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Review Article

Tandam mass spectrometry instrumentation and application in pharmaceutical analysis

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ABSTRACT

Proteins and peptides can be analyzed using mass spectrometry (MS) using a range of techniques, including matrix-aided laser desorption ionization-mass spectrometry (MALDI-MS) and electrospray ionization-mass spectrometry (ESI-MS). These techniques make it possible to determine a protein's mass as an intact molecule or to identify a protein using peptide-mass fingerprinting that is produced during enzymatic digestion. The amino acid sequence of proteins (top-down and middle-down proteomics) and peptides (bottom-up proteomics) can be ascertained by fragmenting the proteins and peptides using tandem mass spectrometry (MS/MS). Furthermore, post-translational modifications (PTMs) of proteins and peptides can be identified using tandem mass spectrometry. In this article, we go over the use of MS/MS in biomedical research and provide concrete examples of how to identify proteins, peptides, and their PTMs as useful biomarkers for diagnosis and treatment. In numerous applications, tandem mass spectrometry (MS/MS) has shown to be a practical and efficient analytical method for the direct detection of target compounds in food samples. It combines the power of MS/MS as an identification and confirmation approach with the separation capabilities of chromatography when used with chromatographic techniques.

1. Introduction

Tandem mass spectrometry (MS-MS) is a related technology in which compounds are separated by molecular weight by one mass spectrometer, fragmented as they exit, and identified on the basis of their fragments by a second mass spectrometer. Tandem mass spectrometry, also known as MS/MS or MS^2. main principle of Tendam mass spectrometry of involves multiple steps of mass spectrometry selection, with some form of fragmentation occurring in between the stages. In a tandem mass spectrometer, ions are formed in the ion source and separated by mass-to-charge ratio in the first stage of mass spectrometry (MS1).Ions of a particular mass-to-charge ratio (precursor ions) are selected and fragment ions (product ions) are created. The resulting ions are then separated and detected in a second stage of mass spectrometry (MS2).

J. J. Thomson made this statement about mass spectrometry, but it may be even more apt in describing tandem mass spectrometry. As such, besides being the father of mass spectrometry, Thomson can also be considered the forefather of tandem mass spectrometry. In fact, to demonstrate experimentally the processes of neutralization and collisional ionization, Thomson built the first MS/MS instrument, which consisted in a serial arrangement of two magnets, with the field on one magnet oriented perpendicular to the other.(Busch, Glish et al. 1988) Tandem mass spectrometry (MS/MS) can be considered as any general method involving at least two stages of

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mass analysis, either in conjunction with a dissociation process or in a chemical reaction that causes a change in the mass or charge of an ion.(Hoffmann and Stroobant 2007) In the most common tandem mass spectrometry experiment a first analyser is used to isolate the precursor ion (mp +), which then undergoes fragmentation (this could be achieved either spontaneously or by making use of some activation technique) to yield product ions (mf +) and neutral fragments (mn) which are then analysed by a second mass analyser. This reaction is depicted in equation 1. mp +→mf + + mn (1) An activation barrier must be surmounted before the general reaction depicted in equation 1 can occur. The energy to overcome this barrier can come from one of two sources (Busch, Glish et al. 1988): i. By the excess energy deposited onto the precursor ion by the ionization process. Nevertheless, this is valid only we dealing with electron ionization at high energies. ii. By means of activation methods such as collision activated/induced dissociation (CAD and CID), infrared multiphoton dissociation (IRMPD), electron capture/electrontransfer dissociation (ECD and ETD) and surface induced dissociation (SID), for which the fundamental aspects will be discussed in section 2 of this chapter. The principle of MS/MS is illustrated in Figure 1. Tandem mass spectrometry can be conceived in two ways: in space by the coupling of two mass spectrometers, or in time by an appropriate sequence of events in an ion storage device. This consequently leads to two main categories of instruments that allow for tandem mass spectrometry experiments: tandem mass spectrometers in space or in time.(Hoffmann and Stroobant 2007)

1.1. Instrumention

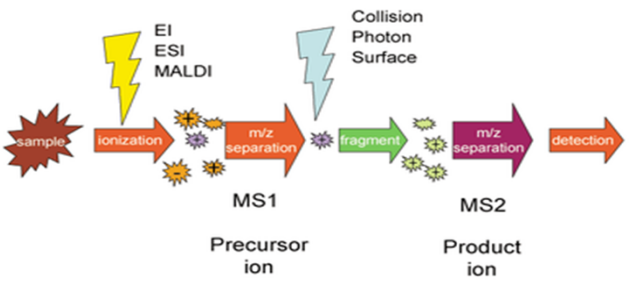


Figure 1: Schematic representation of tandem- MS/MS

Tandem mass spectrometry, also known as MS/MS (mass spectrometry/ mass spectrometry), is a process by which gas-phase ions of an analyte resulting from an initial ionization undergo separation according to their m/z values, using a first iteration of mass spectrometry. Ions of individual m/z values (precursor ions) are then subjected to collisional activation so that these precursor ions will fragment, producing product ions of different m/z values. These product ions then are separated and detected by a second iteration of mass spectrometry; thus the name tandem mass spectrometry or MS/MS. The process of fragmenting precursor ions is called collisionally activated dissociation (CAD) or collision-induced dissociation (CID). These terms are used interchangeably. CAD is a result of an inelastic collision between an ion of a specific m/z value (the precursor ion) and an inert atom or molecule such as He, Ar, Xe, or N2.

