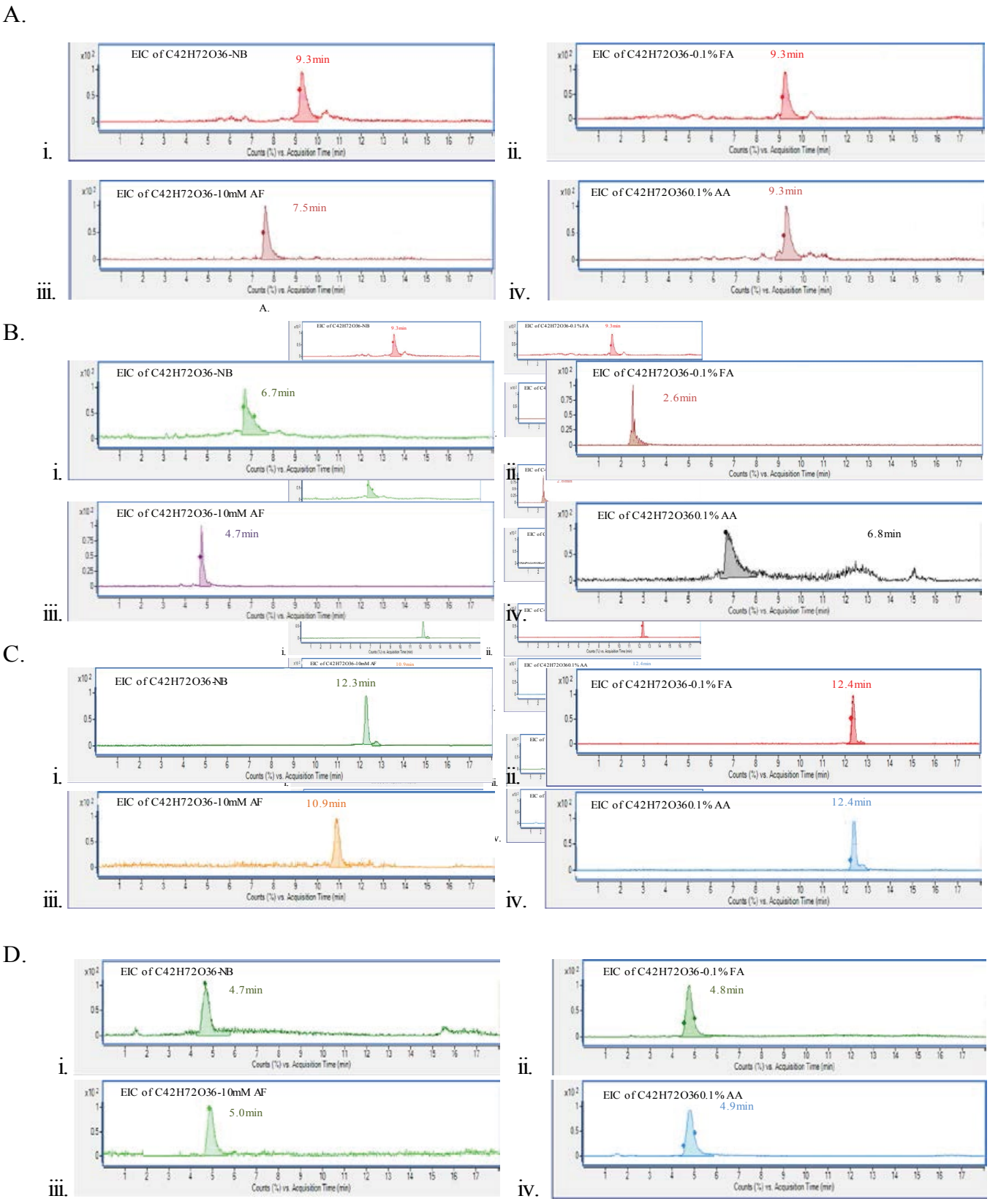
	Parameters			
Column types	**Altima HP HILIC (AH)**	**Poroshell120 HILIC (PH)**	**Prevail Carbohydrate (PC)**	**Hypercarb (TH)**
Particle size (µ)	3	2.7	5	3
Column Size (mm)	2.1 x150	2.1 x 150	4.6 x 150	2.1 x 100
Injection volume (µl)	1	1	1	0.5
Flow rate (mL/min)	0.2	0.3	0.7	0.15
Gradient type	Normal phase	Normal phase	Normal phase	Reverse phase
Column temperature (°C)	25	25	30	25

Table 1: Parameters setup for different types of columns (as reported by column packing note)



A. Altima HILIC (AH) column; B. Poroshell120 HILIC (PH) column; C. Prevail Carbohydrate (PC) column; and D. Hypercarb (TH) column with the presence of (i) No buffer (NB), (ii) 0.1% formic acid (FA), (iii) 0.1% acetic acid (AA) and (iv) 10mM ammonium formate (AF) in mobile phases.

