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Mass Fragmentation Patterns as Fingerprints for Positive Identification of Polyphenolic Compounds in a Crude Extract

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Abstract : Sixteen compounds of *Neobalanocarpus heimii* were successfully identified directly from their plant extract using a triple quadrupole LC-MS/MS system. In order to fulfil the objectives of this work, a series of stilbene oligomers of various degrees of condensation were isolated and their structure are characterized. Out of these, four are resveratrol dimers, three trimers, and nine tetramers. The isolation process was done on a fully automated semi-preparative HPLC system. Their structures were elucidated on the basis of 1D- and 2D-NMR as well as MS data. The mass fragmentation patterns of the compounds were recorded and a retrievable in-house library was built to keep the data. In order to demonstrate the potential of this approach, the polyphenolic crude extract was analysed with the LC-MS/MS system and the MS/MS spectra extracted for each chromatographic peak of interest. The fragmentation patterns were compared with those of anticipated pure compounds that were previously recorded. All compounds were successfully identified. It is therefore believed that the LC-MS/MS potential for dereplication of structurally similar compounds in a crude mixture was thus firmly established.

Key words : LC-MS/MS, ESI, triple-quadrupole, *Neobalanocarpus heimii,* oligostilbenes

Introduction

The conventional methods for analysis of plant sample usually involve preliminary sample treatments such as solvent extraction and appropriate derivatization followed by chromatographic separation.[1] The solvent extraction process, however, is tedious and often leads to insufficient recovery of the extractives with large molecular sizes from plant.[2] Traditionally, phytochemistry studies rely on the fractionation, isolation and purification as well as structural elucidation of compounds from crude extracts.[3-5] In this strategy several spectroscopic methods are necessary to provide information about the compound structures. This process is often time consuming, expensive and it may end up with disappointing outputs when losing the marker compound(s) throughout the long isolation process. For this reason, there is great pressure on phytochemists to develop a procedure to identify at least known compounds from the wood extractives quickly and efficiently at the level of the crude extract. This information is important to avoid replication in isolation and purification of known compounds. Thus, quick and easy methods for dereplication of plant extracts are essential.

Dereplication reduces the amount of work and sample consumption when compounds are identified at a very early stage without the need of large scale isolation project. Therefore, efforts are oriented toward the discovery of novel and/or pharmacological significant compounds as well as identification of marker compound(s) in authenticating plant species. Some examples of the practice are the analysis of the secondary metabolites constituents of the genus *Hypericum*,[6] identification of quinolinone alkaloids of *Haplophyllum acutifolium*[7] and investigation of bioactive phytochemicals in the leaves of *Melicope vitiflora*.[8] In addition, some studies adopt this strategy to determine adulteration in herbal preparation,[9] characterize herbal formulas[10] as well as detects natural and synthetic steroid in environmental waste.[11] By implementing this approach we can be assured that resources are spent wisely and economically on fingerprinting plant species, hence increasing the potential for quick authentication of herbal samples.

In dereplication technique, a screening technology is

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designed to distinguish between known secondary metabolites and new molecules directly in crude plant extracts.[12] Typically, a dereplication strategy employs a combination of separation science, spectroscopic methods and database searching. Usually, the approaches make use of hyphenated techniques. The hyphenation may be as simple as HPLC with MS detection[13] to as extended as HPLC-APCI-MS-SFC,[14] capillary electrophoresis with ICP-MS[15] and HPTLC-UV/Vis/FLD-ESI/MS.[16]

We report herein the potential of an HPLC coupled with triple quadrupole MS system with electrospray ionization (ESI) interface in dereplicating oligostilbene in plant sample. In addition, we prove the concept by reanalyzed the chromatographic profile of the isolated compound and compared them with those in crude extract.

Experimental

General experimental procedures

Mass spectra were aquired on an Agilent 6410 triple quadrupole LC-MS/MS system. The system is equipped with electrospray ionization (ESI) interface. The system is controlled by MassHunter^{TM} software. The collision-induced dissociation (CID) were executed by nitrogen gas at 30 V collision energy. The nebulizer pressure was set at 45 psi and fragmenter voltage at 100 V for all experiment.

