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# Distinguishing Isomeric Anthraquinone by LC-MS

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**Abstract:** Six anthraquinone derivatives with similar substitutions were introduced into an ion trap LC- MS system to obtain their mass fragmentation data. Every sample contains two hydroxyl groups that differ in *ortho*, *meta* and *para* position. The samples are divided into two isomeric groups, with and without methyl group respectively. Their fragmentation patterns are analysed in order to assess the capability of LC-MS in distinguishing the structural isomers of anthraquinone. Each compound produced different ionization patterns, despite their similarities in molecular formula and molecular weight. The anticipated compound fragments are proposed based on the m/z value of product ions. This study concludes that an Ion Trap LC-MS is able to distinguish those closely related isomeric compounds.

**Key words:** Tandem MS · Fragmentation · Dihydroanthraquinone · Dihydromethylanthraquinone

## INTRODUCTION

Anthraquinone, chemically known as anthracene-9,10-dione (Fig. 1) is rather small group of compounds. They distributed in small group of plant family, such as Leguminosae [1], Rubiaceae [2-4], Polygonaceae [5], Guttiferaceae [6], Liliaceae [7,8] and a few other families. Detection of anthraquinones can be done directly from plant crude extract using ammonia to give rose-pink color as positive indication [9].

The compounds are believe to be the major active component in rhubarb and aloe vera. The medicinal values of the compounds are due to their purgative activity, that makes them good laxative [10]. Plants with high content of anthraquinone are used in many traditional preparation medicine, from traditional Chinese medicines (TCM) [10,11] to Ayurvedic preparation [12]. In North Western Himalaya, *Rumex nepalensis* is a high value medicinal herb due to its high anthraquinone content [13].

Even though they are widely marketed as laxative, many other bioactivity studies have been conducted on anthraquinone-rich plants and they show good activity especially on antioxidant and antimicrobial.

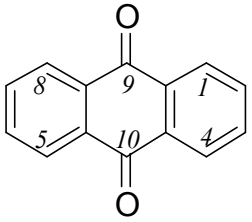


Fig. 1: Anthraquinone structure

A study on extracts from different parts of *Cyperus rotundus* showed reducing power of 0.711-0.851 at the concentration of 2.5-10 mg/ml [14]. In two separate studies, Zovko *et al*. demonstrated activities of *Rhamnus alaternus, R. fallax, R. intermedia* and *R. pumila* extracts against *S. aureus, P. aeruginosa, E. coli, C. albican, A. niger and M. gypseum* with MIC values below 2.5 mg/ml [15]. *Frangula rupestris* and *F. alnus* bark extracts demonstrated similar MIC values [16]. Isolated anthraquinones showed antiplasmodial activities against *P. falcifarum* with $IC_{50}$ values of 1-4µg/ml [17,18]. Other potential usage of anthraqunones includes as mosquitocidal [19], photosensitizer [20] and food preservation to retard discoloration, rancidity or

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deterioration due to antioxidant activity [21]. Anthraquinone is also used in the production of paper from kenaf [22].

The usage of chromatographic and spectroscopic techniques are well known in identification of chemical constituents in plant materials and plant products. Determination of drug content in pharmaceutical preparation can be easily done by a simple spectrophotometer [23,24]. For volatile component, gas chromatography mass spectrometry (GCMS) would do an excellent job [25]. In high performance liquid chromatography (HPLC), biologically active substance can be detected from plant leaves [26].

As small compounds, anthraquinones has quite limited structural variation, which means they form many isomers especially stereoisomers. In spectroscopic methods, stereoisomers that are similar in structures and sharing the same molecular formula are difficult to be identified and distinguished from one another. The aim of this study is to develop and validate spectrometric method to distinguish very similar structures of anthraquinones. An LC-MS system with ion trap is used due to its sensitivity and capability to produce tandem mass spectra. The mass of all isomeric anthraquinones would be the same, thus the need of tandem mass spectra. Despite closely similar structural skeleton, their fragmentation pattern in $MS^2$ and $MS^3$ would be different and this will provide finger prints for every compound [27].

