



(51) International Patent Classification:

H01J 49/00 (2006.01) *B01D 59/44* (2006.01)
B01D 15/08 (2006.01) *G01N 31/00* (2006.01)

(21) International Application Number:

PCT/US2016/034293

(22) International Filing Date:

26 May 2016 (26.05.2016)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/168,015 29 May 2015 (29.05.2015) US

(71) Applicant: **WATERS TECHNOLOGIES CORPORATION** [US/US]; 34 Maple Street, Milford, MA 01757 (US).(72) Inventors: **ASTARITA, Giuseppe**; 60 Hayden Rowe, Hopkinton, MA 01748 (US). **GEROMANOS, Scott**; 2 Crest Road, Middletown, NJ 07748 (US).(74) Agents: **VERNON, Deborah, M.** et al.; Mccarter & English, LLP, 265 Franklin Street, Boston, MA 02110 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM,

DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

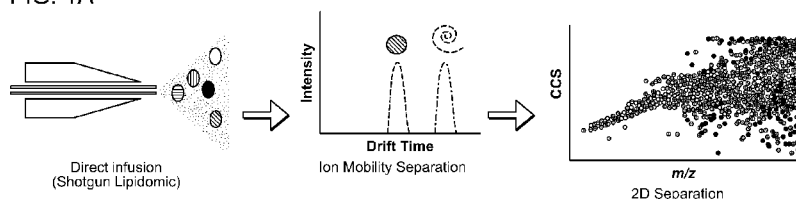
- as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))
- as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))

Published:

- with international search report (Art. 21(3))

(54) Title: MASS SPECTROMETRY WITH QUADRUPOLE AND ION MOBILITY SEPARATION CAPABILITIES

FIG. 1A



(57) Abstract: The present disclosure relates generally to metabolomics and lipidomics, and to the analysis of metabolite and lipids in simple and complex mixtures. More specifically, the present disclosure relates to a mass spectrometry system using data independent acquisition and having quadrupole and ion mobility separation capabilities for the identification and quantification of metabolites and lipids. In some embodiments, an optional chromatographic separation can also be included.

MASS SPECTROMETRY WITH QUADRUPOLE AND ION MOBILITY SEPARATION CAPABILITIES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of and priority to U.S. Provisional Patent Application No. 62/168,015, filed on May 29, 2015, the entire contents of which is incorporated herein by reference.

TECHNICAL FIELD

[0002] The present disclosure relates generally to metabolomics and lipidomics, and to the analysis of metabolite and lipids in simple and complex mixtures. More specifically, the present disclosure relates to a mass spectrometry system using data independent acquisition and having quadrupole and ion mobility separation capabilities for the identification and quantification of metabolites and lipids. In some embodiments, an optional chromatographic separation can also be included.

BACKGROUND OF THE INVENTION

[0003] Metabolomics is the study of chemical processes involving metabolites. It is the study of the unique chemical fingerprints that specific cellular processes leave behind. The metabolome represents the collection of all metabolites in a biological cell, tissue, organ or organism, which are the end products of cellular processes. Metabolomics also involves the comprehensive characterization of these metabolites in biological systems. It can provide an overview of the metabolic status and global biochemical events associated with a cellular or biological system.

[0004] Lipidomics is the study of pathways and networks involving cellular lipids in biological systems. The lipidome represents the complete lipid profile within a cell, tissue or organism and is a subset of the metabolome. Lipidomics involves the comprehensive characterization of the thousands of cellular lipid molecular species and their interactions with other lipids, proteins, and other metabolites. Lipidomics examines the structures, functions, interactions, and dynamics of cellular lipids and the changes that occur during perturbation of the system.

[0005] Identification and quantification of metabolites and lipids in biological samples is a fundamental problem in both disciplines. In the past, analyses were performed using a direct infusion “shotgun” method into a mass spectrometer. These methods suffered from, at least, ion suppression effects and the lack of additional separation techniques. Analyses were also performed using on-line, two dimensional liquid chromatography mass spectrometry (LC/MS). In LC/MS, complex samples can be separated into classes (using normal phase chromatography) or species (using a combination of normal phase chromatography coupled with reverse phase chromatography) prior to introduction into the mass spectrometer. The quadrupole mass selection can be varied as function of time which can be coupled with unique data-dependent and data-independent acquisition modes, including MS^E, HDMS^E, HDDDA or HDMLDA to the align precursor and product ions.

[0006] These methods, however, suffer from a lack of specificity of fragmentation, complex data interpretation and limited identification capabilities.

SUMMARY OF THE INVENTION

[0007] The present disclosure relates to the analysis of metabolites and lipids in simple and complex mixtures using a quadrupole selector and ion mobility separation in combination with mass spectrometry using data independent acquisition. An optional chromatographic separation can also be included. In some embodiments, the present disclosure relates to a method for the direct analysis of the metabolome and lipidome using a stepwise quadrupole selection coupled with ion mobility-MS^E in mass spectrometry instruments having quadrupole and ion mobility separation capabilities. In other embodiments, the present disclosure relates to a method for the direct analysis of the metabolome and lipidome using normal phase-like chromatographic separation of lipid classes followed by a stepwise quadrupole selection coupled with ion mobility-MS^E in mass spectrometry instruments having quadrupole and ion mobility separation capabilities.