NMR data were obtained from a Bruker Avance III 500 MHz. Chemical shift are reported on δ scale and the coupling constant given in Hz.

HPLC grade MeOH and acetonitrile were purchased from Merck. Distilled and deionized water were obtained from the central instrument center (Analytical Unit, Faculty of Pharmacy, UiTM) and used throughout the study. Trifluoroacetic acid (TFA) was obtained from Sigma-Aldrich. Others solvents and reagents were of analytical grade. The purities of compounds were determined to be greater than 90% by normalization of the peak areas detected by HPLC analyses.

The plant sample was collected and positively identified by certified botanist.

HPLC apparatus and chromatographic conditions

The chromatographic system for quantitative analysis consisted of a 306 pump, 811C dynamic mixer, UV/VIS-156 detector, 231 XL sample injector, and UniPoint data processor (all from Gilson, USA). The chromatographic separation of analyses for crude extract was carried out on Genesis-C18 (Jones Chromatography; 4 μm, 10 × 250 mm), whereas purification process was on an Agilent Eclipse XDB-C18 (Agilent; 5 μm, 4.5 × 150 mm). Data was collected and analyzed using Gilson Unipoint software. The mobile phase consisting of 0.1% TFA in water (A) and acetonitrile (B) was run with gradient elution at a flow rate of 5.0 mL/min (semi-preparative) and 1.0 mL/min (analytical scale). The injection volume was 50 and 10 μL, respectively. UV absorption was monitored at 215 and 283 nm. The column temperature was maintained at 30^{o}C.

Solvents and chemicals

For HPLC experiments, HPLC-grade acetonitrile and trifluoroacetic acid (TFA) from Fischer were used together with HPLC grade water, purified at 15 MΩ using PURELAB® Option water purification system from Elga, and filtered through Agilent cellulose membrane. NMR experiments were carried out in deuterated methanol, CD_3OD (99.8%), and acetone, $(CD_3)_2CO$ (99.5%), both without TMS from Merck and Fischer.

Preparation of reference compounds

The pure compounds were obtained by isolation and purification of *N. heimii* wood extract. The collected fractions were repetitively re-fractionated until pure compounds were obtained. The chromatographic conditions and solvent systems were individually determined for each fraction prior to the process. The isolated compounds were characterized by spectroscopic methods including nuclear magnetic resonance (NMR) and mass spectrometry (MS).[17]

Setting-up compounds database

The identified compounds were then analyzed by mass spectrometry to determine their mass value as well as the fragmentation of their structures.

The compounds were dissolved in methanol and automatically injected into the MS system and the Total Ion Current (TIC) was recorded. An MS scan experiment provides information about the m/z value of the protonated compound. In order to obtain fragmentation information on the compounds, an MS/MS Product Ion experiment was carried out. Likewise, the TIC of product ions can be recorded and it contains information on the compound fragmentation. The fragmentation behavior of these compounds was observed in collision-induced dissociation (CID) product ion spectra of the compounds under electrospray-ionization (ESI) conditions.

The product ions spectra of each compound were kept for reference.

Preparation of plant crude extract

The plant crude extract was obtained by succesive extraction of the plant material using hexane and acetone-water (3:1) at room temperature. Hexane was used to remove fatty materials from the sample. A classical method of repetitive maceration and lixiviation was used in the extraction process until the presence of phenolics compounds was tested negative. The extract was dried under vacuum and kept in -18^{o}C until analysis.

Mass spectrometric analysis of crude extract

In performing the LC-MS/MS analyses on the crude

extract, the precursor ions chosen were m/z 471, representing dimeric stilbenes, m/z 681 for trimeric and m/z 907 for tetrameric stilbenes. The fragmentation patterns extracted from the experiments would be compared with those of the pure compounds, which were previously isolated.

Results and discussion

The database and compound identification

Seventeen compounds were succesfully isolated from *N. heimii* wood extract: heimiols A and B, balanocarpol, ampelopsin A, coppaliferol A, vaticanol A, ampelopsin C, vaticaphenol A, heimiol D, hemsleyanol D, ampelopsin H, heimiol E, hopeaphenol, isohopeaphenol, hopeaphenol A and isohopeaphenol A. All the structures are shown in Figure 1.