Some of the kinetic energy of the ion is transformed into internal energy, and the activated ion will then dissociate. MS/MS can be accomplished in a temporal or spatial domain. Spatial domain MS/MS is called tandem-in-space mass spectrometry. Temporal domain MS/MS is called tandem-in-time mass spectrometry. In the world of GC/MS, tandem-in-space mass spectrometry is carried out in a double-focusing instrument as described in the double-focusing GC-MS section of this chapter or in a triple quadrupole mass spectrometer, schematically illustrated. Other types of tandem-in-space MS/MS instruments exist, but they currently have no role in GC/MS. In this same world, tandem-in-time mass spectrometry is carried out in the QIT. Tandem-in-time MS/MS can also be carried out in what is known as a Fourier transform ion cyclotron resonance mass spectrometer, but these instruments are really not very often, if at all, found in GC/MS. The term triple quadrupole is somewhat misleading. The name implies that there are three QMFs in tandem; however, the instrument has two QMFs separated by a collision cell that was constructed from a quadrupole device used in the rf-only mode in the original design of the instrument. This instrument was developed at Michigan State University in the mid-1970s.[1] The development of the triple quadrupole had a significant impact on the field of mass spectrometry. Today, although still often referred to as the triple quadrupole, the collision cell can be any number of devices from an octupole to a hexapole to a traveling-wave device. Very few, if any, manufacturers use a quadrupole as the collision cell; therefore, a more appropriate description would be the tandem quadrupole mass spectrometer. What all of these devices have in common is that they have entrance and Tandem MS Instruments: exit lenses to speed up and slow down the ions entering and leaving the collision cell, respectively. The ion velocity is increased to increase its kinetic energy. The product ions will have the same velocity when they reach the exit of the collision cell as the precursor ions had upon entering. The product ions that are formed can also undergo CAD because they have kinetic energies as they are formed. The resulting spectrum can exhibit peaks representing product ions of the precursor ions and product ions of the product ions. The ions exiting the collision cell must be slowed so that the effect of the quadrupole field of the second QMF will be optimum for ion separation. The pressure in the collision cell is ~10−1 Pa, whereas the pressure in the two QMFs is ~10−3 Pa.

The operational pressure of QIT mass spectrometers is ~10−1 Pa; therefore, after isolation of the precursor ion of a specific m/z value in the ion trap, the energy of the ion is increased by applying waveforms to the endcap electrodes to bring about collisions with the already present He atoms. These wave forms can be specific for a very narrow m/z range of ions; therefore, product ions that are formed and stored during the energizing of the precursor ion will not undergo collisional activation. However, it should be noted that because of the need to use rf voltages to both energize and store ions, the product ions that have an m/z value that is less than one-third of the m/z value of the precursor ions will not be sufficiently stored. In the double-focusing GC-MS used for MS/MS, the collision cell is mounted between the ion source and the first field. This means that the ion will have the kinetic energy provided by the accelerating voltage, which is between 1 keV and l0 keV. This results in a single high-energy collision. In the QIT and triple quadrupole, CAD occurs for ions with <40 eV kinetic energy. This is called low-energy CAD. The difference is in the number of collisions. MS/MS in a triple quadrupole will result in <10 collisions; MS/MS in a QIT will result in a much larger number of collisions, several hundred to a few thousand.

1.2. In tandem-in-space mass spectrometry

MS/MS can take several forms. The first analyzer can be set to allow ions of only a specific m/zvalue to pass into the collision cell. The second analyzer is scanned to obtain a product-ion mass spectrum exhibiting ions of all m/z values resulting from CAD. This is called a product-ion analysis, and the results are a product-ion mass spectrum. The second m/z analyzer can be set to allow ions of only a single m/z value to pass to the detector. The first analyzer is scanned. This results in a signal for any ion of any m/z value that produces product ions of a specific m/z value. This is called a precursor-ion analysis, and the results are a precursor-ion mass spectrum for a specific product ion. The two m/z analyzers can be scanned simultaneously with the second m/z analyzer having a lower starting m/z than the first. This will result in a signal for ions of any m/z value passing the first analyzer that undergo a loss of a specific mass (a neutral loss) in the collision cell. This is called a common-neutral-loss analysis, and the results are called a neutral-loss spectrum of a specified offset. The most widely used form of MS/MS is in a process called selected reaction monitoring (SRM).* SRM is a process where an ion of a lesser m/z value is allowed to pass the second m/z analyzer when ions of a specified higher m/z value pass the first analyzer. This process is analogous to SIM; however, because it involves the transition from an ion formed by an analyte to a fragment of that ion, it provides for a higher degree of selectivity. Manufacturers have said that SRM is a more sensitivetechnique than SIM. Of course this is not possible for two reasons. The first is that there is a certain amount of ion loss going through each of two m/z analyzers associated with the instrument used for SRM, which will be twice the loss experienced when the ions pass through a single analyzer in SIM. The second is that all of the precursor ions may not be transformed into the product ion. The limit of detection or limit of quantitation can be much larger in SRM than in SIM because of the increased specificity associated with SRM. In a single acquisition cycle of a tandem-in-space instrument, there can be multiple transition pairs. Just like SIM, too many pairs can defeat the increase in signal strength that will result from monitoring ions for longer periods. It is possible for transition pairs to have separate dwell times in an acquisition cycle. Different transition pairs can be implemented for different time segments in the analysis.