[0008] For example, a complex biological sample can be infused into the system via a syringe, a flow injection analysis using an autosampler, or similar. A first quadrupole can select packages of ions in a stepwise fashion, or otherwise, and transfer these selected ions for ion mobility separation, or vice versa, before MS^E acquisition. The system and method of the present disclosure can systematically, e.g., sequentially, isolate precursor ions in unit or higher mass resolution windows using a quadrupole. The quadrupole can isolate ions in a stepwise manner from a low to a high m/z ratio. The selected or isolated ions can be further

separated by ion mobility. The first quadrupole and ion mobility separation can be performed in any order before transmitting the ions into a collision cell and acquiring accurate measurements. Accurate mass measurement can be obtained at low voltages (e.g., precursor spectra) and high voltages (e.g., full product ion spectra) for each mass window at each step of the quadrupole.

[0009] Automated post-acquisition interpretation of the full product ion spectra can effectively simulate an unlimited number of precursor and neutral-loss scans in a single analysis. As such, little to no prior knowledge of the metabolite or lipid ions of interest is required for the present disclosure to generate rapid metabolomics and lipidomics analyses.

[0010] In one embodiment, the present disclosure relates to a system including a quadrupole or quadrupole selector (Quad), an ion mobility spectrometry (IMS) and a mass spectrometer (MS), wherein the components are coupled together as Quad-IMS-MS or IMS-Quad-MS.

[0011] In another embodiment, the present disclosure relates to a system further including a normal phase chromatography separation system. The components of the composition can be coupled together as Chrom-Quad-IMS-MS or Chrom-IMS-Quad-MS. The normal phase chromatography system can include a supercritical fluid chromatography (SFC), carbon dioxide-based chromatography (i.e., CO₂ is included within the mobile phase), or a hydrophilic interaction chromatography (HILIC).

[0012] The quadrupole can be scanned, or otherwise used, to select packets of ions in small m/z intervals of less than about ± 2.5 Daltons. The quadrupole is capable of selecting these intervals across absolute m/z values from about 50 Daltons to about 1600 Daltons. The quadrupole can also select these intervals across smaller sets of m/z values that have a width of less than about 200 daltons, e.g., from about 600 daltons to about 800 daltons.

[0013] The mass spectrometer can acquire data in an independent mode. The mass spectrometer can include a collision cell operable in a first mode wherein at least a portion of said ions are fragmented to produce daughter ions, and a second mode wherein substantially less ions are fragmented, a mass analyzer, and a control system which, in use, repeatedly switches the collision cell back and forth between the first and the second modes. In the first mode, the control system can arrange to supply a voltage to the collision cell selected from the group consisting of ≥ 15 V. In the second mode, the control system can arrange to supply a voltage to the collision cell selected from the group consisting of ≤ 5 V. The control system

can automatically switch the collision cell between the at least two modes at least once every 0.5 seconds.

[0014] In another embodiment, the present disclosure relates to a method of determining analytes of a complex sample including isolating or selecting the analytes using a quadrupole, separating the analytes using an ion mobility spectrometer, and analyzing the analytes using a mass spectrometer. The method step of isolating or selecting using a quadrupole can occur before or after the method step of separating the analytes using an ion mobility spectrometer.

[0015] In another embodiment, the present disclosure relates to a method of determining analytes of a complex sample including initially separating the analytes using a normal phase chromatography system, isolating or selecting the analytes using a quadrupole, separating the analytes using an ion mobility spectrometer, and analyzing the analytes using a mass spectrometer. Again, the method step of isolating or selecting using a quadrupole can occur before or after the method step of separating the analytes using an ion mobility spectrometer.

[0016] The method can also feature passing ions to a fragmentation means including a collision cell, operating the fragmentation means in a first mode wherein at least a portion of the ions are fragmented to produce daughter ions, recording a mass spectrum of ions emerging from the fragmentation means operating in the first mode as a high fragmentation mass spectrum, switching the fragmentation means to operate in a second mode wherein substantially less ions are fragmented and recording a mass spectrum of ions emerging from the fragmentation means operating in the second mode as a low fragmentation mass spectrum. These steps can be repeated a plurality of times.

[0017] The present disclosure has many advantages over the prior art, including better specificity of fragmentation since precursor ions are better isolated, simplifying data interpretation resulting from more specific fragments derived from better isolated precursors, and enhanced confidence for identification since there is less manual interpretation, more specific fragments and less false positive identifications. In general, the use of the additional chromatographic step allows the quadrupole to target an eluting family or class of compounds and reduce the duty cycle and scanning of the quadrupole.

BRIEF DESCRIPTION OF THE DRAWINGS

[0018] The foregoing and other features and advantages provided by the present disclosure will be more fully understood from the following description of exemplary embodiments when read together with the accompanying drawings, in which:

[0019] Figure 1 shows a system using direct infusion (e.g., shotgun lipidomic) into a mass spectrometer having ion mobility separation capabilities (1A) and a system using a chromatographic separation into a mass spectrometer having ion mobility separation capabilities (1B).

[0020] Figure 2 shows the chemical complexity of a biological sample. A sample analyzed using the system shown in Figure 1A generates multiple overlapping analytes having the same collision cross-section, e.g., drift time, and m/z values. Similar overlapping analytes are observed for biological samples analyzed using the system shown in Figure 1B.

[0021] Figure 3 shows an exemplary embodiment of the present disclosure. Figure 3A shows a system using direct infusion of co-eluting lipid classes into a IMS - mass spectrometer capable of data independent acquisition, e.g., HDMS^E. The fragments transferred to the mass spectrometer include overlapping analytes and fragmentation patterns making identification difficult. The high energy fragmentation has over 100 fragments. Figure 3B shows a system of the present disclosure using direct infusion of co-eluting lipid classes into a mass spectrometer capable of data independent acquisition and having quadrupole and ion mobility separation capabilities. The stepwise quadrupole filtering, e.g., 0.5-5 Daltons from m/z 50 to 1500, acts as an additional separation step, similar to a chromatographic step. The ion mobility separation further disperses the analytes by collision cross section (or drift time). The fragments transferred to the mass spectrometer are not overlapping, or significantly less overlapping, less complex and allows for simple identification.