Heimiol A, heimiol B, balanocarpol and ampelopsin A are dimeric stilbenes with m/z value of 471; coppaliferol A, vaticanol A and ampelopsin C are trimer (m/z = 681) and Hopeaphenol, isohopeaphenol, hopeaphenol A, isohopeaphenol A, ampelopsin H and heimiol E are tetramers (m/z = 907). The MS/MS data of all compounds were kept for reference.

The identification of the compounds in the crude extract were made based on their similarities in fragmentation patterns with those of isolated compounds. The analyses were done separately based on their oligomeric degrees as (Figures 2b-2d).

MS2 scan and product ion analyses

Twenty peaks are observed in the TIC (Figure 2a). The mass spectra of each peak were extracted. The mass spectra of peaks 1, 2, 4 and 5 exhibited intense signal at m/z 471, corresponding to protonated ions $[M+H]^+$ of dimer stilbenes. Peaks 3 and 14 were produced by ions at m/z 681, correspoding to protonated ion $[M+H]^+$ of trimeric stilbenes. Peaks 7, 8, 9, 10, 11, 12, 15, 16, 17, 18, 19 and 20 were observed to obtain ions at m/z 907, corresponding to protonated ion $[M+H]^+$ of stilbene tetramers. Peaks 6, 7 and 13 displayed ion signals at both m/z 681 and 907, corresponding to co-eluting trimeric and tetrameric stilbenes. Peak 12 and 13 are overlapping peaks for compounds with close retention times.

After considering the co-elutions and overlapping peaks, 22 compounds were expected to be isolated. However, only 16 compounds were successfully isolated. LC/MS analysis is a powerful tool to determine the number of compounds present in an extract. The LC-MS could facilitate the isolation process by targeted chromatographic operation.

The next step was to perform LC-MS/MS analyses on the crude extract. The precursor ions chosen were m/z 471, representing dimeric stilbenes, m/z 681 for trimeric and m/z 907 for tetrameric stilbenes. The fragmentation patterns extracted from the experiments would be compared with those of the pure compounds, those previously isolated.

LC-MS/MS analyses on the crude extract for the precursor ions at m/z 471 resulting chromatogram as shown in Figure

