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Short communication

Use of high resolution LC–MS^n analysis in conjunction with mechanism-based stress studies: Identification of asarinin, an impurity from sesame oil in an animal health product

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ABSTRACT

During analysis of certain stability batches of an animal health product, an unknown peak was found at a level above the identification thresholds set by VICH. This unknown species is extremely labile in the gas phase under normal electrospray ionization (ESI) mass spectrometric condition. Multiple ions were detected with no clear indication of which one is the molecular ion. To overcome this challenge, we utilized tandem MS/MS analysis and multiple MS instruments. The slightly different ionization processes between the two different instruments provided strong, complementary evidence leading to the identification of the correct molecular ion. Based on the formula thus determined, the unknown species was found to be related to sesame oil, which is one of the major excipients used in this drug product. The unknown species was eventually identified as asarinin using high resolution LC–MS^n in conjunction with mechanism-based stress studies, in which the unknown species was generated based on the degradation chemistry of sesamin as revealed by the LC–MS^n analysis. This overall approach in combining LC–MS^n analysis along with mechanism-based stress studies can be used as a general strategy for identification of unknown pharmaceutical impurities, especially the degradants related to the active pharmaceutical ingredient (API) and excipients.

1. Introduction

Pharmaceutical impurities, when exceeding certain thresholds, may negatively impact the efficacy and safety of the drug products. Therefore, the levels of these impurities need to be controlled and monitored starting from the manufacturing processes and throughout the shelf lives of the drug products. When the levels of impurities in a drug substance or a drug product exceed the limits set by certain regulatory requirements, such as the International Conference on Harmonization (ICH) or Veterinary International Conference on Harmonization (VICH) guidelines [1–3], identification of the impurities is required.

Among the various analytical techniques used for impurity identification, LC–MS has become the most effective and widely used technique [4]. LC–MS analysis can not only provide the molecular weight or chemical formula of an unknown impurity if accurate mass is available, but also reveal the structural information of a molecule via tandem MS/MS analysis. For the majority of the LC–MS analyses, electrospray ionization (ESI) has become a prominent ionization technique because of its ability to generate intact gas phase ions which facilitates the determination of the molecular ions of unknown impurities. Despite of the mildness in the ESI process, fragmentation does occur for some labile molecules. Although fragmentation is useful to obtain the structural information, it can also hinder the determination of the molecular ion of an unknown impurity when multiple fragments at various levels are present in the mass spectrum. In such cases, fragmentation pathways from different ions, among which the molecular ion is likely present, need to be determined by tandem MS/MS analysis and then compared. Based on a comparative analysis of individual ions' fragmentation pathway, the molecular ion may be identified. Another strategy is to use a different mass spectrometer of different configuration by a different manufacturer; complementary information obtained between the two different instruments should facilitate the determination of the molecular ion. It has been reported that the mass spectrum of an analyte can be different when analyzed by a different instrument [5]. This may be due to a slightly different ionization process caused by the different configuration of the ion source.

Frequently, analytical techniques complementary to LC–MS such as NMR spectroscopy are required for unambiguous structure identification of an unknown impurity. Nevertheless, structure characterization by NMR can be quite challenging in such cases, even with the advent of LC-NMR, since the levels of these unknown species present in the drug substances or drug products are usually

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quite low and the quantities of the samples are usually limited. In our laboratory, we have utilized a strategy that combines LC–MS^n analysis with mechanism-based stress studies (forced degradation) in which adequate amount of the desired impurity may be produced. This strategy has enabled us to rapidly identify unknown impurities in the drug substances and drug products at a high confidence level without resorting to NMR characterization [6,7]. A typical mechanism-based stress study is a mini-scale organic reaction that is specifically designed (mostly likely based on initial LC–MS analysis results) to generate the impurity to be identified, which is then used to confirm the proposed structure and degradation mechanism. The structures identified through this approach are of very high confidence level and we have validated this approach via subsequent full characterization by NMR analysis [7]. This strategy is particularly effective in the identification of drug substance related impurities, such as drug degradants and process impurities. Nevertheless, the same principle and process can also be used to facilitate the identification of excipient-related impurities. In this paper, we report the identification of an unknown impurity arising from sesame oil using this strategy. This unknown impurity eluting at a relative retention time (RRT) of 0.91 was found in certain stability batches of an animal health product at a level above the identification threshold set by VICH. When analyzed by ESI-MS, multiple fragments and cluster ions were observed for this unknown species while the molecular ion was not or barely detected in one of the two instruments used. Various attempts such as adjusting various source parameters to minimize source fragmentation, conducting tandem MS/MS analysis, changing LC conditions, and using a different mass spectrometer were made in order to determine the molecular ion. When the MS^n results, in particular the high resolution MS^n results, obtained from both LC–MS systems were evaluated, we were able to determine the molecular ion which led to the formula of the unknown species. An examination of the product formulation suggested that the unknown species relates to one of the main excipients, sesame oil. The formula of the unknown species was then found to match that of sesamin and asarinin (an epimer of sesamin, also known as episesamin) that can be extracted from sesame oil [8]. Using an acidic stress condition known in the literature [8], asarinin was generated from sesamin via epimerization; the retention time and MS^n fragmentation patterns of the stress generated asarinin match those of the unknown peak, confirming the proposed structure for the latter. This case study has again demonstrated that the overall approach in combining LC–MS^n including high resolution MS/MS analysis with the mechanism-based stress studies can be effectively used as a general strategy for the positive identification of unknown pharmaceutical impurities, especially the degradants that are related to the APIs and excipients in pharmaceutical products.