[0022] Figure 4 shows an exemplary embodiment of the present disclosure. The addition of an additional chromatographic separation, such as a normal phase chromatographic system, e.g., HILIC or SFC, can add yet another separation dimension to the system. For example, the application of normal phase chromatography to a complex lipid sample can separate the lipids into classes. As shown in Figure 4, the Ceramide species (Cer) elute early, whereas the PC species elute later.

[0023] Figure 5 shows an exemplary table of lipid classes separated by normal phase chromatography having defined retention time windows. As shown in Figure 5, the FA species (or class) elutes between 0-1 minute and have defined molecular weight range between 200-400 m/z. Similarly, the Cer species (or class) elutes between 0-2 minutes and have a different defined molecular weight range between 500-800 m/z. One of the advantages of separating the biological sample by class wherein the classes have defined molecular weight ranges is the stepwise scanning of the quadrupole can be targeted or focused to the m/z region of interest for the corresponding elution time. By reducing the scanning range of quadrupole, the duty cycle is reduced, leading to an increase in sensitivity and more reliable quantification (e.g., more data points acquired per unit of time).

[0024] Figure 6 shows an exemplary embodiment of the present disclosure. Figure 6A shows a system using chromatographic introduction of co-eluting lipids into a IMS-mass spectrometer capable of data independent acquisition, e.g., HDMS^E. The fragments transferred to the mass spectrometer include overlapping analytes and fragmentation patterns making identification difficult. The high energy fragmentation have over 100 fragments. Figure 6B shows a system of the present disclosure using chromatographic introduction of co-eluting lipids into a mass spectrometer capable of data independent acquisition and having quadrupole and ion mobility separation capabilities. The stepwise quadrupole filtering, e.g., 0.5-5 Daltons over selected m/z windows at specific retention times, acts as an additional separation step. The ion mobility separation further disperses the analytes by collision cross section (or drift time). The fragments transferred to the mass spectrometer are not overlapping, or significantly less overlapping, less complex and allows for simple identification.

[0025] Figure 7 shows an exemplary configuration of the components in the present disclosure including an IM separation device, a quadrupole and segmented collision cell prior to the TOFMS.

[0026] Figure 8 shows an exemplary configuration of the components in the present disclosure, including an IM separation device, a quadrupole and segmented collision cell prior to the TOFMS. Ions can be accumulated in the trap travelling-wave (T-Wave) and periodically released into the T-Wave IM where they separate according to their mobility. The IMS includes a travelling wave RF ion guide, which incorporates a repeating sequence of transient DC pulses to propel ions through the guide in the presence of N₂ bath gas. Upon

exiting the IMS cell, ions can be selected with the quadrupole and undergo CID for structural elucidation prior to detection with the TOFMS.

[0027] Figure 9 shows three different exemplary configurations of the components in the present disclosure, including an ion source, IM separation device, a quadrupole and segmented collision cells prior to the IMS and the TOFMS.

DETAILED DESCRIPTION

[0028] The present disclosure relates to metabolomics and lipidomics, and to the screening of the metabolites and lipids present in biological samples.

[0029] In one embodiment, the present disclosure relates to a composition including a quadrupole or quadrupole selector (Quad), an ion mobility spectrometer (IMS) and a mass spectrometer (MS), wherein the components are coupled together as Quad-IMS-MS or IMS-Quad-MS.

[0030] **Quadrupole**

[0031] The quadrupole selector can be any quadrupole instrument capable of isolating or selecting at least one fraction of ions for further analysis. The fraction of isolated or selected ions can have the substantially the same m/z values. The fraction of isolated or selected ions can also have m/z values within a defined interval, such as all ions can be within about 5 Daltons window of each other, i.e., the difference between the highest m/z and the lowest m/z of the ions in the fraction is about 5 Daltons. In some embodiments, the fraction can be centered around a specific m/z value. The same fraction having an about 5 Dalton difference between the highest and lowest values can also be described relative to the specific m/z value, i.e., ± 2.5 Daltons. For example, a fraction of ions centered around 500 Daltons and having a 5 Dalton window, i.e., 497.5 to 502.5 m/z , can also be described as having a ± 2.5 Dalton window.

[0032] The fraction of isolated or selected ions can have m/z values within about 5 Daltons, 4.5, 4, 3.5, 3, 2.5, 2, 1.5, 1, 0.5, 0.25 or about 0.1 Daltons of each other. These values can also define a range, such as between about 3 and about 0.5 Daltons. Similarly, the fraction of isolated or selected ions can have m/z values within about ± 2.5 Daltons, 2.25, 2, 1.75, 1.5, 1.25, 1, 0.75, 0.5, 0.25, 0.125, 0.1 and about ± 0.05 Daltons. These values can also define a range, such as between about ± 2 Daltons and about ± 0.05 Daltons.