1.3. In tandem in time mass spectrometry

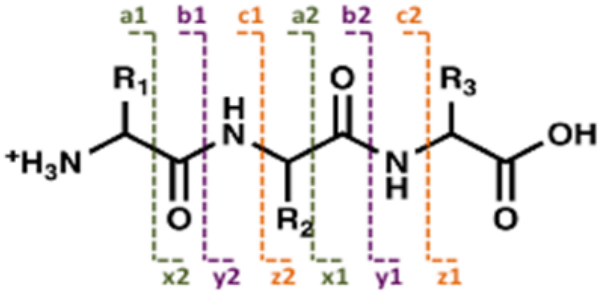
The separation with ions trapped in the same place, with multiple separation steps taking place over time. Aquadrupole ion trap or FTMS instrument can be used for such an analysis. Trapping instruments can perform multiple steps of analysis, which is sometimes referred to as MSn (MS to the n). Tandem-in-time mass spectrometry only allows for product-ion analyses. A process can be carried out that will pass for SRM in the QIT. The triple quadrupole GC-MS/MS has become somewhat obscure in recent years. Several years back, Waters and Thermo Scientific discontinued offering these instruments. This left only the Varian 1200. Both companies have now reintroduced their instruments as improved models; Agilent has introduced a GC/MS/MS instrument as of the 2008 Riva Del Garda 32nd Symposium on Capillary Chromatography. There is a company (CHROMSYS, LLC, the U.S. subsidiary of the German company CHROMTECH, GmbH) that has a third-party add-on for the Agilent GC/MS systems. This renewed interest is partially driven by interest in specificity for analytes in the area of food safety. The QIT GC/MS instruments from Varian and Thermo Scientific continue to be popular for MS/MS.

2. Applications of MS/MS or Tandam mass spectrometry

2.1. Peptides

Tandem mass spectrometry can be used for protein sequencing.When intact proteins are introduced to a mass analyzer, this is called "top-down proteomics" and when proteins are digested into smaller peptides and introduced into the mass spectrometer, this is called "bottom-up proteomics". Shotgun proteomics is a variant of bottom up proteomics in which proteins in a mixture are digested prior to separation and tandem mass spectrometry.Tandem mass spectrometry can produce a peptide sequence tag that can be

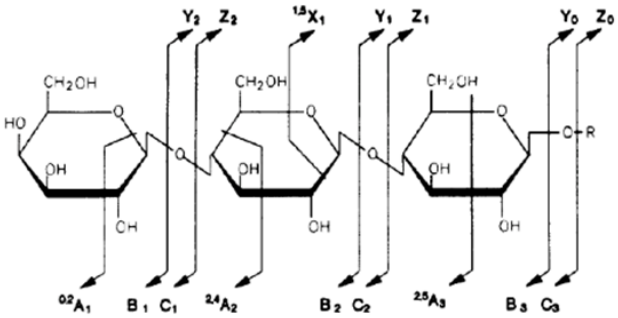
used to identify a peptide in a protein database.



A notation has been developed for indicating peptide fragments that arise from a tandem mass spectrum. Peptide fragment ions are indicated by a, b, or c if the charge is retained on the N-terminus and by x, y or z if the charge is maintained on the C-terminus the subscript indicates the number of amino acid residues in the fragment. Superscripts are sometimes used to indicate neutral losses in addition to the backbone fragmentation, for loss of ammonia and for loss of water. Although peptide backbone cleavage is the most useful for sequencing and peptide identification other fragment ions may be observed under high energy dissociation conditions.These include the side chain loss ions d, v, w and ammonium ions and additional sequence-specific fragment ions associated with particular amino acid residues.

3. Oligosaccharides

Oligosaccharides may be sequenced using tandem mass spectrometry in a similar manner to peptide sequencing. Fragmentation generally occurs on either side of the glycosidebond (b, c, y and z ions) but also under more energetic conditions through the sugar ring structure in a cross-ring cleavage (x ions).



Nomenclature for oligosaccharide fragments (including glycolipids, when R = ceramide) Again trailing subscripts are used to indicate position of the cleavage along the chain. For cross ring cleavage ions the nature of the cross ring cleavage is indicated by preceding superscripts.

4. Oligonucleotides

Tandem mass spectrometry has been applied to DNA and RNA sequencing.A notation for gas-phase fragmentation of oligonucleotide ions has been proposed.

5. Newborn Screening

Newborn screening is the process of testing newborn babies for treatable genetic, endocrinologic, metabolic and hematologic diseases. The development of tandem mas spectrometry screening in the early 1990s led to a large expansion of potentially detectable congenital metabolic diseases that affect blood levels of organic acids.

6. Drug and Biomedical Analysis Using Liquid Chromatography Tandem Mass Analysis

Methods: The applications of these instruments include analysis of nine corticosteroids illegally adulterated in traditional Chinese medicines, identification of drug metabolites of roxithromycin, propafenone and some glucuronide conjugates and the quantitative determination of plasma samples containing amlodipine, nitrendipine, flupirtine and their major metabolites.

6.1. The analysis of water-soluble vitamins in Italian pasta.

A sensitive and selective liquid chromatography tandem mass spectrometry (LC–MS/MS) method for the determination of several water-soluble vitamins, namely vitamins B_1, B_2 , B_6 (pyridoxine, pyridoxal, and pyridoxamine), and PP (nicotinamide and nicotinic acid), pantothenic acid, and folic acid was developed and validated. The analytes were characterized by means of their electrospray (ESI) and atmospheric pressure chemical ionization (APCI) mass spectra In general, the positive ion spectra were 100- to 1000-fold more intense than the corresponding negative ion ones. Chromatography of water-soluble vitamins was obtained by using a reversed-phase C16 Amide (15 cm, 5 mm) column and a mobile phase made of ammonium formate buffer (20 mM, pH 3.75)/methanol under gradient elution conditions.

The LC–ESI-MS/MS method was applied to the quantitative analysis of the natural content of vitamins in typical Italian pasta samples, as well as in fortified pasta samples produced for the US market.