2. Experimental

2.1. Materials

All reagents used to prepare mobile phases for the HPLC analyses were of HPLC grade, obtained from Fisher Scientific (Pittsburgh, PA, USA). Sesamin and other chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Sample preparation

To a portion of the animal health drug product (~1.25 g paste) was added approximately 20 mL of methanol in a 50 mL volumetric flask. The mixture was sonicated for 8 min and then heated in a water bath for 15 min, during which time the flask was continuously swirled so that the contents in the flask were gently boiling and the oil phase was clear in appearance. The flask was then allowed to cool to room temperature. This heating and cooling process was repeated until the oil drops became clear upon cooling. The mixture was diluted to volume with methanol and 5 mL of the supernatant was transferred into a 100 mL volumetric flask followed by dilution to volume with the diluent. The diluent consisted of mobile phase A and mobile phase B (refer to Section 2.3) in a ratio of 3:2 (v/v). A portion of the sample solution was centrifuged for 10 min at 2500 rpm and the supernatant was sampled for HPLC and LC–MS^n analyses.

2.3. HPLC analyses

A Waters Alliance 2695 HPLC system equipped with a PDA detector was used for sample analysis. The mobile phase consisted of (A) 10 mM aqueous ammonium formate with 10% acetonitrile adjusted to pH 3 with formic acid and (B) acetonitrile. The gradient started from 40%B to 60%B in 25 min followed by equilibration at 40%B for 10 min. A Waters Symmetry Shield RP8 column (150 mm × 4.6 mm ID, 3.5 μm particle size) was used with a flow rate of 1.0 mL/min.

A Thermo Fisher Scientific Surveyor HPLC system equipped with a PDA detector was also used for sample analysis with a modified LC method. The mobile phase consisted of (A) 0.1% formic acid in water and (B) acetonitrile. The gradient started from 45%B to 55%B in 15 min followed by equilibration at 45%B for 10 min. An Agilent Eclipse XDB C8 column (150 mm × 4.6 mm ID, 5 μm particle size) was used with a flow rate of 1.5 mL/min.

2.4. LC–MS^n (n = **1**–**5**) analyses

LC–MS^n experiments were performed on two LC–MS systems. The first one is a Waters QTof Premier mass spectrometer equipped with a Waters 2695 Alliance separation module. The LC flow was split 10:1 prior to the mass spectrometer. Approximately 100 μL/min of the LC flow was directed into the ion source of the mass detector. Positive ESI mode was used and the source was operated under the following conditions: electrospray voltage 3.5 kV, cone voltage 30 V, source temperature 100 °C, ion sabre probe temperature 250 °C, cone gas flow 60 L/h, and desolvation gas flow 600 L/h. The QTof mass analyzer was operated at the V mode and the calibration was performed externally with a sodium cesium iodide solution. Time-of-flight (TOF) MS and MS/MS spectra were acquired at 1 scan/s scanning rate and 0.1 s inter-scan time. A LockSpray source was used for high resolution MS^n experiments. A suitable reference solution ($C_{19}H_{21}N_2O_3$) was infused into the reference channel of the LockSpray source via the embedded syringe pump at a flow rate of 3–5 μL/min. The intensity of the reference peak was adjusted to 100–200 counts/s by tuning the reference spray cone voltage and changing the reference solution concentration. The LockSpray interval was set to 5 s during LC–MS acquisition. The accurate masses of the analyte peaks were obtained by mass measurement against the standard peak in the reference channel.