Figure 2: Total Ion Chromatogram (TIC) of lentinan standard at 2.5 mg/mL



A. Altima HILIC (AH) column; B. Poroshell120 HILIC (PH) column; C. Prevail Carbohydrate (PC) column; and D. Hypercarb (TH) column with the presence of (i) No buffer (NB), (ii) 0.1% formic acid (FA), (iii) 0.1% acetic acid (AA) and (iv) 10mM ammonium formate (AF) in mobile phases.

Figure 3: Retention times of lentinan at 2.5 mg/mL standard concentration

is one of the most important parts in the control of retention time of compounds [13,15]. These suggested that the different pH level of the mobiles phases has resulted to different reaction to the lentinan which then affected it retention time.

Development of optimum MS/MS spectra of lentinan is necessary to study the fragmentation pattern as well as structural characteristic of this compound. Lentinan is a β-D-glucan polysaccharide of 1151 m/z that consists mainly and only repeating units of glucose molecules. The cleavage of the lentinan at specific chains releases glucose units (molecular mass of 162) and resulted to generation of product ions of 989 m/z, 827 m/z, 665 m/z, 503 m/z, 341 m/z and 161 m/z (341 m/z minus 179 [glucose with OH group from water]). Further optimization to fragment the lentinan was done by applying different collision energies or Collision Induced Dissociation (CID) to the compound. Figure 4 showed the MS/MS spectra (fragmentation patterns) of 2.5 mg/mL lentinan that obtained from CID of 0.0 V, 10.0 V, 20.0 V and 40.0 V. As summarized in Table 2, the numbers of product ions of the lentinan generated were different at some CID levels. Optimum MS/MS spectra with all the six targeted products ions (989 m/z, 827 m/z, 665 m/z, 503 m/z, 341 m/z and 161 m/z) was successfully obtained at CID of 20.0V for lentinan that passed through Altima HILIC column with No Buffer (AH; NB), 0.1% Formic Acid (AH;0.1% FA) and with 0.1% Acetic Acid (AH; 0.1% AA) respectively, Poroshell120 HILIC without any buffer (PH; NB) and with 10 mM Ammonium Formate (PH;10 mM AF) respectively, Prevail Carbohydrate with No Buffer (PC; NB), with 0.1% Formic Acid (PC; 0.1% FA) and 0.1% Acetic Acid (PC; 0.1% AA) respectively and also through Hypercarb column with No Buffer (TH; NB), 0.1% Formic Acid (TH; 0.1% FA) and 0.1% Acetic Acid (TH; 0.1% AA) respectively. Collision energies of 0.0 V, 10.0 V and 40.0 V under these column and buffer combinations were able to generate one to five product ions only. However, some columns and buffer were also able to generate the optimum fragmentation at CID of 10.0 V such as at AH; 0.1% AA, PH; 10 mM AF, PC; NB and PC; 0.1% AA. This shown that the optimum MS/MS CID that generated all six product ion of lentinan at most of combinations of column and buffer was at 20.0 V. There were certain column and buffer combinations that unable to generate optimum MS/MS spectra for lentinan at any CID level. This can be seen at lentinan standard that passed through AH; 10mM AF, PH; 0.1% FA, PH; 0.1% AA, PC; 10mM AF and TH; 10 mM AF. No product ion of lentinan was generated at 10.0 V for the standard that passed through PH; 0.1% FA. The presence of 10mM ammonium formate in mobile phases through Prevail Carbohydrate (PC) column was totally failed to generate MS/MS spectra at any collision energy even at high concentration of the standard. These results suggested that the presence of buffers which changed the pH condition of mobile phases have affected the ionic level or conformation shift of the lentinan. It then has reflected the sensitivity of certain column to retain the lentinan and probably led to induction or reduction on peak efficiency and ion transmission level of the lentinan that affected the ion abundance threshold required for MS/MS responses. Therefore it suggested that only combinations of AH; NB, AH; 0.1% FA, AH; 0.1% AA, PH; NB, PH; 10 mM AF, PC; NB, PC; 0.1% FA, PC; 0.1% AA, TH; NB, TH; 0.1% FA and TH; 0.1% AA were able to meet the ion abundance threshold for MS/MS generation at optimum fragmentation pattern at 20.0 V CID.