6.2. The determination of amphetamine-type stimulants in blood and urine

Amphetamine, methamphetamine, phentermine,3,4methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), and 3,4

methylenedioxy-N-ethylamphetamine (MDEA) are the most popular amphetamine-type stimulants. The use of these substances is a serious societal problem worldwide, In this study, a method based on gas chromatography–tandem mass spectrometry (GC–MS/MS) with simple and rapid liquid-liquid extraction (LLE) and derivatization was developed and validated for the simultaneous determination of the six aforementioned amphetamine derivatives in blood and urine

The detection of all compounds was based on multiple reaction monitoring (MRM) transitions. The most important advantage of the method is the minimal sample volume (as low as 200 L) required for the extraction procedure. The validation parameters The procedure was successfully applied to the analysis of real blood and urine samples examined in 22 forensic toxicological cases. This is the first work presenting the use of GC–MS/MS for the determination of amphetamine-type stimulants in blood and urine.

6.3. Protien identification using ms/ms data

An enzyme, often trypsin, digests the proteins to peptides, in most cases; one or more stages of chromatography are used to regulate the flow of peptides into the mass spectrometer. Peptides are selected one at a time using the first stage of mass analysis, Each isolated peptide is then induced to fragment, possibly by collision, and the second stage of mass analysis used to capture an MS/MS spectrum.For each MS/MS spectrum, software is used to determine which peptide sequence in a database of protein or nucleic acid sequences gives the best match. Each entry in the database is digested, in silico, using the known specificity of the enzyme, and the masses of the intact peptides calculated. If the calculated mass of a peptide matches that of an observed peptide, the masses of the expected fragment ions are calculated and compared with the experimental values. Some search engines also predict and compare the relative intensities of the fragment ions.

7. Applications of MS/MS in the dereplication of NPs

7.1. Quiterpene lactones (STL)

Sesquiterpene lactones (STL) are an important class of natural products that occur in several plant families, mainly in Asteraceae. These compounds are interesting due to their several biological activities and relevance as chemotaxonomic markers. The biosynthesis of STLs is initiated by condensation of three isoprene molecules followed by oxidation, and their chemical structures are divided into groups according to their carbocyclic skeleton (e.g., germacranolides, eudesmanolides, guaianolides, glaucolides, cadinanolides, hirsutinolides, and furanoheliangolides). The fragmentation pattern of the protonated STL belonging to the goyazensolide type of

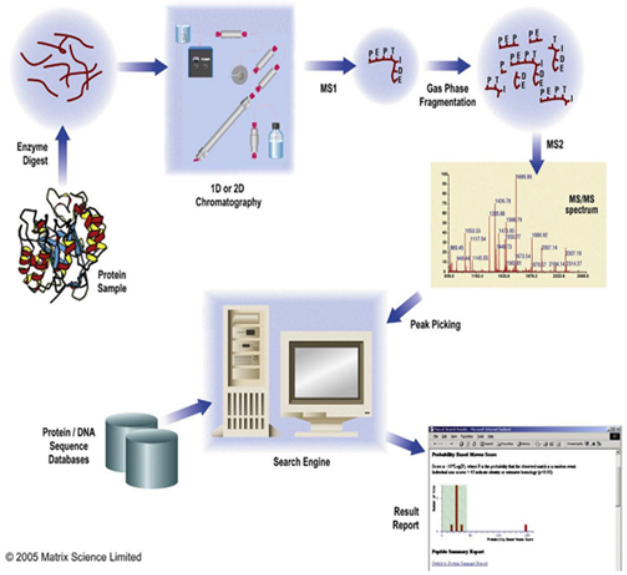


Figure 2:

furanoheliangolides has been investigated by Crotti and co-workers using ESI-MS/MS.

Firstly, the authors selected ten STL standards exhibiting the same structural core, but differing in terms of the presence/absence of a hydroxyl group at C-15, a single/double bond between C-4 and C-5, and the presence of an acyloxy group or hydroxyl group at C-8. All the MS/MS spectra were obtained at 10 eV, as optimized by varying the collision energies between 5 and 50 eV.

The authors reported that the fragment ion [M+H-R2CO2H] + is diagnostic for compounds that exhibit an acyloxy group at C-8. Whereas the fragment ion [M+H-CO2] + indicates the presence of a hydroxyl group at C-8. The acylium ion R2 +, which is formed for compounds, were useful for identification of the ester bound at C-8. Moreover, the relative configuration of C8 of centratherin and budlein A could also be identified on the basis of the relative intensity of the fragment ion [M+H-R2CO2H]+.

These data were further used by Gobbo-Neto & Lopes, in combination with retention times and UV-DAD spectra, for the online identification of 36 compounds in the methanol extract of leaves from Lychnophora ericoides (Asteraceae), including STLs.

7.2. Phenylpropanoids

Phenylpropanoids is one of the largest and most important classes of NPs. It includes a vast range of phenolic compounds, from small and simple forms to complex molecular structures. Moreover, from a biosynthetic viewpoint phenylpropanoids are the precursors of important secondary metabolites, such as aromatic

acids, benzoic acids, cinnamic acids, lignins, lignans, phenylpropenes, coumarins, styrylpyrones, flavonoids, stilbenes, flavolignans, isoflavonoids, terpenoid quinones, and tannins, among others.