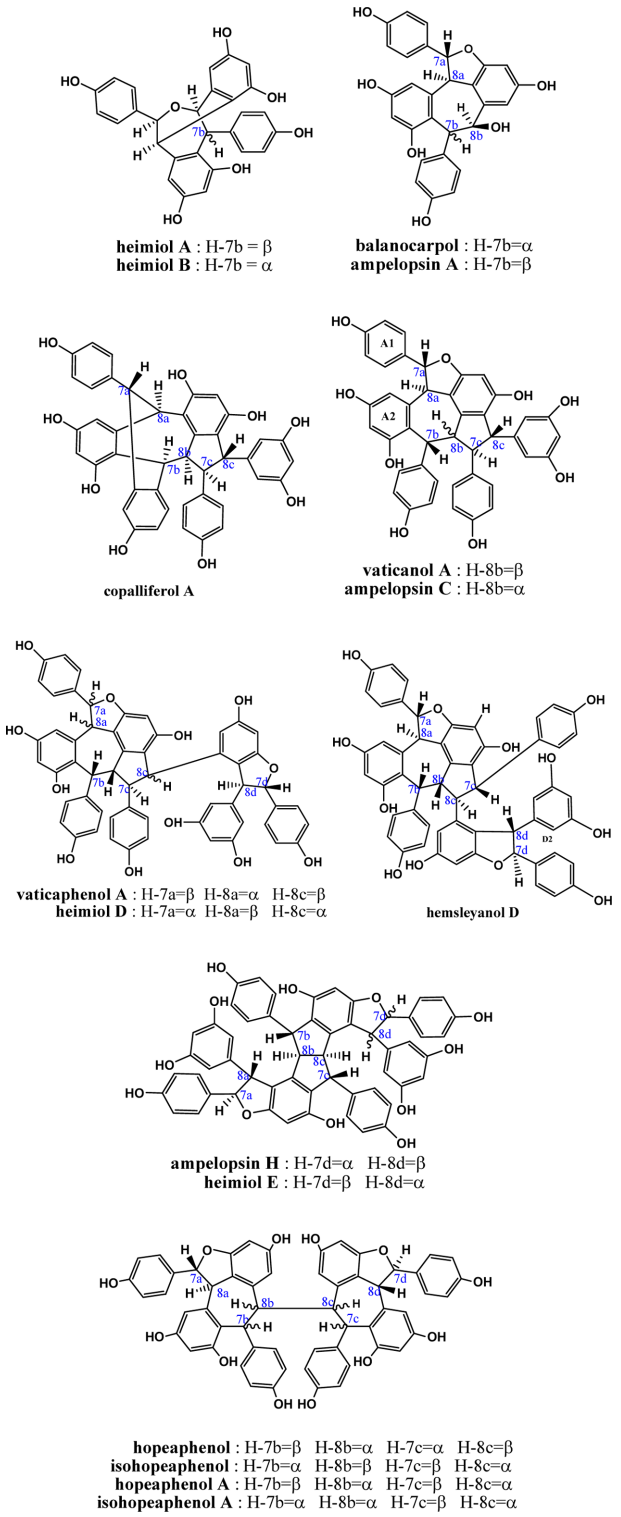

Figure 1. Oligostilbenes from *Neobalanocarpus heimii*.

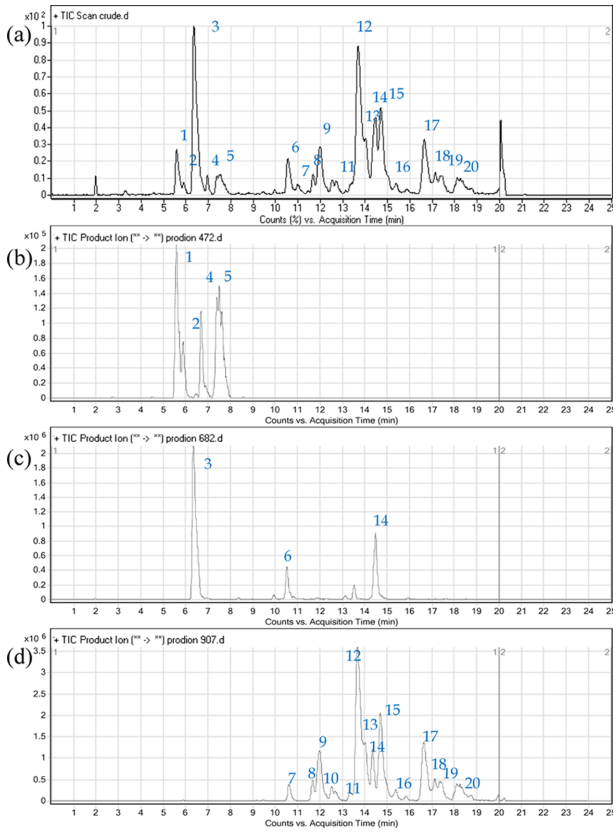

Figure 2. Total Ion Current (TIC) for (a) MS2 scan of the crude extract; (b) Product Ion for precursor ion $[M+H]^+$ at *m/z* 471; (c) Product Ion for precursor ion $[M+H]^+$ at *m/z* 681 and (d) Product Ion for precursor ion $[M+H]^+$ at *m/z* 907.

2b and displays four peaks consist of three major peaks and one small peak (associated with the first peak).

The chromatogram of product ion analysis for molecular ions $[M+H]^+$ at m/z 681 showed four peaks at RT 6.4, 10.5, 13.5 and 14.5 min. The signal at 13.5 appear as a quite small peak. It corresponds to a stilbene trimer that was not isolated and identified. The chromatogram is shown in Figure 2c.

LC-MS/MS analyses were performed on the crude extract for the precursor ions of m/z 907 and the chromatogram is shown in Figure 2d. The chromatogram showed that all the protonated compounds with m/z 907 were eluted after 10 minutes of chromatographic run-time. Fourteen peaks were observed with some of them highly overlapping. In this study, nine compounds with an MS of 906 were isolated and all the structures were successfully elucidated.