A Thermo Fisher Scientific Surveyor HPLC system equipped with a PDA detector and a LTQ-Orbitrap mass spectrometer was also used for the LC–MS^n analyses. The LC flow was split 10:1 prior to the mass spectrometer. Approximately 150 μL/min of the LC flow was directed into the ion source of the mass detector. The electrospray mass spectrometry was operated in positive ion mode, with the spray voltage at 4.5 kV. The capillary voltage and tube lens were set at 43 and 75 V, respectively. The capillary temperature was set at 300 °C, the sheath and auxiliary gas flow rates were at 40 and 5 (arbitrary units), respectively. The mass accuracy was calibrated at the beginning of a working day using a mixture of polytyrosine. Full MS scan was acquired in the mass range of 100–800 Da with a resolving power of 30,000 at m/z 400. High resolution MS/MS exper-

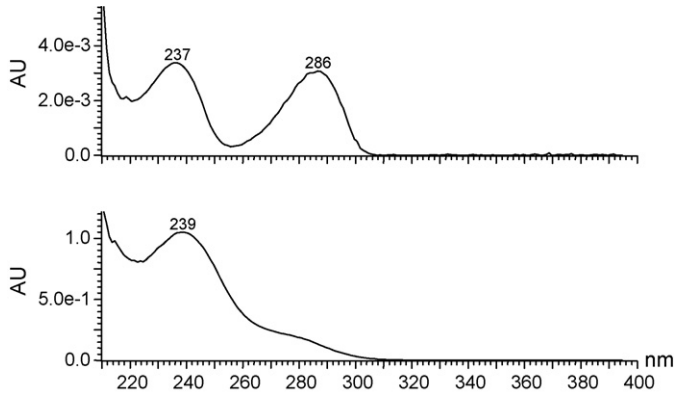
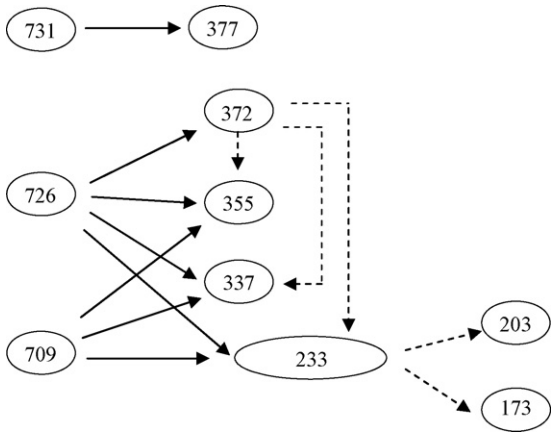


Fig. 1. UV spectra of the unknown peak at RRT 0.91 (upper) and the API of the drug product (lower).



Scheme 1. Summary of the fragmentation pathways obtained on Waters QTof Premier mass spectrometer. Dotted arrows indicate the results were obtained from MS/MS experiments of ions formed in in-source fragmentation (pseudo MS^3 experiments).

iments were performed in Orbitrap using helium as collision gas, with a parent ion isolation width of 2.0 Da and normalized collision energy of 28. The mass resolution was set at 15,000 and 7500 for the precursor and product ions, respectively. Low resolution MS^n (n = 2–5) experiments were performed in LTQ starting from the m/z 337 ion produced in in-source fragmentation, with a parent ion isolation width of 2.8 Da and normalized collision energy of 28.

2.5. Generation of asarinin from sesamin via epimerization under acidic stress

Approximately 2 mg of sesamin was dissolved in 5 mL of a mixture consisting of conc. HCl and ethanol (10/90, wt/wt). The solution was stirred under refluxing (about 85 °C) and was monitored by HPLC until equilibrium was reached. At every hourly interval, ~100 μL of the reaction solution was taken out and injected into the LC–MS system.

3. Results and discussion

3.1. LC-PDA–MS^n analysis of the unknown peak at RRT 0.91

An unknown peak at RRT 0.91 was observed in certain batches of the animal health product at a level above 1% that exceeds the VICH identification threshold for impurities in drug products for animal health. One of these batches was analyzed by LC-PDA–MS^n in order to identify the RRT 0.91 unknown species.

The UV profile for the RRT 0.91 peak was quite unique with two absorbance maxima at 237 and 286 nm, which is very different from that of the API in the drug product which has only one maximum at 239 nm (Fig. 1). The TOF mass spectrum of this unknown peak (Fig. 2) showed four major ions at m/z 233, 372, 726 and 731. In addition, some minor ions at m/z 173, 337 and 709 were also observed. If the ion at m/z 709 is assigned as the protonated molecular ion, the ions at m/z 726 and 731 could be assigned as the ammonium and sodium adducts of the molecular ion, respectively. Nevertheless, ions at m/z 372, 233 and 173 could not be assigned. Although the ion source parameters were adjusted and tuned in order to minimize the in-source fragmentation, these ions remained, suggesting that they may correspond to co-eluting species, cluster ions or the fragments from an extremely labile molecule. Therefore, the molecular ion could not be determined based on the mass spectrum of the unknown peak alone.

To examine if the ions at m/z 372, 233 and 173 are from possible co-eluting species or fragments of the ions at m/z 709, 726 and 731, tandem MS/MS experiments were conducted on these ions. The complete fragmentation pathways of the observed ions are summarized in Scheme 1.