7.2.1. Aromatic acids

Gómes-Romero and co-workers have identified several phenol derivatives in foods used in diets (e.g., propolis, lemon, borage, cabbage-broccoli, garlic, onion, etc) by using a MS/MS library previously established from commercially available standard phenolic compounds. The authors reported that the negative ion mode electrospray ionization was more adequate than the positive ion mode for this purpose, although the latter was also utilized when necessary. They described that loss of CO_2 (44 Da) or H_2O (18 Da) from the [M-H]- ion, which was used as precursor ion in MS/MS experiments, are the major fragmentation routes for the selected compounds. However, they did not result in diagnostic fragment ions (DFI). Thus, in order to adjust the intensity of some specific and diagnostic fragment ions, the collision energy values were varied. Samples of these foods were freeze-dried, powdered, and extracted with methanol 80% in ultrasonic bath, which was followed by centrifugation, filtration, and dilution with water/acetonitrile 1:1 (v/v). After that, samples were analyzed by LC coupled with a diode array detector (DAD) set at 254 nm, and a quadrupole orthogonal acceleration time-of-flight mass spectrometer (micrOTOF-Q^{TM}) equipped with an electrospray (ESI) ion source. The authors compared the MS/MS data of each peak of the chromatogram.

Tandem Mass Spectrometry – Applications and Principles previously established library in both the positive and negative ion modes, and they identified nine hydroxybenzoic acid derivatives, nine cinnamic acid derivative, and six simple phenolic compounds, apart from eleven flavonoids. Vanillic and syringic acids were identified on the basis of their DI m/z 152 and 182, respectively, as well as on the DI m/z 92, which is common for both compounds in the negative ion mode. Moreover, syringic acid produced the ions m/z 166 and 123 as DI, via direct loss of two CH3

1. From the deprotonated molecule, and loss of CH3
2. Elimination followed by CO2 elimination.

Transcinnamic , caffeic, and three coumaric acid isomers were also shown to eliminate CO_2 from the deprotonated molecule (Gómez-Romero et al., 2011).

7.3. Caffeoylquinic acid (CQA) and feruloyl quinic (FQA) derivatives

Miketova and co-workers have published a study on the fragmentation of protonated and deprotonated 3,5- and 4,5-dicaffeoylquinic acid (di-CQA) derivatives using electrospray ionization tandem mass spectrometry (Miketova et al., 1999). They demonstrated that both the positive and negative ion modes of analysis allow for identification of the ester groups bound at the quinic acid moiety, although the negative ion mode is the most informative method of analysis for the free compounds. The presence of a phenolic hydroxyl at the ortho or para position was proposed to be important for the formation of some diagnostic ions in the negative ion mode. Gobbo-Neto & Lopes have reported on a sensitive analytical method for the dereplication of various classes of secondary metabolites found in the L. ericoides leaf extracts, including caffeoylquinic acid (CQA) and feruloyl quinic (FQA) derivatives (Gobbo-Neto & Lopes, 2008). The authors employed a methodology based on HPLC coupled with a diode array detector (HPLC-DAD) and HPLC coupled with electrospray ionization tandem mass spectrometry (ESI-MS/MS) using collision-energy values ranging between 10 and 25 eV, in combination with co-injection of authentic standards and accurate-mass measurements.

For chlorogenic acids the authors utilized the ion with m/z 353 as precursor ion and reported that the fragment ion at m/z 173, which is a result of water elimination from the quinic acid moiety, is diagnostic for CQA derivatives esterified at position 4. The CQA derivatives substituted at position 5 and di-CQA isomers (3,5- and 4,5-substituted) were confirmed by co-elution with authentic standards. HPLC-DAD analysis was used in combination accurate mass measurements for identification of feruloylquinic acids (FQA) and feruloyl-caffeoylquinic acids (FCQA). The ion at m/z 367 was used as the precursor ion, yielding the fragment ion with m/z 173, which is diagnostic for 4-FQA derivatives. 3-FQA and 5-FQA were identified on the basis of the ions m/z 193 and 191, Electrospray Ionization Tandem Mass Spectrometry as a Tool for the Structural Elucidation and Dereplication of Natural Products: An Overview 603 by comparison with previously reported studies (Clifford et al., 2003; Clifford et al., 2005). On the other hand, the FCQA isomers could not be identified, even when DAD, ESI-MS/MS in the positive and negative ion modes, and accurate mass data were employed.

7.4. Lignans

Lignans are a group of secondary metabolites consisting of compounds containing two phenylpropanoid units linked by oxidative coupling between C-8 and C-8' in their structures (Willfor et al., 2006). Several biological activities, such as anti-tumoral, fungicide, and anti-viral actions, have been attributed to the presence of these compounds, (Ayres & Loike, 1990; Botta et al., 2001; Pan et al., 2009). Shengmai San (SMS), a prescription comprised of Panax ginseng, Schisandra chinensis, and Ophiopogon japonicus, has long been used in traditional Chinese medicine. Wang

and coworkers have employed HPLC-DAD-MS/MS for identification of the multiple SMS constituents (Wang et al., 2011). These authors identified 53 compounds, including 21 lignans, 6 steroidal glycosides, and 12 homoisoflavonoids. Tandem Mass Spectrometry – Applications and Principles identified by comparison of their retention times, MS data, and UV spectra with those of authentic compounds, and the other twenty-six were tentatively identified by comparing their UV spectra, molecular weights, and structural information from MS/MS spectra with those previously published in the literature. [1–15]

Studies on the dereplication of lignans in the ethanol extract from the fruits of Schisandra chinensis have also been conducted by He and co-workers using high-performance liquid chromatography coupled with a photodiodearray detector and an electrospray ionization ion source. The authors identified seventeen dibenzylcyclooctadiene lignans on the basis of the relative intensities of protonated and cationized molecules, their UV spectra, and the respective retention times (He et al., 1997). Zheng and co-workers have utilized HPLC coupled with an ESI ion source and a hybrid ion IT-TOF mass analyzer for dereplication of lignans in Panax ginseng, Radix ophiopogonis, and Schisandra chinensis baill extracts, which are used in traditional Chinese medicine for treatment of tumoral diseases, coronary atherosclerosis, and some other cardiopathies (You et al., 2006; Yu et al., 2007). [16–20]