[0033] The quadrupole can also be capable of isolating or selecting multiple fractions of ions for further analysis. The fractions of ions can be isolated or selected in any fashion or manner. They can be isolated by discrete selection of one or more specific m/z value of interest. Then can also be isolated by scanning the quadrupole over a range of m/z values. The range of absolute m/z values that can be scanned can vary from about 50 to about 1600 m/z values. The range of absolute m/z values can be narrower, such as for example, to analyze a known class of lipids having a defined range of m/z range. The narrower range can be any subset range within the about 50 to about 1600 m/z values, in 1 m/z intervals, such as from about 200 m/z to about 390 m/z , or from about 400 m/z to about 602 m/z , or from about 600 m/z to about 950 m/z . For example, a known class of lipids have a range of m/z values from about 450 to about 575. The range of absolute m/z values scanned can vary from about 450 m/z to about 575 m/z . These values can vary up to about 1, 5, or 10% depending on the confidence of the known range of values for the lipids.

[0034] The scanning of the quadrupole over the absolute range of m/z can be performed in a stepwise fashion in defined steps. The magnitude of each step can be less than about 10 Daltons. The magnitude of each step can be less than about 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0.09, 0.08, 0.07, 0.06, 0.05, 0.04, 0.03, 0.02 or about 0.01 Daltons. These values can also define a range, such as from about 2 to about 0.02 Daltons. In some embodiments, one or more of the multiple fractions of ions can overlap. For example, a quadrupole can scan over a defined range in about 2 Dalton steps and selecting m/z values within about a 2 Dalton window (± 1 Dalton). A first selection at 500 m/z would select ions having 498 m/z – 502 m/z . A second selection at 502 m/z would select ions having 500 m/z – 504 m/z . In other embodiments, the one or more of the multiple fractions of ions do not overlap.

[0035] In one embodiment, the quadrupole isolates and selects ions by scanning in a stepwise manner at a m/z interval of less than ± 2.5 Daltons. In other embodiments, the quadrupole isolates and selects ions by scanning from about 50 Daltons to about 1600 Daltons. In still other embodiments, the quadrupole isolates and selects ions by scanning in over a narrower mass range having a width of less than about 500, 450, 400, 350, 325, 300, 275, 250, 225, 200, 175, 150, 125, 100, 75 or about a 50 Daltons. These values can also be used to define a range, such as about 200 to about 100 Daltons.

[0036] The scanning of the quadrupole over a defined absolute range can be vary throughout a scan in both the window of ion selected or isolated, e.g., +/- 2 Daltons, and in the magnitude of each step.

[0037] The quadrupole can be capable of isolating or selection analytes or ions introduced from an infusion source or chromatography system, or after ion mobility separation. The analytes of interest can be referred to as analytes, ions or precursors in the present disclosure. Examples of the resolving quadrupoles include 4KDa, 8KDa or 32KDa mass range options for MS/MS of small to macromolecular species.

[0038] **Ion Mobility Mass Spectrometer**

[0039] The ion mobility spectrometer can be any IMS instrument capable of separating ions. IMS involves the interaction, or motion, of gas-phase ions in the presence of gas, e.g., nitrogen, within a pressurized chamber, e.g., between 1 and 760 Torr. IMS can analyze ionized analytes by their mobility, which is the function of ion charge, mass and shape. The IMS can include an ion source for ionization of introduced analytes, an ion gate to form short ion packets, a gas filled drift tube for ion separation in electrostatic fields, and a collector to measure time dependent signal. IMS can be coupled to the other components of the present disclosure to add an additional dimension of analytical separation.

[0040] Ion mobility spectrometry separates gas phase ion on the basis of ion mobility (K) that determines ion drift velocity (v) under the influence of an electric field (E) via $v=KE$ (equation 1). The raw mobility is often converted to standard temperature and pressure conditions (e.g., pressure in Torr and temperature in Kelvin) by defining the reduced mobility (K_0) via $K_0=K(P/760) \times (273/T)$ (equation 2) where P is the buffer gas pressure. The mobility is related to orientationally averaged collision cross-section Ω_{avg} of the ion and gas molecule via the Mason-Schamp equation (K_0) via $K_0=(3q/16N) \times (2\pi/\mu kT)^{1/2}/\Omega_{avg}$ (equation 3) wherein q is the ion charge, N is the gas number density, μ is the reduced mass of the analyte ion/gas molecule pair, k is the Boltzman constant and T is the gas temperature. A theoretical collision cross-section, Ω , can be calculate using models, such as the MOBCAL Trajectory Method.

[0041] The IMS can separate analytes or ions introduced based on differences in drift time or collision cross section values. The IMS can operate with a pressurized chamber having an pressure between about 1 and about 760 Torr. In some embodiments, the speed of the separation is increased by lowering the pressure. The IMS can be operated at a pressure

less than about 700 Torr, 650, 600, 550, 500, 450, 400, 350, 300, 250, 200, 150, 100, or about 50 Torr. These values can also be used to define a range, such as about 400 to about 50 Torr.

[0042] The IMS can operate with at a variety of temperatures. In some embodiments, the temperature can affect the speed and quality of the separation. Cold temperature can reduce the degrees of freedom of flexible molecules such as lipids containing acyl groups, which can facilitate the separation of isomeric species. The IMS can be operated at a source temperature of greater than about 50, 60, 70, 80, 90, 100, 100, 120, 130, 140 or about 150 degree C. These values can also be used to define a range, such as about 100 to about 120 degree C. The IMS can be operated at a desolvation temperature of greater than about 100, 150, 200, 250, 300, 350, 400, 450, 500, 550 and 600 degree C. These values can also be used to define a range, such as about 200 to about 520 degree C.