As shown in Scheme 1, three ions at the higher mass range, m/z 709, 726 and 731, followed the same fragmentation pattern under the collision induced dissociation (CID), i.e., losing a neutral molecule with a mass of 354. Furthermore, the ion at m/z 355 is the common fragment resulting from the ions at m/z 726 and 709. If 354 is the molecular weight (M) of the unknown species, these three ions in the higher mass range could be assigned as the cluster ions of $[2M+H]^+$, $[2M+NH_4]^+$, and $[2M+Na]^+$, respectively. In addition, the m/z 372 ion could be assigned as the ammonium adduct ion $[M+NH_4]^+$, since ammonium formate was used in the mobile phase. This assignment was also supported by the observation that a loss of 17 from m/z 372 occurred to form the fragment ion at m/z 355 under CID. Because of the relatively loose structures of these cluster ions formed in the gas phase, they should be quite susceptible to losing one neutral molecule upon collision. Several ions at lower mass range were observed: m/z 233 and 337 are the common fragments from m/z 726, 709 and 372, while the ion at m/z 173 is one of the fragments from m/z 233. However, the protonated molecular ion at m/z 355 was very low in abundance and barely detected, suggesting it could be quite fragile and readily susceptible to fragmentation in the ion source.

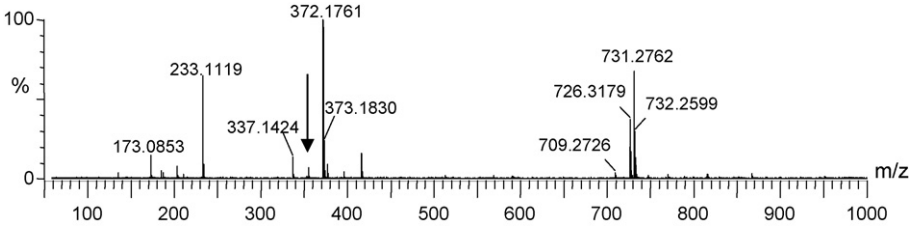


Fig. 2. Full scan TOF mass spectrum of the unknown peak at RRT 0.91 obtained on QTof Premier mass spectrometer. The arrow indicates the m/z 355 protonated molecular ion.

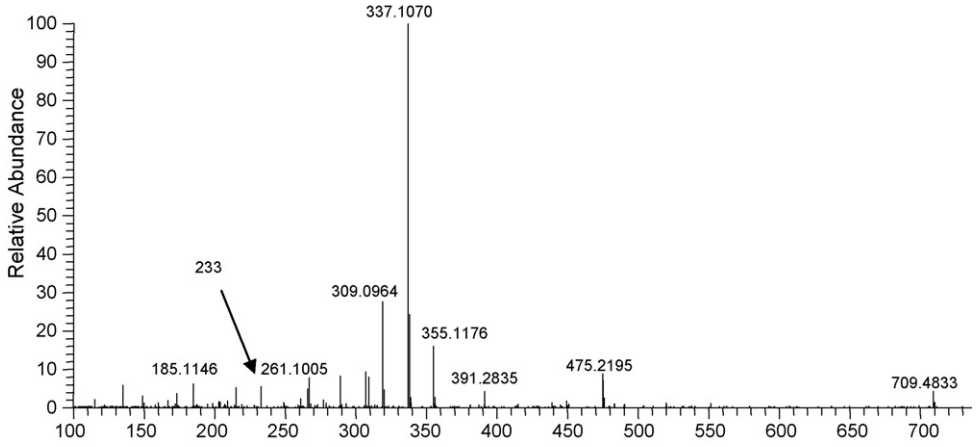
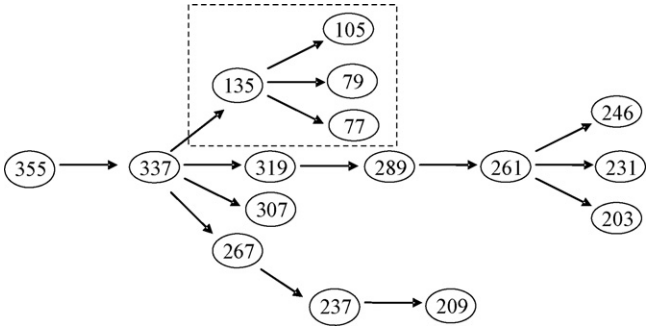


Fig. 3. High resolution, full scan mass spectrum of the isolated unknown peak at RRT 0.91 obtained on LTQ-Orbitrap mass spectrometer.



Scheme 2. Summary of the fragmentation pathways obtained on LTQ-Orbitrap. Ions in the box are the most informative fragments.