Fragmentation patterns analyses for compound identification

When the LC-MS/MS analyses were performed on the crude extract for the precursor ions at m/z 471, the mass spectra containing the daughter ions were extracted from each of these four peaks and are shown in Figure 3. In the same figure, MS/MS spectra previously obtained from pure heimiol A, ampelopsin A, heimiol B and balanocarpol are displayed for comparison purpose. From the experience gained in the isolation work, these compounds were expected to elute at early retention time.

The similarity in fragmentation pattern between chromatographic peaks and anticipated pure compound is very high. Therefore, peak 1 is likely to correspond to heimiol A, peak 2 to ampelopsin A, peak 4 to heimiol B and peak 5 to balanocarpol. However, before any final positive identication could be made, it was necessary to check on the retention times of these pure compounds in the exact same conditions as those used for the analysis of the crude extract. Observed retention times are compiled in Table 1.

LC-MS/MS analyses were also performed on the crude extract for the precursor ions of m/z 681. The mass spectra were extracted from each peak and their fragmentation patterns were analyzed. The patterns were compared with those of isolated trimeric stilbenes, copalliferol A, vaticanol A and ampelopsin C, as shown also in Figure 3. From the experience gained in the isolation work, these compounds were expected to elute later than the dimeric stilbenes but earlier than the tetramers.

The similarity in fragmentation pattern between chromatographic peaks and anticipated pure compound suggesting peak 3 is likely to correspond to copalliferol A, peak 6 to vaticanol A and peak 14 to ampelopsin C. The chromatograms of the above mentioned compounds were compared with that of crude extract to determine the similarity of their retention time. The retention times of each peak correspond to those in the chromatogram of the crude extract (Table 1), hence confirm the identification of the peaks.

When the fragment ions from the MS/MS analysis of m/z 907 were extracted from each peak, the fragmentation patterns were compared with those of isolated tetrameric stilbenes (Figure 3 cont.). Only the fragment ions extracted from well-resolved peaks showed similarity in the fragmentation patterns with those of anticipated pure compounds. The high similarity in fragmentation pattern of MS/MS spectra suggested that peak 9 is likely to correspond to hopeaphenol A, peak 12 to hopeaphenol, peak 14 to heimiol D, peak 15 to isohopeaphenol, peak 17 to vaticaphenol A, peak 18 to ampelopsin H and peak 19 to heimiol E.

As a result, seven out of nine isolated tetrameric stilbene compounds were considered because they gave well separated chromatographic peaks in the set conditions.

Peak 14 is correspond to two compounds, ampelopsin C and heimiol D. From the MS scan of the crude extract, the compounds are completely co-eluted, beyond a slight indication of containing more than a single compound. The MS/MS experiment, however succesfully distinguished them due to the dirrefence in their m/z value (681 and 907, respectively).

Two other tetrameric stilbenes, isohopeaphenol A and hemsleyanol D which were also isolated and characterized were not identified using this method. The comparison of their retention times, however is able to confirm the