[0043] The IMS can operate at a variety of voltages. The IMS can be operated at a capillary voltage of greater than about 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.5, 4, 4.5 or 5 kV. These values can also be used to define a range, such as about 2.5 and about 3.0 kV. The IMS can be operated at a cone voltage of greater than about 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 or 60 V. These values can also be used to define a range, such as about 30 to about 50 V.

[0044] The Table below lists additional and representative parameters of an LC-ESI-IMS system for the acquisition of lipids.

Table. Summary of MS Settings used to measure CCS.

ES ⁺	Capillary Voltage (kV)	Cone Voltage (V)	Source Temperature (°C)	Desolvation Temperature (°C)	Desolvation Gas Flow (L/hr)	IMS gas (mL/min)	Wave Velocity (m/s)	Wave Height (V)	EDC Delay Coefficient (V)
MS 1	2.5	40	120	500	600	90	600	40	1.58
MS 2	2.5	30	100	250	200	90	650	37	1.58
MS 3	2.5	30	110	200	400	90	650	40	1.44

ES ⁻	Capillary Voltage (kV)	Cone Voltage (V)	Source Temperature (°C)	Desolvation Temperature (°C)	Desolvation Gas Flow (L/hr)	IMS gas (mL/min)	Wave Velocity (m/s)	Wave Height (V)	EDC Delay Coefficient (V)
MS 1	2.5	30	120	500	1000	90	900	40	1.58
MS 2	2.9	45	100	250	200	90	790	40	1.58
MS 3	3.0	40	110	200	400	80	600	40	1.41

[0045] The IMS can add selectivity to the separation and detection of structural isomers, isobars, and conformers. Derivatization methods and alternative ion-mobility gases (e.g., CO₂) can be used to increase or maximize the separation of isobaric and isomeric lipid species by IMS. Derivatization can increase the CCS of the isomers and affect the interactions of lipid ions with drift gas and improve separation in the ion-mobility cell. In addition, the use of volatile modifiers in the mobility cell (e.g., small percent of isopropanol), the use of IMS gases having different polarizabilities, the use of changes in the pressure of these gases, or combinations thereof, can also be used to affect the separation of lipid isomers.

[0046] The IMS can be capable of separating analytes or ions introduced from an infusion source or chromatography system, or after quadrupole isolation or selection. Currently, MS detection employs one of three, major, IMS-separation approaches: (1) drift-time ion mobility spectrometry (DT-IMS); (2) travelling-wave ion mobility spectrometry (TW-IMS); and (3) field-asymmetric ion mobility spectrometry (FA-IMS), also known as differential-mobility spectrometry (DMS). In DT-IMS, ions migrate through a buffer gas in the presence of an axial, linear, electric-field gradient. In TW-IMS, a sequence of applied voltages generates a “travelling wave” that propels the ions through the buffer gas. Thus both DT-IMS and TW-IMS can allow all or substantially all of the ions to pass through the mobility cell. DMS, however, operates by varying the compensation voltage, filtering selected ions in a space-dispersive fashion.

[0047] In one embodiment, the inlet for such method of acquisition can be an ambient or real-time ionization source including matrix-assisted laser desorption/ionization, desorption electrospray ionization, rapid evaporative ionization mass spectrometry, direct analysis in real time, laser ablation electrospray ionization can be used instead of flow injection analysis (FIA), direct infusion and chromatography.

[0048] In another embodiment, the IMS can be used as a filter to select or scan across specific drift times or CCS values, potentially followed by further quadrupole isolation and MS^E.

[0049] Mass Spectrometer - Data Independent Acquisition (e.g., MS^E)

[0050] The mass spectrometer can be any MS instrument capable of providing accurate mass determination for both parent and daughter peaks, and capable of data independent acquisition. Data independent acquisition provides a further increase to the

specificity of fragmentation for identification purposes. The present disclosure incorporates by reference U.S. Patent Nos. 6,717,130 and 6,586,727 which fully describe a mass spectrometer having data independent acquisition.

[0051] Conventional techniques use separation / mass spectrometer instrumentation and data dependent selection and fragmentation of precursor ions. For example, a mass spectrometer can select precursors in a first mass spectrometer, can collisionally fragment the selected precursors in a collision cell, and analyze the resulting fragments in a second mass spectrometer. Using data dependent analyses parent-daughter peak groupings are made based solely on precursor ion selection in the first mass spectrometer. Multiple precursors, however, can overlap and have m/z values that lie within the transmission window of the first mass spectrometer. In such a case the fragmentation spectrum obtained in the second mass spectrometer will contain fragments from multiple precursors. As a result, conventional techniques can inadvertently group ions that in fact come from two or more distinct precursors.

[0052] In one embodiment, ion mobility can be coupled with data dependent acquisition (HD-DDA). DDA can select ions for MS/MS acquisition in real time, as components elute from a chromatographic system. Embedded algorithms can rapidly interrogate MS survey spectra and co-eluting precursor ions can be selected for MS/MS analysis based on threshold intensity, charge state, pre-defined exact mass include/exclude lists, or combinations thereof. The collision energy for each spectrum can be optimized according to precursor charge state and m/z .

[0053] In one embodiment, pathway-dependent acquisition (PDA) can be coupled with ion mobility (HD-PDA). This mode of acquisition can be used to adjust the quadrupole selection based on the masses isolated in the previous scan and to adjust the quadrupole selection based on known biochemical pathways.

[0054] In one embodiment, machine-learning-dependent acquisition (MLDA) can be coupled with ion mobility (HD-MLDA). This mode of acquisition can be used to adjust the quadrupole selection based on the masses isolated in the previous scan, which can be recognized as part of predetermined networks, not necessarily belonging to known biochemical pathways. This mode of acquisition can rely on cloud based data to calculate potential networks of masses to be monitored.