Six different concentrations of lentinan standard (0.078 mg/mL, 0.156 mg/mL, 0.312 mg/mL, 0.625 mg/mL, 1.25 mg/mL and 2.50 mg/mL) were evaluated at CID 20.0 V to determine the lowest level of the lentinan concentration can be fragmented to produce all the six product ions of lentinan through different combinations of column and buffer. Supplemenatry Files (1-4) showed the MS/MS spectra of different lentinan concentrations at the column and buffer combinations that generated optimum MS/MS spectra in previous analysis. Each lentinan concentration showed a peak of 1151.3731. However, the lowest concentration of lentinan that successfully generated MS/MS spectra was different for different combinations of buffer and column.

As shown in Table 3, there was no MS/MS spectra generated for lentinan standard at concentration of 0.078 mg/mL. The lowest concentration of lentinan that generated MS/MS spectra with all the six product ions was at 0.156 mg/mL which only showed by AH; NB and TH; 0.1% AA combination respectively. MS/MS spectra for lentinan standard that used PC; NB, PC; 0.1% FA, PC: 0.1% AA, TH; 0.1% FA combinations were started to be generated at 0.312 mg/ml standard concentration. Generation of MS/MS spectra for lentinan that passed through Altima HILIC with the presence of 0.1% FA and 0.1% AA respectively was only be obtained for the highest three concentrations which were at 0.625 mg/mL, 1.25 mg/mL and 2.5 mg/mL. This was same for the standard that passed through Poroshell120 HILIC without buffer and with 10 mM AF. Lentinan that passed through TH: NB has shown the generation of MS/MS spectra at only 2.5 mg/mL of the standard. The results shown that the combinations of column and buffer were probably had influence the differences on MS/MS responses through alteration of column and lentinan characteristics at different pH condition which might be reflected the sensitiveness of the columns to retain the lentinan and hence the peak efficiency. These presented data have suggested that the best combinations found in this study was Altima HILIC column without any buffer in mobile phase (AH; NB) and Hypercarb column with the present of 0.1% acetic acid in mobile phase (TH; 0.1% AA) respectively as the combinations have given good sensitivity to enhance peak intensity of lentinan at low concentration that required for MS/MS spectra generation.

Conclusion

Compound analysis by Liquid-Chromatography Mass Spectrophotometry is based on mass to charge ratio (m/z). β-D-glucan such as lentinan, schizophyllan and krestin is a highly polar compound that consists of long chain polysaccharides. The number of charges for this big compound (such as Lentinan with 400000 Da, 1151 m/z) could be more than one. Analysis of lentinan through LC/MS that based on m/z ratio could produce the same m/z ratio as other types of polysaccharides such as oligosaccharides which are also polar compound and consist of long chain polysaccharides. Optimization of the MS/MS response is so important in order to determine the actual characteristics of lentinan in term of molecular weight, retention time and also the product ions for structure confirmation purpose. Selection of suitable collision energy or CID during fragmentation of lentinan for generation of all the related product ions should be made. However, the successfulness of the fragmentation process through MS/MS mode is also depending on the level of ionization intensity of the particular compound. Enhancement of peak efficiency through suitable combination of column and buffer is so crucial in order to enhance the detection sensitivity of the LC/MS to meet certain MS/MS requirement. It could be done by strengthening the ionic condition of the compound and increasing the columns competency to retain the compound through modification of pH condition by certain buffers. As the conclusion, this study has found that the optimum performance of MS/MS response for lentinan fragmentation was at 20.0 V CID. Combinations of Altima HILIC with no buffer and Hypercarb column with 0.1% acetic acid were found to be the best combinations in this study as the MS/MS spectra able to generate all the six lentinan product ions at the lentinan concentration as low as 0.156 mg/mL. Use of