3.2. High resolution LC–MS analysis of the unknown peak at RRT 0.91

To support the hypothesis/assignment of these ions, the unknown species was isolated, concentrated and analyzed using a different LC–MS system with a modified LC method to eliminate the possibility of the presence of the co-eluting species and cluster ions. In the modified LC method, a different C8 column and mobile phases devoid of ammonium formate were used as described in Section 2.3. A Thermo Fisher Scientific Surveyor HPLC system equipped with a PDA detector and a LTQ-Orbitrap mass spectrometer was used for the analysis. Only one peak was observed corresponding to the isolated unknown peak in the UV chromatogram (data not shown), suggesting there was no co-eluting species. The mass spectrum of the isolated unknown peak is shown in Fig. 3. Similar to that obtained from the Waters QTof Premier mass spectrometer, common ions such as *m*/*z* 337 and 709 were also observed. However, *m*/*z* 709 was the only cluster ion observed in the higher mass range (Fig. 3). The *m*/*z* 372, 726 and 731 ions were not observed. The absence of the *m*/*z* 372 and 726 ions, assigned as the ammonium adducts in the TOF mass spectrum, is consistent with the fact that ammonium formate is not present in the mobile phase. On the other hand, the absence of the *m*/*z* 731 ion, assigned as the sodium adduct in the TOF mass spectrum, may be due to the different source configuration in the current instrument used. In addition, the proposed molecular ion at *m*/*z* 355 was observed at a relatively higher abundance than that in the TOF mass spectrum, while *m*/*z* 233 (fragment from *m*/*z* 709) and *m*/*z* 173 (fragment from *m*/*z* 233) were seen in low abundance. It appears that the ion source in the LTQ-

Table 1
Summary of the accurate masses for RRT 0.91 determined on LTQ-Orbitrap.

Analyte ion	Experimental mass	Theoretical mass	Formula	Mass error (ppm)
m/*z* 319	319.0964	319.0965	$C_{20}H_{15}O_4$	0.3
m/*z* 337	337.1070	337.1071	$C_{20}H_{17}O_5$	0.3
m/*z* 355	355.1176	355.1176	$C_{20}H_{19}O_6$	0.04
m/*z* 709	709.2274	709.2280	$C_{40}H_{37}O_{12}$	0.75

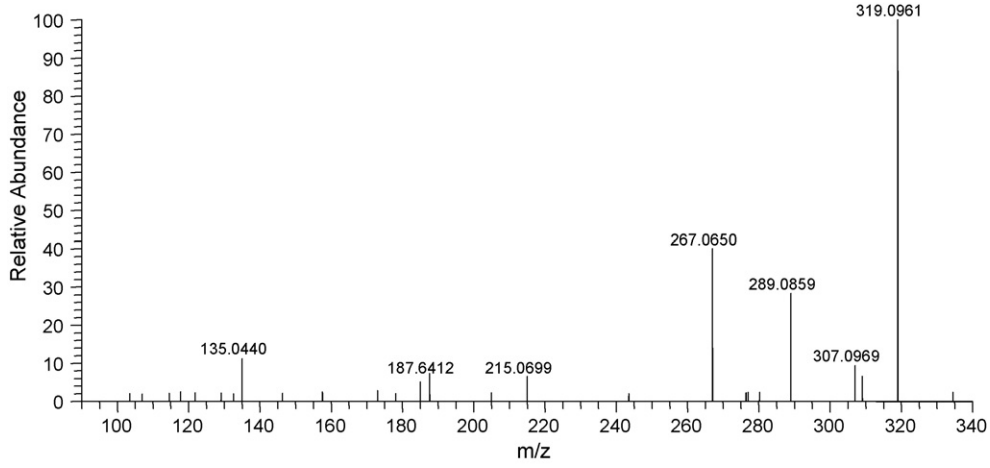


Fig. 4. High resolution, product ion spectrum of the ion at *m*/*z* 337 obtained on LTQ-Orbitrap mass spectrometer.

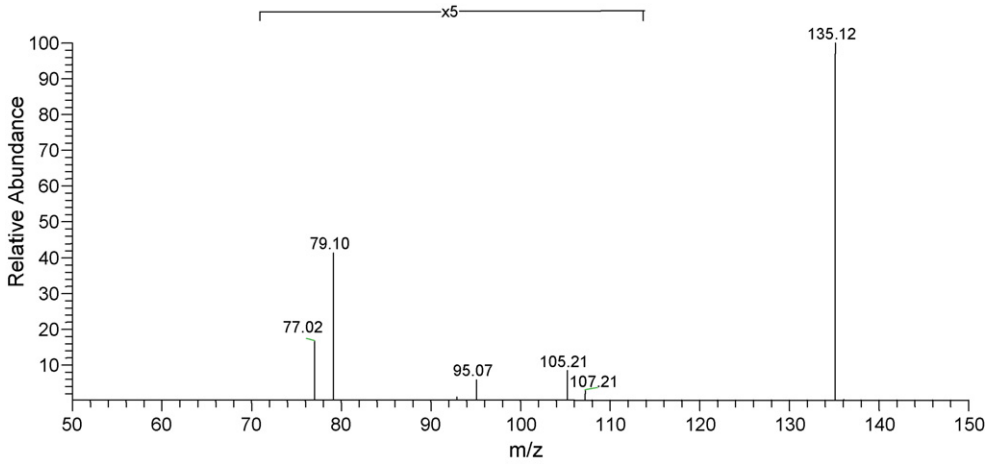


Fig. 5. Unit resolution, pseudo MS^4 spectrum of the product ions at *m/z* 135 from *m/z* 337 obtained on LTQ.