[0055] Data independent acquisition involves the use of a collision cell that alternates low and high collision energy before MS detection. The low-energy spectra can contain ions primarily from unfragmented precursors, while the high-energy spectra can contain ions primarily from fragmented precursors. The alternating energy protocol can collect spectra from the same precursor in two modes, a low-energy mode and a high-energy mode.

[0056] Thus, the output of the instrument using data independent acquisition is an inventory, or list, of precursor and fragment ions, each ion can be described by its retention time, drift time, isolated/selected m/z , determined m/z , intensity, etc. The low-energy mode can produce a list of ions that contains primarily unfragmented precursor ions. The high-energy mode can produce a list of ions that contains primarily fragmented precursor ions. As described in U.S. Patent Nos. 6,717,130 and 6,586,727, the parent-daughter peaks can be grouped upon these descriptions, e.g., drift time. These groupings can assist in structural elucidation.

[0057] Post acquisition processing can also be used to perform the peak groups and identification during or shortly after data acquisition. For example, analyses having long acquisition times, e.g., about 5 minutes per sample, can use post acquisition processing to provide structural elucidation while the data is being acquired.

[0058] In one embodiment, the system of the present disclosure includes a quadrupole, IMS and a Q-ToF coupled together to separate and determine metabolites, lipids, etc. in a complex biological sample based on the mass selection of the quadrupole, the drift time / ccs of the IMS and the independent data acquisition and accurate mass measurement of the QToF. The signals obtained can be aligned or correlated based on drift time and mass filtering to determine which of the precursors, or which fragmentation signals, correspond to each other.

[0059] The mass spectrometer can include a collision cell operable in a first mode wherein at least a portion of said ions are fragmented to produce daughter ions, and a second mode wherein substantially less ions are fragmented, a mass analyzer, and a control system which, in use, repeatedly switches the collision cell back and forth between the first and the second modes. In the first mode, the control system can arrange to supply a voltage to the collision cell selected from the group consisting of $\geq 15V$; $\geq 20V$; $\geq 25V$; $\geq 30V$; $\geq 50V$; $\geq 100V$; $\geq 150V$; and $\geq 200V$. In the second mode, the control system can arrange to supply a voltage to said collision cell selected from the group consisting of $\leq 5V$; $\leq 4.5V$; $\leq 4V$;

$\leq 3.5\text{V}$; $\leq 3\text{V}$; $\leq 2.5\text{V}$; $\leq 2\text{V}$; $\leq 1.5\text{V}$; $\leq 1\text{V}$; $\leq 0.5\text{V}$; and substantially 0V . These sets of values can also be used to define a range, such as between about 20 and 30 V, or about 5 and 3 V.

[0060] The control system can automatically switch the collision cell between the two modes in a sufficiently short time to allow at least one of the analytes, precursors or ions to be exposed to each mode. The control system can automatically switch the collision cell between the two modes every about 5, 4, 3, 2.5, 2, 1.5, 1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1 or 0.05 seconds. These values can also be used to define a range, such as about 5 to about 0.5 seconds.

[0061] The mass spectrometer can also be capable of determining an accurate mass for parent or precursor ions and daughter ions after fragmentation. Examples of mass spectrometry instruments include a High-Resolution Mass Spectrometry (HRMS) for accurate mass measurements, such as a Waters SYNAPT® G2-S Quad-ToF MS System (Waters Technologies Corporation, Milford, MA), travelling wave IM, drift tube IM, FAIMS DMS. Figures 7, 8 and 9 show different arrangements of the components for the present disclosure.

[0062] In another embodiment, the present disclosure is related to a method of determining analytes of a complex sample comprising isolating the analytes using a quadrupole, separating the analytes using an ion mobility spectrometer, and analyzing the analytes using a mass spectrometer. The steps can be coupled together, or otherwise performed, as isolating using quadrupole – separating using ion mobility spectrometer – analyzing using a mass spectrometer, or separating using ion mobility spectrometer – isolating using quadrupole - analyzing using a mass spectrometer. The steps of isolating, separating and analyzing can be performed using the various components and conditions as described herein.

[0063] For example, analyzing the analytes using the mass spectrometer can include passing the ions to a fragmentation means including a collision cell, operating the fragmentation means in a first mode wherein at least a portion of the ions are fragmented to produce daughter ions, recording a mass spectrum of ions emerging from the fragmentation means operating in the first mode as a high fragmentation mass spectrum, switching the fragmentation means to operate in a second mode wherein substantially less ions are fragmented, recording a mass spectrum of ions emerging from the fragmentation means

operating in the second mode as a low fragmentation mass spectrum, and repeating these steps a plurality of times.

[0064] SAMPLE INTRODUCTION

[0065] The sample can be introduced to the system, e.g., a Quad-IMS-QToF using a variety of approaches, including a shotgun approach or infusion. The sample introduction can also be by flow injection analysis using an autosampler, or by chromatography separation.

[0066] In one embodiment, a metabolomics/lipidomics experiment (e.g., shotgun) can be divided into categories of top-down and bottom-up. In a top-down analysis, lipid species can be identified by their accurate mass-to-charge ratio (m/z), determined on high resolution mass spectrometers such as an Tof, FT-ICR (Fourier transform ion cyclotron resonance mass spectrometry) or an orbitrap. Although individual molecular species are not identified by MS/MS, top-down lipidomics can demonstrated potential in high-throughput screens.