They reported that elimination of CH3 OCH3 from the or protonated molecule is diagnostic of a methoxyl group at rings A or B of lignans, and that there is not an oxygen atom on ring C, as in the case of schizandrin A. Elimination of C5H10 by cleavage of the eight-membered ring to produce a five-membered ring was also reported. On the other hand, elimination of a water molecule (loss of 18 Da) produces the diagnostic product ion (DPI) for lignans that have a hydroxyl group at C-6 or C-7 of ring C, such as shizandrol A. Lignans that have a hydroxyl group at C-7 of ring C and an ester function at rings A or B, such as angeloyl gomisin H , have been reported to fragment by elimination of H2O and an olefinic ketene (C5H6O or C7H4O), the latter being associated with the presence of the ester at rings A or B. Elimination of the phenol ester as an olefinic ketene from lignans using electron ionization (EI) has been previously reported by Zhai & Cong (Zhai & Cong, 1990). In addition, the authors described that the formation of the sodiated molecule ([M+Na]+) is a more favored ionization process, as compared to protonation, for lignans that have a hydroxyl group at C-7 and an ester bound at C-6 of ring C, such as schisantherin A. In the case of compound schisantherin an elimination of the corresponding carboxylic acid (e.g., benzoic acid, C7H6O2) is the major fragmentation process (Zheng et al., 2009). [21–30]

7.5. Flavonoids

Flavonoids are an important class of secondary metabolites that are biosynthesized by plants. They are related to protection against predators and UV-Vis light, attraction of www.intechopen.com Electrospray Ionization Tandem Mass Spectrometry as a Tool for the Structural Elucidation and Dereplication of Natural Products: An Overview 605 pollinators, and antioxidant and hormonal control, among other functions (Dewick, 2004). They also have economic importance because they can be used as pigments, tanning substances, nutritional complements, and food flavors. Moreover, they display pharmacological properties, such as anticarcinogenic, anti-inflammatory, allergen, antiviral, and anti-ulcerogenic actions, among others (Simões et al., 2004). The fragmentation of aglicone and glycoside flavonoids by MS/MS has been extensively investigated. The major fragment ions result from different retrocyclization cleavages (e.g., retro-Diels-Alder reactions, RDA).

Main retrocyclization cleavages of some classes of flavonoids. Wolfender and co-workers have proposed an LC-MS/MS methodology for the online characterization and dereplication of selected commercial and isolated flavonoids (Wolfender et al., 2000). ESI-MS/MS experiments were carried out in hybrid quadrupole time-of-flight (Q-TOF) and ion trap (IT) mass spectrometers. In the IT mass analyzer, the precursor ions are trapped in a short space for varied periods of time, which allows for an increased number of collisions to take place between the collision gas and the precursor ions, thus raising the internal energy of these ions. On the other hand, in the q-TOF mass analyzer the interactions between the precursor ion and the collision gas occur during short periods of time, so that a spatial separation between the ionization and the collision induced dissociation (CID) process comes into effect, thereby diminishing the collision energy. Due to these differences, the MS/MS spectra obtained using IT and q-TOF mass analyzers are different from each other. In order to improve the efficiency of the transmission energy, the precursor ions are accelerated in a linear beam instrument, promoting formation of different fragment ions in both cases. The authors reported that the higher energy in Q-TOF causes hard fragmentation of flavonoids, so that some fragment ions may not be observed using IT. Tandem Mass Spectrometry – Applications and Principles both techniques for dereplication.

Considering the flavone apigenin described in that work, the authors firstly approached the generation of ring cleavages in the positive ion mode. In the positive IT-MS mode at 35% energy, only the ion with m/z 153 was detected, while the same fragments produced during Q-TOF were achieved when the energy was amplified up to 50%(Wolfender et al., 2000).

Rak and co-workers have put forward a strategy for the dereplication of flavonoids in a sample of commercial black currant juice without any preliminary study about its components. This strategy was based on the fact that most of the flavonoid derivatives have an aglycone part. Firstly, the authors developed a procedure for detection of aglycone flavonoids in the juice chemical constituents using multiple reaction monitoring (MRM) in the negative ion mode. In this type of scan, both the precursor and the product ion are specified for the detection of only one pair at the detector (Sleno & Volmer, 2004). Considering the great number of possible aglycone derivatives, the authors chose apigenin, luteolin, quercetin, myricetin, and naringenin, which are amongst the commonest aglycone flavonoids. In addition, the authors performed the experiment using a high declustering potential (DP) with some standard solutions of aglycone analogues, aiming to minimize formation of cluster ions from solvents. The authors showed that the major compounds in the black currant juice can be identified by selecting the characteristic m/z values, in combination with their retention times. They also conducted full scan MS experiments, which evidenced that odd-electron fragment ions resulting from homolytic cleavages were more abundant than even-electron ion fragment ions when the highest negative DP values were employed. The tendency toward radical fragmentation reactions was confirmed by MRM experiments, which were accomplished in parallel with the chromatographic run (Rak et al., 2010). A third chromatographic run was carried out on a quadrupole linear ion trap apparatus, in order to confirm the structure of the precursor ions on the basis of diagnostic fragment ions. Twelve flavonoid derivatives were identified in the sample of black currant juice, demonstrating the great versatility of these MS techniques for the dereplication of flavonoids (Rak et al., 2010).