Orbitrap is "milder" than that in the QTof Premier. The difference in the ion source designs may contribute to the slightly different ionization processes which provided complementary information in the determination of the molecular ion in this case. These results further support the previous assignments that ions at *m/z* 355, 372, 709, 726 and 731 correspond to $[M+H]^+$, $[M+NH_4]^+$, $[2M+H]^+$, $[2M+NH_4]^+$ and $[2M+Na]^+$, respectively. Based on the accurate mass measurement of the unknown peak on the LTQ-Orbitrap mass spectrometer, with the element selection limited to C, H, O and N, the formula $C_{20}H_{19}O_6$ was matched within a 1 ppm range of the theoretical mass for the ion at *m/z* 355. Therefore, the chemical formula of the unknown species was determined to be $C_{20}H_{18}O_6$. Formulas matched for other ions observed are summarized in Table 1.

The accurate masses of the ions at *m/z* 372 and 337 were also measured previously on the Waters QTof Premier mass spectrometer. Using a Lockspray compound ($C_{19}H_{21}N_2O_3$, 325.1552) as the internal standard, the accurate masses of *m/z* 372 and 337 ion from RRT 0.91 were determined and their formulas were matched with $C_{20}H_{22}NO_6$ and $C_{20}H_{17}O_5$, respectively. These results support the previous assignment of $[M+NH_4]^+$ for the *m/z* 372 ion and further support the assignment of the chemical formula of the unknown species as $C_{20}H_{18}O_6$.

3.3. Identification of the unknown peak at RRT 0.91

MS^n (n = 2–5) experiments were conducted on ions at *m/z* 355 and 337 using LTQ-Orbitrap mass spectrometer in order to probe the structure of this peak. The major fragmentation pathways of the observed ions obtained from the multiple MS^n experiments are summarized in Scheme 2. Fig. 4 shows the high resolution product ion spectrum of the ion at *m/z* 377 obtained on Orbitrap. Based on the accurate mass measurement, five fragments from ion at *m/z* 337 were matched with chemical formulas $C_{20}H_{15}O_4$ (*m/z* 319), $C_{19}H_{15}O_4$ (*m/z* 307), $C_{19}H_{13}O_3$ (*m/z* 289), $C_{16}H_{11}O_4$ (*m/z* 267) and $C_8H_7O_2$ (*m/z* 135), respectively, within a mass error of less than 5 ppm from their theoretical masses. However, little clue, other than loss of small fragments such as H_2O and CH_2O during the fragmentation, could be retrieved from these fragments as to the structure of the unknown peak. Hence, some of the product ions from *m/z* 337 were further fragmented to obtain more structural information. The most informative fragmentation is from the ion at *m/z* 135 that has a formula of $C_8H_7O_2$. Three major fragments at *m/z* 77, 79 and 105 were observed from the *m/z* 135 ion under CID (Fig. 5). This suggests that this ion likely contains a benzene ring, two methylene groups, and two oxygen atoms. The ring-double bond equivalence (RDB) is 5 for this ion, suggesting the presence of an additional double bond or a ring structure beyond the benzene ring. On the other hand, the RDB of the unknown species is 11, indicating a highly unsaturated or multiple ring structure. The losses of H_2O (−18), CH_2O (−30) and CO (−28) were frequently observed for this unknown species as shown in Scheme 2. Up to this point, it was certain that this unknown species contains at least one benzene ring in the struc-

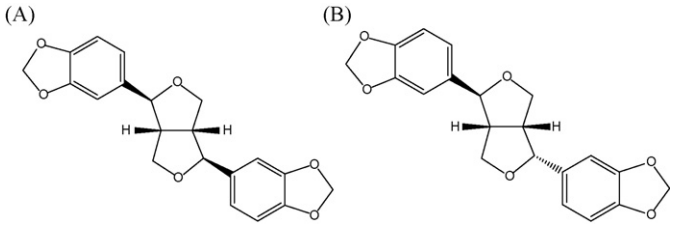


Fig. 6. Structures of sesamin (A) and asarinin (B).