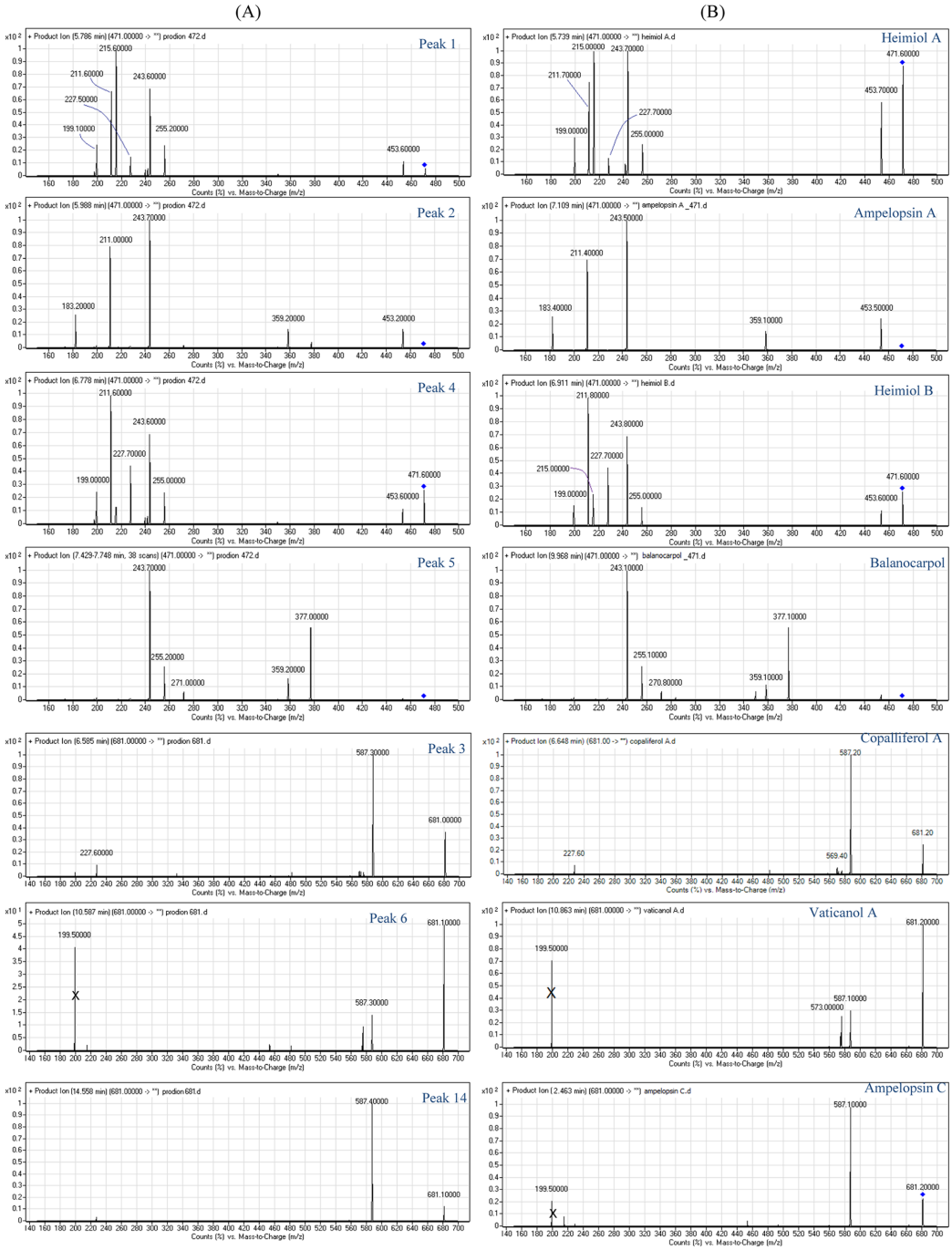

Figure 3. Spectra of product ions analyses for precursor ion $[M+H]^+$ at *m/z* 471 and 681; (A) from the crude extract and (B) from the pure compounds.

identity of each compound.

The objective of the study was to look at the power of the mass spectrometry in positively identifying highly similar compounds from a crude extract, If identification of all compounds in unknown extract was to be completed using mass spectrometry, optimization of similar chromatographic conditions would not be required anymore.

Results validation and verification

It was necessary to check on the retention times of the pure compounds in the exact same conditions as those used for the analysis of the crude extract, in order to make positive identification. For this validation, all pure compounds were analyzed in order to compare their retention times to those in the crude mixture. The HPLC