MS/MS data of protonated and deprotonated apigenin . Electrospray Ionization Tandem Mass Spectrometry as a Tool for the Structural Elucidation and Dereplication of Natural Products

For the online identification of aglycon flavonoids present in the hydroalcoholic extract of L. ericoides leaves, Gobbo & Lopes have compared the retention time, the UV spectra and the accurate mass of each peak of the chromatogram with authentic standards, besides comparing the MS data with those previously published in the literature (Gobbo-Neto & Lopes, 2008). A peak exhibiting absorbance maximum above 340 nm, which is characteristic of flavanones previously identified in L. ericoides (e.g, pinocembrin and pinostrombin) was identified in the MS/MS spectrum of the deprotonated molecule, as well as by comparison with literature results (Cuyckens & Claeys, 2004; Fabre et al., 2001; Ma et al., 1997; Zhang & Brodbelt, 2003).

MS/MS spectrum of the corresponding protonated molecule displayed the base peak at m/z 153, which indicates a dihydroxyl substituent at ring A of pinocembrin. On the other hand, the presence of a fragment ion at m/z 167, together with the absence of diagnostic ion at m/z 153, indicates a hydroxyl and a methoxyl substituent at ring a, indicative of the presence of the flavanone pinostrombin (GobboNeto & Lopes, 2008).

Pinobanksin was identified on the basis of its UV spectra, comparison with retention time of previously isolated standards, and accurate mass data. 3- O-acetylpinobaskin was elucidated by comparison with pinobanksin ([M+H]+ at m/z 313), being the mass difference due to the methyl group atom bound at the phenol oxygen. Clearly, the substance was assigned as 3-O-acetylpinobaskin not only because of the difference of 14 mass units at PI, but also because of the appearance of the same product ions in 3-O-acetylpinobaskin, which differs by 14 mass units from the pinobaskin product ions (e.g., the diagnostic product ion of pinobaskin, m/z 153, presented a difference of 14 mass units from 3-O-acetylpinobaskin, m/z 167, thus confirming the presence of an acetyl group instead of a hydroxyl group in ring A).

Waridel and co-workers have proposed an LC-MS/MS methodology for the differentiation between C-6 and C-8 glycoside flavonoids (Waridel et al., 2001). Firstly, the authors analyzed standards of selected flavonoids in a reverse-phase C18 column using isocratic elution with acetonitrile/water 4:1 containing 0.5% acetic acid. Low energy CID experiments were performed on ion-trap (IT) and hybrid quadrupole time-of-flight (Q-TOF) instruments. ESI and APCI were used in both the positive and negative ion modes of analysis. The optimal CID collision energy was chosen so that the same MS/MS spectral profile would be generated and the relative intensities and m/z values of the fragment ions could be compared as a result of the structural differences. The authors postulated the distinction between C-8 and C-6 glycoside flavonoids on the basis of the relative intensity of some peaks of the MS/MS spectra.

Taking into account the stabilities of the fragment ions, which were considered to be associated with their respective relative intensities, the authors reported that loss of H_2O and formation of m/z 379 are processes that are more favored for C-6 isomers (e.g., isovitexin and isoorientin,) than for C-8 isomers (e.g., vitexin, and orientin). Another proposition to distinguish between C-6 and C-8 glicoside Tandem Mass Spectrometry – Applications and Principles flavonoid isomers was based on the intensity of the product ion [M+H–120]+, which had low intensity when the skimmer voltage (to produce in source dissociation) was not employed, but was the base peak when the skimmer voltage was used. The authors selected the ion [M+H–120]+ as the precursor ion in Q-TOF and used the multiple-stage mass spectrometry (MSn) in IT. The results revealed a large

difference between the isomers for the collision energy at 30 eV or 50%, which enabled distinction by hard fragmentation and some elimination reactions, specific for each isomer.

In the case of Q-TOF, the product ion m/z 283, which results from CH2O loss from [M+H-120]+, was the base peak in the MS/MS spectra of both C-6 and C-8 isomers? On the other hand, when IT was employed, the product ion at m/z 283 was the base peak for C-8 isomers, whereas the product ion at m/z 295 [M+H120-H2O]+ was the base peak in the MS/MS spectrum of C-6 isomers. The authors also reported that differentiation between C-6 and C-8 glycoside flavonoid isomers in the negative ion mode was not possible when the MS/MS experiments were performed in the ion trap equipment, once CO elimination from [M-H-120]- is the only fragmentation process for both isomers.(Waridel et al., 2001). Nevertheless, the main difficulty reported by the authors was method standardization, because screening and online identification of these compounds requires previous optimization of the parameters of both IT and q-TOF apparatus.

One of the most important steps in dereplication studies of natural products by LC-MS is the chromatographic separation process. Resolution (e.g, the distance between two adjacent peaks in the chromatogram) is essential for the correct interpretation of the subsequent UVDAD, MS and MS/MS data. The addition of formic or acetic acid to the LC mobile phase (usually methanol/water or acetonitrile/water) has been the most used analytical strategy to improve the chromatographic resolution in HPLC. Shi and co-workers have identified glycoside flavonoid isomers in crude extracts of Fructose aurantii and Fructose aurantii immaturus using HPLC-UV-MSn (Shi et al., 2007). The chromatographic separation between the compounds 48-50 on reverse phase C18 column was achieved by using a mixture of acetonitrile and water/formic acid (0.1%) and applying a linear gradient from 10% and 95% of water/formic acid (0.1%). The differentiation between the compounds was made on the basis of the comparison of retention times, maximum UV wavelengths, and MS and MS/MS data of each individual peak with those of authentic standards. MS/MS spectrum of deprotonated(m/z 593) displayed the fragment ion [M-H-308]- , suggesting elimination of www.intechopen.com Electrospray Ionization Tandem Mass Spectrometry as a Tool for the Structural Elucidation and Dereplication of Natural Products: An Overview 609 Main ions obtained by CID-MS/MS of the [M+H]+ of compounds.