## MATERIALS AND METHODS

**Instrumentation:** An Agilent LC/MS system, which is an HPLC with mass detector, was used to record mass data. The chromatographic component system consists of a quaternary pump equipped with a vacuum degasser, system controller, auto sampler, column thermostats and a multi-wavelength UV detector. The mass spectrometric components include electrospray ionization (ESI) as ion source and ion trap as mass analyzer.

**Chemicals:** Acetonitrile ($CH_3CN$) and ultra-purified water ($H_2O$) were used as mobile phase. Acetonitrile is of HPLC grade supplied by RCI Labscan Limited. Water was purified using ELGA water purification system (18.2MΩ.cm, ±31 °C).

**Sample Materials:** Six anthraquinone isomers were selected for this study. The compounds are differing by the positions of hydroxyl group at C-4, which are *ortho*, *meta* and *para* to hydroxyl group at C-1. Three compounds contain a methyl group at C-6, which divided the anthraquinones into two groups, dihydroxyanthraquinone and dihydroxymethylanthraquinone. The compounds of each groups share the same molecular formula, thus m/z value of 240 and 255 respectively. Fig. 2 shows the list of samples with their chemical structures.

**Sample Preparation:** Each anthraquinone sample was dissolved in acetonitrile and sonicated to complete dissolution. The samples were filtered individually through 0.45µm PTFE (polytetrafluoroethylene) membrane before transferred into their respective vials to be injected into an LC-MS system.

**HPLC Condition:** Mobile phase is an isocratic elution of water: acetonitrile (30:70) with flow rate of 0.7 ml/min. UV detection was carried out at 215nm, 220nm and 283nm.

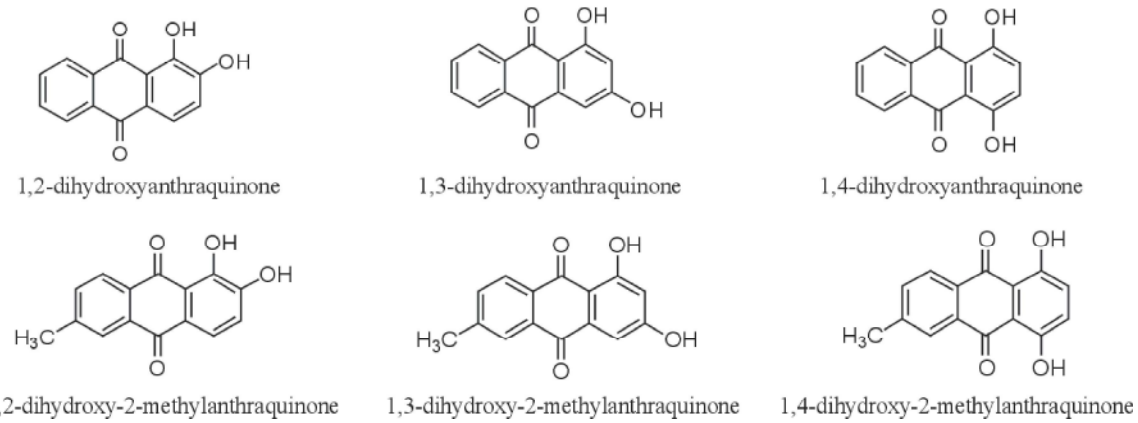



Fig. 2: Isomers of dihydroxynthraquinones and methyldihydroxyanthraquinones.

The Synergi™ C-18 column (150 x 4.6 mm, Phenomenex) was used for the HPLC method development, with guard column. The temperature of column compartment was at 36°C. For all injections, the volume of samples was 5.0µL.

**MS Condition:** Samples were scanned at a range of 100 to 400 m/z. The fragmentation step was set automatically until $MS^5$, with positive and negative ionization modes. Prior to the experiment, the system had been calibrated and tuned similar to the working condition.

**LC-MS Experiment:** Two types of experiment were carried out in order to achieve objectives of this study. The first experiment is to select the preference of ion polarity. The selection was based on the abundance of ions determined by the intensity of the peaks. In the second experiment, the preferred ion polarity was used for fragmentation study. Tandem MS experiment was carried out for five fragmentation steps. The selection of ion to be further fragmented was automatically performed by the system.