[0067] In a bottom-up analysis, all lipid precursor ions can be subjected to MS/MS analysis, and identified by m/z of characteristic fragments or neutral losses on triple quadrupole instruments. Tens of precursor-ion and neutral-loss scans could be monitored simultaneously to detect various classes of lipids for a more untargeted analysis. In some embodiments, most lipid species represent linear combinations of a relatively few building blocks. Those building blocks can include glycerol, sphingoid bases, polar head groups, and fatty acyl substituents, which can be released from the precursor ions upon collision-induced dissociation (CIO) with an inert gas, e.g., argon or helium. For example, using ESI in positive mode, precursor-ion scans for m/z 184.1 (phosphocholine) or neutral loss scans for m/z 59.1 (trimethylamine) can identify the $[M+H]^+$ ions that correspond to sphingomyelins, phosphatidylcholines and lysophosphatidylcholines. Using ESI innegative mode, precursor-ion scans for m/z 97.0 (sulfate) can identify the $[M-H]^-$ ions of sulfatides whereas neutral loss scans for m/z 87.0 (serine) can be used to identify phosphatidylserines. One of the advantages top down / bottom up analysis is high throughput screening of metabolites and lipids in biological samples.

[0068] Shotgun lipidomics on a triple quadrupole instrument can be a low-mass resolution approach, e.g., 1-2 Da. In some embodiments, only a single precursor or neutral-loss scan can be performed at a time. Hybrid, tandem mass spectrometers built on Tof, FT-ICR, and orbitrap technology can be used to acquire high resolution tandem mass spectra

from hundreds of lipid precursors. For example, a quadrupole-ToF instrument can be used to sequentially isolate precursor ions in unit or higher mass resolution windows by using a quadrupole working stepwise from a low to a high m/z ratio, transmit ions into a collision cell, and acquire accurate measurements of full product ion spectra for that window at each step of the quadrupole. The addition of ion mobility to a Q-ToF instrument can enhance the analysis and provide a separation post- or pre- quadrupole, according to the geometry of the instrumentation (whether the quadrupole is before or after the ion mobility cell). The automated post-acquisition interpretation of the full product ion spectra can emulate an unlimited number of precursor and neutral-loss scans in a single analysis. Using the modes of acquisition of the present disclosure, no prior knowledge of the lipid ions of interest is required, which can be ideal for the rapid analysis of unknown lipid mixtures in biological samples.

[0069] **Normal phase chromatography**

[0070] In another embodiment, the present disclosure relates to a system further including a normal phase chromatography separation system. A chromatography system or step can be used to separate the complex sample into groups of similar components, and to add a further dimension or simplification step to the analysis of a complex biological sample. The normal phase chromatography system can include a supercritical fluid chromatography (SFC) or a hydrophilic interaction chromatography (HILIC).

[0071] One of the advantages of incorporating a separation step is distinguishing chemical isomers having identical masses and similar fragmentation profiles. Also, simultaneous introduction of multiple analytes into the ionization source which can induce undesirable effects such as ion-suppression is reduced.

[0072] For example, a normal phase separation can be performed before introduction into the quadrupole to grossly segregate the lipids into families. In one embodiment, HILIC can be used to separate the lipids into families of lipids prior to analysis. Similar to HILIC, supercritical fluid chromatography can also be used to generate a similar lipid separation profile which is suitable for stepwise data independent acquisition.

[0073] One of the main challenges for a lipidomics analysis is the separation of the wide array of lipid species present in biological samples. Normal-phase chromatography can separate lipids into classes that can elute as single peak. Such peaks can be further resolved using ion mobility, isolated via quadrupole selection, both, as described herein. Normal-

phase separation of lipids can mimic a slow infusion of lipid classes, allowing the necessary duty cycle for conduction stepwise quadrupole selection, e.g., 0.5-1 Daltons steps, of lipids according to selected mass ranges. For example, phosphatidylethanolamines eluting between 4-6 minutes have a mass range from about 600 m/z to about 800 m/z

[0074] “Duty cycle” refers to the time it takes to acquire signals in a given setting. “Duty cycle” as used herein refers the percentage of time in which a particular ion is getting through the quadrupole and reaching the detector know as duty cycle.

[0075] The use of a chromatography system or separation step to simplify the analytes introduced, e.g., by separating into molecular weight classes or lipid classes, can increase the duty cycle of the quadrupole by about 5%, 10, 20, 30, 40 or about 50%. One of the benefits of an increased duty cycle is the ability to acquire more data point per unit of time, leading to better quantification and increased signal-to-noise.

[0076] The quadrupole stepwise selection in combination with ion mobility separation can provide a synergistic effect. The quadrupole can act as a separation filter and the ion mobility too, increasing the overall peak capacity and ability to deconvolute the final information using the quadrupole coordinate together with the drift time information.

[0077] In one embodiment, the components of the present disclosure can be arranged, coupled or otherwise linked together in the following order: normal phase chromatography system - quadrupole - ion mobility spectrometer - mass spectrometer. In another embodiment, the components of the present disclosure can be arranged, coupled or otherwise linked together in the following order: normal phase chromatography system - ion mobility spectrometer - quadrupole - mass spectrometer.

[0078] Examples of the normal phase chromatography systems or instruments include high performance liquid chromatography, ultra high pressure liquid chromatography (e.g. UPLC® and UPC²®), supercritical fluid chromatography and carbon dioxide based chromatography.