Fragmentation map proposed for the differentiation between C-6 and C-8 glicoside flavonoid isomers (Waridel et al., 2001). A rutinose molecule, whereas its MS^3 spectrum showed the same profile as that of the aglycone of poncirin.

Moreover, the retention time of didymin (tR=46,1 min) in the total ion chromatogram was higher than hespiridin (tR=32,9 min), thus indicating a structure with fewer oxygen atoms as compared to hespiridin.

Alkaloids comprise a vast group of secondary metabolites found mainly in higher plants, such as angiosperms (Cordell et al., 2001). Although there is no general definition that www.intechopen.com 610 Tandem Mass Spectrometry – Applications and Principles encompasses the great structural diversity of this class of NPs, it is known that these compounds exhibit alkaline properties due to the nitrogen atoms found in their cyclic skeleton. Another chemical property of alkaloids is their solubility in aqueous solutions when salt complexes are formed in the presence of mineral acids (Cordell et al., 2001; Dewick, 2004).

Zhou and co-workers have proposed a methodology involving ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) for the screening of some pyrrolizidine alkaloids exhibiting toxic effects against human beings, such as fetal problems and hepatotoxic and tumorigenic effects (Zhou et al., 2010).

The pyrrolizidine alkaloids were extracted directly from the powdered plant using diluted hydrochloric acid, followed by mixed-phase cation exchange (MCX) and solid-phase extraction (SPE) and elution with methanol/ammonia 3:1 (v/v). After solvent removal, the residue was analyzed by UPLC coupled with DAD and triple quadrupole tandem mass spectrometry, equipped with an electrospray ion source operating in the positive ion mode. The authors demonstrated that the fragment ions with m/z 150 and m/z 168 are diagnostic for pyrrolizidine alkaloids belonging to the Otonecine type group, whereas the fragment ions m/z 120 and m/z 138 are diagnostic for compounds of the Retronecine types,

Some pyrrolizidine alkaloids analogs (e.g., compounds senkirkine; and clivorine) that have fragment ions in common could not be distinguished from each other on the basis of their precursor ion spectra only. In this case, dereplication was achieved by combining data from the spectra of the precursor ion with those of multiple reaction monitoring (MRM) experiments.

Pivatto and co-workers have utilized accurate mass electrospray ionization tandem mass spectrometry for the offline dereplication of selected piperidine alkaloids from flowers and fruit extracts of Senna spectabilis, which is commonly used in traditional medicine as antiinflammatory, analgesic, laxative, antimicrobial, and antiulceral agent in some countries (Samy & Ignacimuthu, 2000; Viegas Jr. et al., 2004).

The authors obtained the ethanol extract from powdered dried flowers and green fruits, followed by redissolution with aqueous www.intechopen.com Electrospray Ionization Tandem Mass Spectrometry as a Tool for the Structural Elucidation and Dereplication of Natural Products: An Overview 611H_2SO_4 and washing with n-hexane. The acid residue was basified with NH4OH (pH 9) and then extracted

with dichloromethane. The analysis showed that some protonated compounds have common mass losses (e.g., loss of H2O) and known fragment patterns (e.g., the charge-induced fragmentation that forms the acylium ion in p-coumaroyl derivatives), the authors reported that the extracts obtained from flowers contained more oxygen atoms than those obtained from fruits. On the other hand, the formation of some ions as evidenced in the spectrum of the precursor ion could not be explained on the basis of their ESI-MS/MS data, but they can also be of great importance when this technique is used in combination with NMR (Pivatto et al., 2005).

Fragmentation of piperidine alkaloids identified in Senna spectabilis by ESI-MS/MS. The dereplication of alkaloids has also been achieved by means of UV-Vis spectroscopy in combination with tandem mass spectrometry (MS/MS). Fabre and co-workers have studied a direct and fast characterization of isoquinoline alkaloids from the aerial parts of Eschscholtzia californica, which is used in European folk medicine as analgesic, anodyne, diaphoretic, diuretic, soporific, and spasmolytic agent (Fabre et al., 2000). Firstly, the authors analyzed authentic standards of the isoquinoline alkaloids berberine and papaverine by HPLC and ESI-MS/MS using direct infusion and collision-induced dissociation, aiming to identify possible diagnostic ions for each compound. After that, the powdered aerial parts of E. californica were extracted with MeOH for 15 min at 60°C. The residue obtained after solvent elimination was dissolved in aqueous HCl (pH 1), then dodecylsulfate sodium salt was added and further extracted with chloroform (three times). The organic phase was concentrated and dried, then suspended in MeOH and analyzed by high www.intechopen.com 612 Tandem Mass Spectrometry – Applications and Principles performance liquid chromatography coupled with a diode array detector (HPLC-DAD) and HPLC-ESI-MS/MS.

8. Conclusion

The mass spectrometer portion of the GC-MS is a complex device and must be treated with a great deal of care. Instrument performance is important and must be verified regularly. The quality of the data must be verified, and regular maintenance of the instrument is important. The GC-MS is not a black box to be used when needed. It must be thoroughly understood and watched over. The use of electrospray ionization tandem mass spectrometry (ESI-MS/MS) for the online and offline dereplication of natural products (NPs) has been discussed herein. Online identification using ESI-MS/MS coupled with liquid chromatography with UV-DAD as first detection has shown to be a more powerful technique as compared to the sole use of ESIMS/MS. Structural determination is usually achieved on the basis of a combination between retention times, UV and MS/MS spectra and comparison with data of a previously established library of authentic standards. However, this strategy is still limited because previously isolated (or commercially acquired) standards are necessary, not to mention the difficulty in establishing reliable libraries using MS/MS data acquired on different equipments. These are the main challenges to be overcome in coming years in this research field.

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None.

10. Conflict of Interest

None.

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