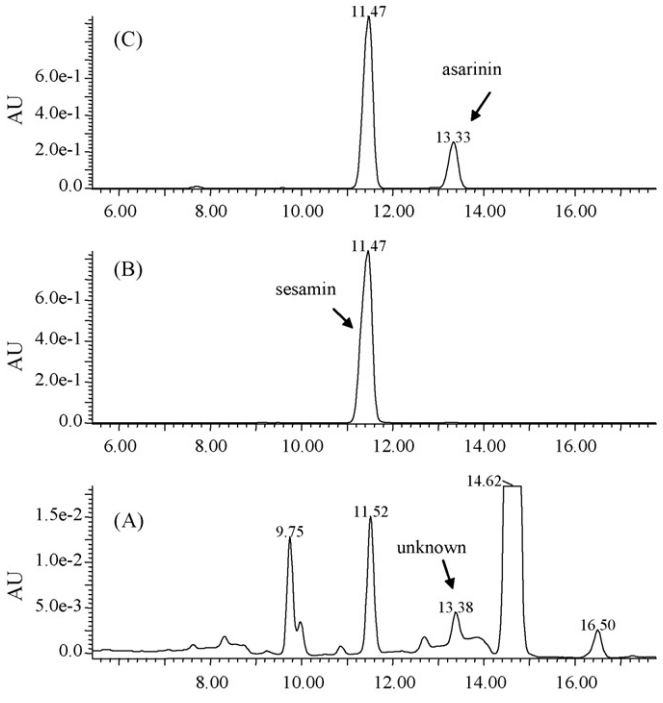


Fig. 7. UV chromatograms of stability sample of the animal drug product (A), sesamin (B), and reaction mixture sampled after 1 hour refluxing at 85 °C (C).

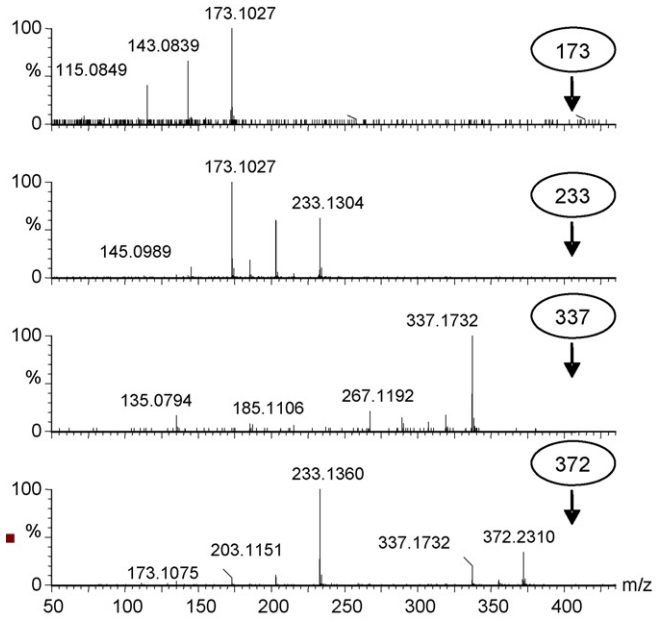
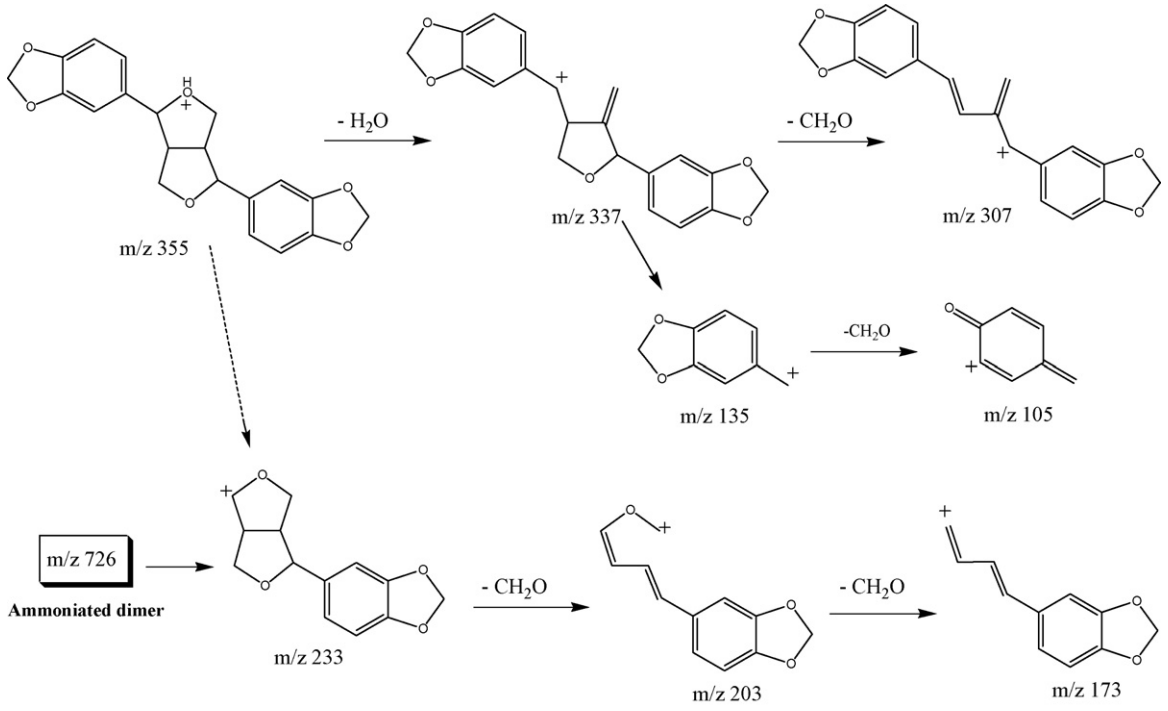