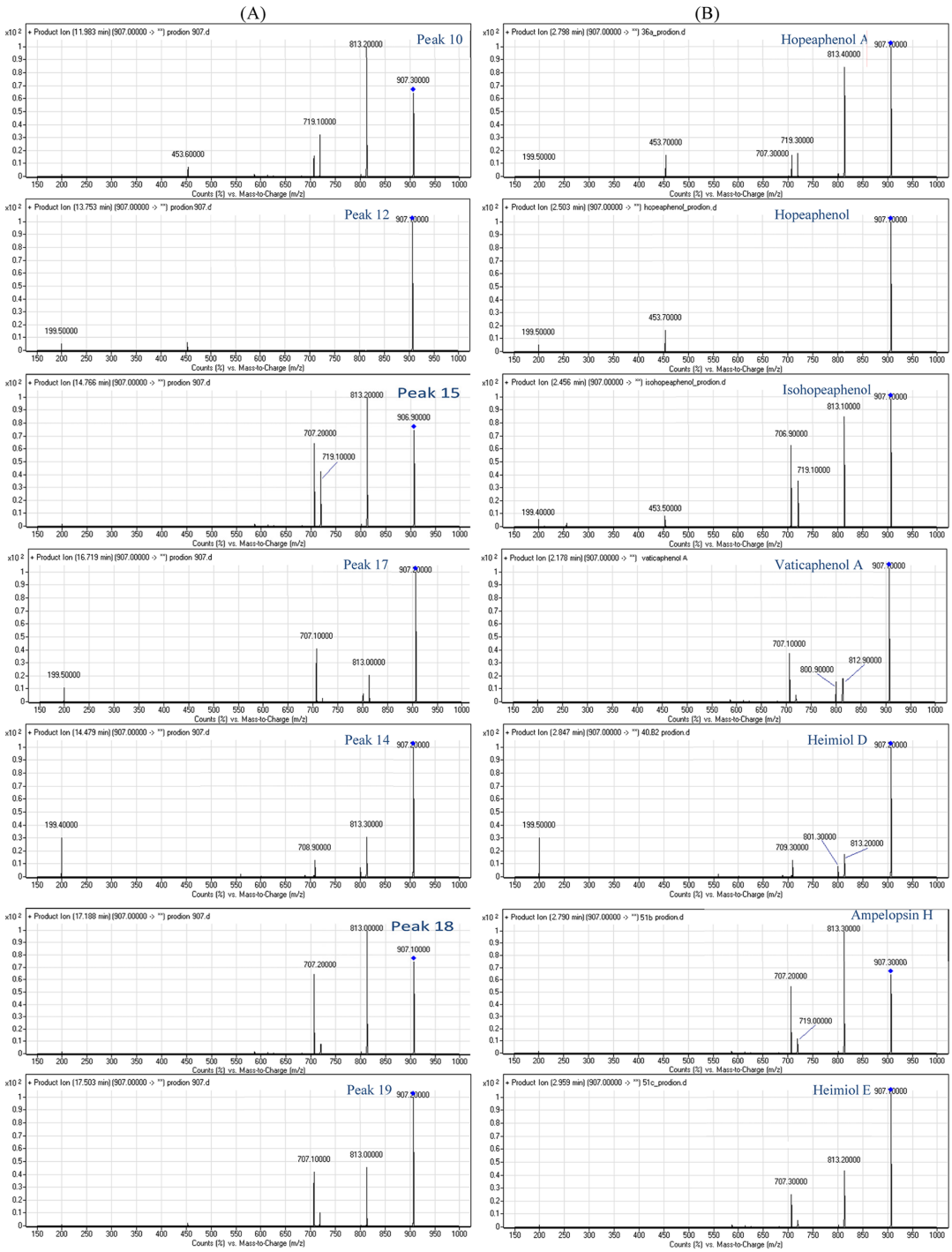

Figure 3 (cont.): Spectra of product ions analyses for precursor ion $[M+H]^+$ at *m/z* 907; (A) from the crude extract and (B) from the pure compounds.

analyses on the same chromatographic conditions verified the identification of compounds in their crude extract. The retention times of all compounds and their corresponding peaks in the chromatogram of crude extract are shown in Table 1. All chromatographic peaks are positively identified.

Conclusions

The LC-MS/MS experiment was successful in identifying the oligostilbenes directly from a crude extract. The system was recognized as being able to successfully identify a known compound solely from its fragmentation

Table 1. Retention times for the elution of crude extract and the pure compounds.