## RESULTS AND DISCUSSION

**Fragmentation Patterns Of Dihydroxyanthraquinone Isomers:** Fragmentation data were recorded similar to the technique used for oligostilbenes [27]. Three anthraquinones with different substitution positions (*ortho*, *meta* and *para*) were subjected to tandem LC-MS experiment to determine the capability of LC-MS system in differentiating them. For each compound, five steps of fragmentations were performed, the numbers of fragmentation step are, however, according to the availability of their ions.

For positive ionization of 1,2-dihydroxyanthraquinone, the parent ion (241 m/z) was fragmented into m/z 195 and 213 at $MS^2$, then further fragmented into m/z 133 in $MS^3$. Figs. 3 and 4 show the mass fragmentation spectra for 1,2-dihydroxyanthraquinone and the illustration of its anticipated ion fragments.

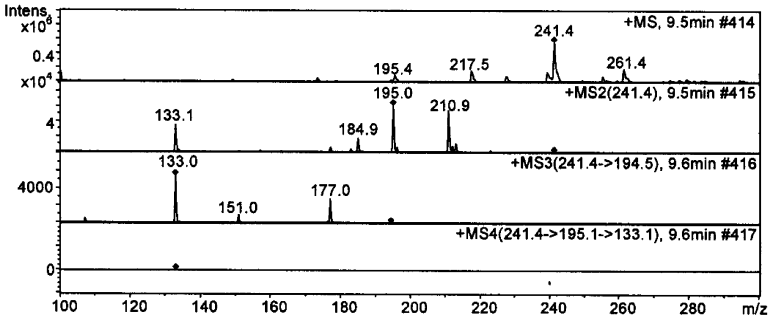



Fig. 3: MS, $MS^2$ and $MS^3$ spectra for 1,2-dihydroxyanthraquinone.

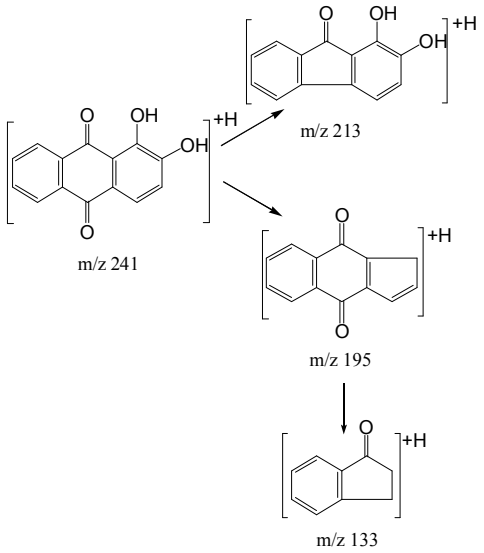



Fig. 4: Anticipated ion fragmentations of 1,2-dihydroxyanthraquinone.

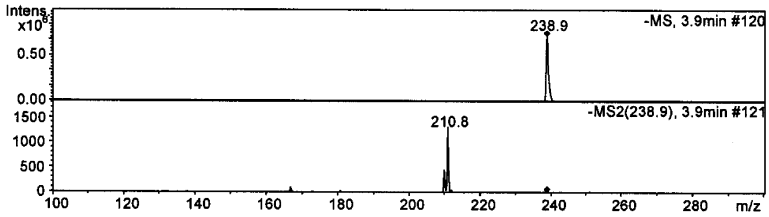
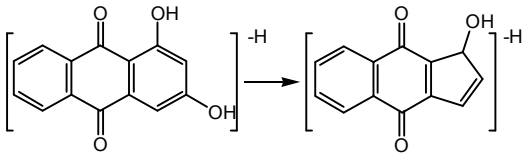



Fig. 5: MS and $MS^2$ spectra for 1,3-dihydroxyanthraquinone.




m/z 329 m/z 211

Fig. 6: Anticipated ion fragmentations of 1,3-dihydroxyanthraquinone.