Fig. 8. Product ion spectra of the four major ions at *m*/*z* 372, 337, 233 and 173 from the unknown peak at RRT 0.91 obtained on Waters QTof Premier mass spectrometer.

ture. However, the complete structure of this unknown species still could not be determined based on the chemical formulas and the corresponding fragmentation pathways (Schemes 1 and 2).

Since the chemical formula, UV profile, and the fragmentation pathways of the unknown species $C_{20}H_{18}O_6$ are distinctly different from those of the API, it is very likely that the unknown species is not related to the API. With the structure knowledge already obtained for the unknown species, we turned our attention to the excipients used in the drug product to look for some clues. Sesame oil, one of the main excipients used in the product formulation, caught our attention. It turned out that sesamin, one of the most abundant lignans in sesame seeds, has the same chemical formula as the unknown species and it contains two benzene rings in the structure (Fig. 6A). In addition, sesamin can be extracted from sesame oil [8]. To confirm if sesamin is the unknown species, an authentic compound of sesamin was obtained from a commercial source and analyzed by LC-PDA–MS^n. Under the original HPLC conditions for the drug product, sesamin was found to elute at RRT 0.77, earlier than the unknown species. However, its UV spectrum was identical to that of the unknown peak at RRT 0.91. In addition, its TOF full scan mass spectrum also showed multiple fragment ions that are identical to those observed for the unknown peak at RRT 0.91 (data not shown). This observation, along with the fact that sesamin has several chiral centers in its structure, suggested that the unknown species could be an isomer of sesamin.

Upon a search of the literature, it was found that asarinin (also known as episesamin, Fig. 6B), an epimeric isomer of sesamin, can also be extracted from sesame oil. The relative amounts of sesamin and asarinin extracted depend on different extraction methods [8]. In addition, both sesamin and asarinin can epimerize towards each other under acidic conditions as reported by Li et al. [8]. To confirm if the unknown species is asarinin, a stress study was conducted to convert sesamin to asarinin under an acidic condition since the latter is not readily available through commercial sources. Hence, a solution of sesamin was refluxed in acidic methanol and the reaction was monitored by LC-PDA–MS^n on Waters QTof Premier mass spectrometer. After 1 h refluxing, the asarinin peak was observed (Fig. 7) and it eluted at the same retention time as that of the unknown peak at RRT 0.91. The UV spectrum of asarinin peak is identical to that of the unknown peak at RRT 0.91 with the two maxima at 237 and 286 nm (Fig. 1). In addition, the TOF full scan mass spectrum of asarinin peak is consistent with that of the unknown peak at RRT 0.91 (Fig. 2). Furthermore, the product ion spectra of the four major ions at *m*/*z* 372, 337, 233 and 173 of asarinin are essentially identical to those of the unknown peak at RRT 0.91 (Fig. 8). Based on all these results, the unknown peak at RRT 0.91



Scheme 3. Proposed fragmentation pathways leading to the formation of some major fragments from the protonated asarinin (*m*/*z* 355) and ammoniated dimer (*m*/*z* 726). All fragmentation was effected via CID except for the dotted line pathway which was produced from in-source fragmentation of *m*/*z* 355.

was identified and confirmed as asarinin. The proposed fragmentation pathways for some major fragments from asarinin observed in QTof Premier and LTQ-Orbitap are shown in Scheme 3.

4. Conclusion

An excipient related unknown impurity peak was successfully identified using a strategy that combines LC–MS^n (including high resolution MS analysis) in conjunction with a mechanism-based stress study. The main challenge of this investigation lies in the difficulty in determining the molecular ion of the unknown species due to its lability in the ESI source. To overcome the challenge, we utilized tandem MS/MS analysis on two high resolution LC–MS instruments during the identification process. The slightly different ionization processes between the two different instruments provided strong, complementary evidence in identifying the correct molecular ion. Once the molecular formula and fragmentation pathways were established, we were able to quickly propose the structure of the unknown species and then verified it via a stress study based on the known chemistry in which the unknown species can be generated. This case study has again demonstrated that the overall approach used in this investigation (an effective combination of LC–MS^n and mechanism-based stress studies) can be used as a general strategy for the rapid identification of unknown pharmaceutical impurities, especially the degradants related to the APIs and excipients.

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