Peaks in TIC (crude extract)	Retention time R_t (min)	Pure compound	Retention time R_t (min)
Peak 1	5.65	Heimiol A	5.68
Peak 2	5.97	Ampelopsin A	6.00
Peak 3	6.35	Copalliferol A	6.41
Peak 4	6.98	Heimiol B	6.93
Peak 5	7.44	Balanocarpol	7.56
Peak 6	10.52	Vaticanol A	10.85
Peak 9	11.98	Hopeaphenol A	12.00
Peak 10	12.50	Isohopeaphenol A	12.48
Peak 12	13.73	Hopeaphenol	13.94
Peak 13	14.00	Hemsleyanol D	14.00
Peak 14	14.52	Heimiol D	14.49
Peak 14	14.52	Ampelopsin C	14.55
Peak 15	14.80	Isohopeaphenol	14.87
Peak 17	16.79	Vaticaphenol A	16.82
Peak 18	17.19	Ampelopsin H	17.21
Peak 19	17.50	Heimiol E	17.70

pattern, regardless of the retention time, or other data. This allows to eliminate the dependence on the chromatographic conditions and selection of column. The system worked very well for oligostilbene compounds. Considering that these compounds have uniform functional groups and limited structural features, we believe that it would work even better with other types of compounds with greater structural variation.

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References

1. Holmbom, B.; Sjostrom, E.; Alèn, R. *Analytical Methods in Wood Chemistry, Pulping and Papermaking* **1999**, 125.
2. Ishida, Y.; Goto, K.; Yokoi, H.; Tsuge, S.; Ohtani, H.; Sonoda, T.; Ona. T. *J. Anal. Appl. Pyrolysis* **2007**, 78, 200.
3. Yan, J.-K.; Li, L.; Wang, Z.-M.; Wu. J.-Y. *Carbohyd. Polym.* **2010**, 79, 125.
4. Hsouna, A. B.; Trigui, M.; Culioli, G.; Blache, Y.; Jaoua. S. *Food Chem.* **2011**, 125, 193.
5. Cheng, J.-J.; Lu, M.-K.; Lin, C.-Y.; Chang, C.-C. *Carbohyd. Polym.* **2011**, 83, 545.
6. Alali F. Q.; Tawaha, K. *Saudi Pharm. J.* **2009**, 17, 269.
7. Staerk, D.; Kesting, J. R.; Sairafianpour, M.; Witt, M.; Asili, J.; Emami, S. A.; Jaroszewski, J. W. *Phytochemistry* **2009**, 70, 1055.
8. O'Donnella, F.; Ramachandrana, V. N.; Smytha, T. J. P.; Smytha W. F. *Anal. Chim. Acta* **2009**, 634, 115.
9. Kesting, J. R.; Huang, J. Q.; Sørensen, D. *J. Pharmaceut. Biomed.* **2010**, 51, 705.
10. Liu, L.; Li, Y.-F.; Cheng, Y.-Y. *J. Chromatogr. B* **2008**, 862, 196.
11. Liu, S.; Ying, G.-G.; Zhao, J.-L.; Chen, F.; Yang, B.; Zhou, J.-L.; Lai, H.-J. *J. Chromatogr. A* **2011**, 1218, 1367.
12. Wolfender, J. L.; Ndjoko, K.; Hostettmann, K. *J. Chromatogr. A* **2003**, 1000, 437.
13. Jerz, G.; Wybraniec, S.; Gebers, N.; Winterhalter, P. *J. Chromatogr. A* **2010**, 1217, 4544.
14. Lavison-Bompard, G.; Thiébaut, D.; Beziau, J. F.; Carrazé, B.; Valette, P.; Duteurtre, X.; Tabet, J. C. *J. Chromatogr. A* **2009**, 1216, 837.
15. Petit, J.; Geertsen, V.; Beaucaire, C.; Stambouli, M. *J. Chromatogr. A* **2009**, 1216, 4113.
16. Morlock, G.; Schwack, W. *J. Chromatogr. A* **2010**, 1217, 6600.
17. Bayach, I.; Manshoor, N.; Sancho-Garcia, J. C.; Choudhary, M. I.; Trouillas, P.; Weber, J. F. F. *Chem. Asian. J.* **2015**, 10, 198.