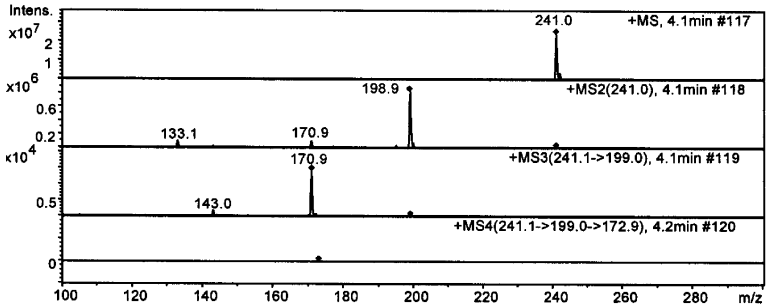



Fig. 7: MS, $MS^2$ and $MS^3$ spectra for 1,4-dihydroxyanthraquinone

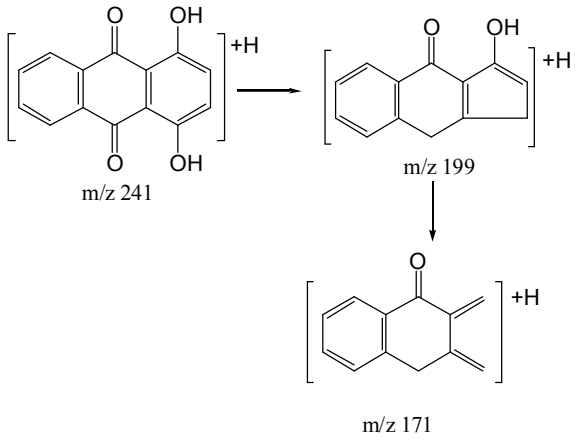



Fig. 8: Anticipated ion fragmentations of 1,4-dihydroxyanthraquinone.

The fragmentation of 1,3-dihydroxyanthraquinone is in negative preference, where the parent ion of 239 m/z was fragmented into m/z 211. For this compound, the highest fragmentation step can be obtained is only up to $MS^2$. This may due to limited number of available ion in negative ionization mode. Figs. 5 and 6 show the mass fragmentation spectra for 1,3-dihydroxyanthraquinone followed by the illustration of its anticipated ion fragments.

The ionization of 1,4-dihydroxyanthraquinone is preferred in positive ion m/z 241. The parent ion was further fragmented into 199 at $MS^2$ and 171 in $MS^3$. Figs. 7 and 8 show the mass fragmentation spectra for 1,4-dihydroxyanthraquinone and its anticipated ion fragments.

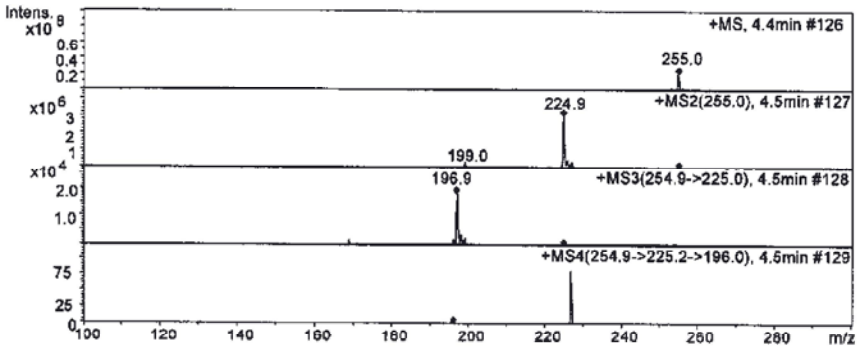



Fig. 9: MS, $MS^2$, $MS^3$ and $MS^4$ spectra for 1,2-dihydroxy-6-methylanthraquinone.