Column & Buffer Combination	Collision energy (CID), v	Generation of product ions (m/z) at 2.5 mg/mL lentinan						
		1151	989	827	665	503	341	161
AH;NB	0	√	√	√	√	√	x	x
	10	√	√	√	√	x	√	x
	20	√	√	√	√	√	√	√
	40t	x	x	√	√	√	√	√
AH;0.1% FA	0	√	√	√	√	√	x	√
	10	√	√	√	√	√	x	x
	20	√	√	√	√	√	√	√
	40	√	x	√	√	√	√	√
AH;10 mM AF	0	√	√	√	√	x	x	x
	10	√	√	√	√	√	√	x
	20	√	√	√	√	√	x	√
	40	x	x	√	√	√	√	√
AH;0.1% AA	0	√	√	√	√	x	x	x
	10	√	√	√	√	√	√	√
	20	√	√	√	√	√	√	√
	40	x	x	√	√	√	√	√
PH;NB	0	√	√	√	x	x	√	x
	10	√	√	√	√	√	x	√
	20	√	√	√	√	√	√	√
	40	√	x	√	√	√	√	√
PH;0.1% FA	0	√	x	x	x	x	x	x
	10	x	x	x	x	x	x	x
	20	√	x	x	√	x	x	x
	40	x	x	√	√	x	x	x
PH;10 mM AF	0	√	√	√	√	√	x	x
	10	√	√	√	√	√	√	√
	20	√	√	√	√	√	√	√
	40	√	x	√	√	√	√	√
PH;0.1% AA	0	√	√	x	x	x	x	√
	10	√	√	√	√	x	x	x
	20	√	√	√	√	x	√	x
	40	x	x	√	√	x	√	√
PC;NB	0	√	√	√	√	√	√	x
	10	√	√	√	√	√	√	√
	20	√	√	√	√	√	√	√
	40	√	x	√	√	x	√	√
PC;0.1% FA	0	√	√	√	x	x	x	x
	10	√	√	√	√	√	x	√
	20	√	√	√	√	√	√	√
	40	x	x	√	√	√	√	√
PC;10 mM AF	0	x	x	x	x	x	x	x
	10	x	x	x	x	x	x	x
	20	x	x	x	x	x	x	x
	40	x	x	x	x	x	x	x
PC;0.1% AA	0	√	√	√	√	x	x	√
	10	√	√	√	√	√	√	√
	20	√	√	√	√	√	√	√
	40	x	x	√	√	√	√	√
TH;NB	0	√	√	√	√	x	x	x
	10	√	√	√	√	x	x	x
	20	√	√	√	√	√	√	√
	40	x	x	√	√	√	√	√
TH;0.1% FA	0	√	√	√	x	x	√	x
	10	√	√	√	√	x	x	x
	20	√	√	√	√	√	√	√
	40	x	x	√	√	√	√	√
TH;10 mM AF	0	√	√	√	x	x	x	x
	10	√	√	√	√	x	√	√
	20	√	√	√	√	√	x	x
	40	x	x	√	√	√	√	√
TH;0.1% AA	0	√	√	√	x	x	x	x
	10	√	√	√	√	x	√	√
	20	√	√	√	√	√	√	√
	40	√	x	√	√	√	√	√

Table 2: Summary of product ions of 2.5 mg/mL lentinan generated at different CID levels for various combinations of column and buffer

Column & Buffer Combination	MS/MS spectra generation at different concentration (mg/mL) of lentinan					
	0.078	0.156	0.312	0.625	1.25	2.5
AH;NB	x	√	√	√	√	√
AH;0.1% FA	x	x	x	√	√	√
AH;10 mM AF	x	x	x	x	x	x
AH;0.1% AA	x	x	x	√	√	√
PH;NB	x	x	x	√	√	√
PH;0.1% FA	x	x	x	x	x	x
PH;10 mM AF	x	x	x	√	√	√
PH;0.1% AA	x	x	x	x	x	x
PC;NB	x	x	√	√	√	√
PC;0.1% FA	x	x	√	√	√	√
PC;10 mM AF	x	x	x	x	x	x
PC;0.1% AA	x	x	√	√	√	√
TH;NB	x	x	x	x	x	√
TH;0.1% FA	x	x	√	√	√	√
TH;10 mM AF	x	x	x	x	x	x
TH;0.1% AA	x	√	√	√	√	√

Table 3: MS/MS response (spectra generation of six lentinan product ions) of different lentinan concentrations at 20.0V CID

ammonium formate was not suitable as there was no MS/MS spectra generated at any lentinan concentration for any columns used except for Poroshell 120 HILIC column. However the MS/MS spectra obtained from this column were at the highest three concentrations only.

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