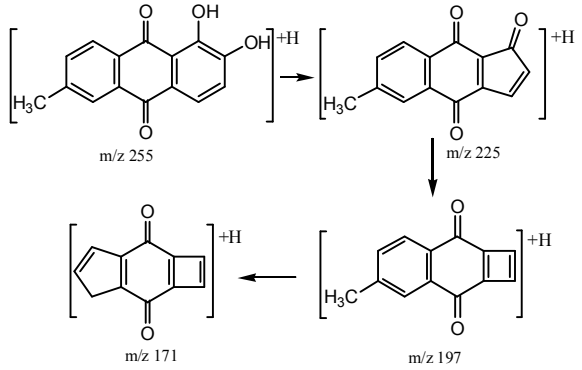



Fig. 10: Anticipated ion fragmentations of 1,2-dihydroxy-6-methylanthraquinone.

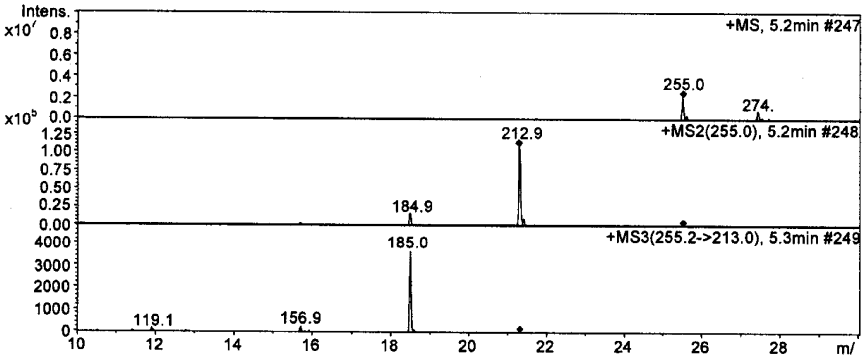



Fig. 11: MS, $MS^2$ and $MS^3$ spectra for 1,3-dihydroxy-6-methylanthraquinone.

**Fragmentation Patterns of Dihydroxymethylanthraquinone Isomers:** Three isomeric anthraquinones, with a methyl group at position carbon-6 and two hydroxyl groups at *ortho*, *meta* and *para* positions were subjected to tandem LC-MS analysis. Similar to the previous group, five fragmentation steps were performed and the numbers of fragmentation step are according to their availability of ions.

For 1,2-dihydroxy-6-methylanthraquinone, its tandem MS spectrum can be analyzed up to $MS^4$. From m/z 255 in MS, the compound fragmented into m/z 225 in $MS^2$, m/z 197 in $MS^3$ and m/z 171 in $MS^4$. Figs. 9 and 10 show the mass fragmentation spectra for 1,2-dihydroxy-6-methylanthraquinone followed by the illustration of its anticipated ion fragments.

Spectrum for 1,3-dihydroxy-6-methylanthraquinone shows the parent ion produced was m/z 255 and the ion was further fragmented into m/z 213, m/z 185 and finally into m/z 157. Figs. 11 and 12 show the mass fragmentation spectra for 1,3-dihydroxy-6-methylanthraquinone followed by the illustration of its anticipated ion fragments.

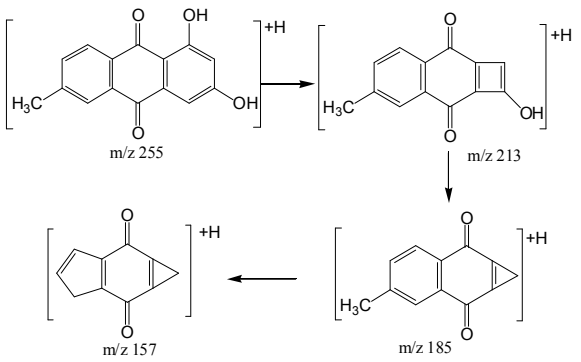



Fig. 12: Anticipated ion fragmentations of 1,3-dihydroxy-6-methylanthraquinone

For the last compound, 1,4-dihydroxy-6-methylanthraquinone, both of the $MS^n$ spectra in positive and negative mode of ionization was poor. This might be due to insufficient ion.

## CONCLUSION

All anthraquinones but one prefers positive ionization. The fragmentation patterns for all anthraquinone isomers are different despite they are structurally similar. Fragmentations in mass spectrometer occur as long as the ion enough to be fragmented. The experiment was successful for very closely related isomers. It is expected that it will be more successful for compounds with different skeleton and varies in functional groups.

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