[0079] In another embodiment, the present disclosure relates to a method of determining analytes of a complex sample including separating the analytes using a normal phase chromatography system, isolating the analytes using a quadrupole, separating the analytes using an ion mobility spectrometer, and analyzing the analytes using a mass spectrometer. The steps of the method of the present disclosure can be coupled, or otherwise connected or arranged as separating using normal phase chromatography system – followed

by, isolating using quadrupole – followed by, separating using ion mobility spectrometer – and followed by analyzing using a mass spectrometer. In another embodiment the steps of the method of the present disclosure can be coupled or otherwise connected or arranged as separating using normal phase chromatography system – followed by, separating using ion mobility spectrometer – followed by, isolating using quadrupole – and followed by, analyzing using a mass spectrometer. The phrase “followed by” refers to coming after the step recited prior to the “followed by” language. “Followed by” does not require directly following (i.e., an intermediate step is possible, but not required as the term can also encompass directly following).

[0080] In other embodiments, the present disclosure can be used to generate molecular maps of lipids present in various samples, such as animal tissues and serum. For example, after HILIC separation, ion mobility further separates lipid species according to their molecular shapes. The molecular landscape visualized using an unbiased 3D representation (e.g., drift time, m/z , intensity) can enhance the detection of many low abundance molecular species that could go unnoticed otherwise, such as by increasing the signal-to-noise ratio and increased peak capacity.

[0081] In one embodiment, the present disclosure and the acquisition mode described herein can be extended for peptides and small proteins, proteomics, carbon clusters, polymers, oligosaccharides and oligonucleotides.

[0082] The disclosures of all cited references including publications, patents, and patent applications are expressly incorporated herein by reference in their entirety.

[0083] When an amount, concentration, or other value or parameter is given as either a range, preferred range, or a list of upper preferable values and lower preferable values, this is to be understood as specifically disclosing all ranges formed from any pair of any upper range limit or preferred value and any lower range limit or preferred value, regardless of whether ranges are separately disclosed. Where a range of numerical values is recited herein, unless otherwise stated, the range is intended to include the endpoints thereof, and all integers and fractions within the range. It is not intended that the scope of the invention be limited to the specific values recited when defining a range.

CLAIMSWhat is claimed is:

1. A system comprising:

- (i) a normal phase chromatography system;
- (ii) a quadrupole;
- (iii) an ion mobility spectrometer; and
- (iv) a mass spectrometer,

wherein components (i) – (iv) are coupled together in order of: normal phase chromatography system - quadrupole - ion mobility spectrometer - mass spectrometer, or in the order of: normal phase chromatography system-ion mobility spectrometer-quadrupole-mass spectrometer.

2. A system comprising:

- (i) a quadrupole;
- (ii) an ion mobility spectrometer; and
- (iii) a mass spectrometer,

wherein components (i)-(iii) are coupled together in order of: quadrupole - ion mobility spectrometer - mass spectrometer, or in order of: ion mobility spectrometer - quadrupole - mass spectrometer.

3. The system of claim 1, wherein the normal phase chromatography system comprises supercritical fluid chromatography (SFC) or hydrophilic interaction chromatography (HILIC).

4. The system of claims 1 or 2, wherein the quadrupole is capable of scanning in a stepwise manner at a m/z interval of less than about ± 2.5 Daltons.

5. The system of claims 1 or 2, wherein the quadrupole is capable of scanning from about 50 Daltons to about 1600 Daltons.

6. The system of claims 1 or 2, wherein the mass spectrometer comprises
- (a) a collision cell operable in a first mode wherein at least a portion of ions are fragmented to produce daughter ions, and a second mode wherein substantially less ions are fragmented; and
 - (b) a mass analyzer, and
 - (c) a control system which, in use, repeatedly switches the collision cell back and forth between the first and the second modes.
7. The system of claim 6, wherein in the first mode the control system arranges to supply a voltage to the collision cell selected from the group consisting of $\geq 15\text{V}$; $\geq 20\text{V}$; $\geq 25\text{V}$; $\geq 30\text{V}$; $\geq 50\text{V}$; $\geq 100\text{V}$; $\geq 150\text{V}$; and $\geq 200\text{V}$.
8. The system of claim 6, wherein in the second mode the control system arranges to supply a voltage to the collision cell selected from the group consisting of $\leq 5\text{V}$; $\leq 4.5\text{V}$; $\leq 4\text{V}$; $\leq 3.5\text{V}$; $\leq 3\text{V}$; $\leq 2.5\text{V}$; $\leq 2\text{V}$; $\leq 1.5\text{V}$; $\leq 1\text{V}$; $\leq 0.5\text{V}$; and substantially 0V .
9. The system of claim 6, wherein the control system automatically switches the collision cell between the at least two modes at least once every 0.5 seconds.
10. A method of determining analytes of a complex sample comprising:
- (i) separating the analytes using a normal phase chromatography system;
 - (ii) isolating the analytes using a quadrupole;
 - (iii) separating the analytes using an ion mobility spectrometer; and
 - (iv) analyzing the analytes using a mass spectrometer,
- wherein the steps are coupled together as separating using normal phase chromatography system – isolating using quadrupole – separating using ion mobility spectrometer – analyzing using a mass spectrometer, or
- separating using normal phase chromatography system – separating using ion mobility spectrometer – isolating using quadrupole - analyzing using a mass spectrometer.

11. A method of determining analytes of a complex sample comprising:

- (ii) isolating the analytes using a quadrupole;
- (iii) separating the analytes using an ion mobility spectrometer; and
- (iv) analyzing the analytes using a mass spectrometer,

wherein the steps are coupled together as isolating using quadrupole – separating using ion mobility spectrometer – analyzing using a mass spectrometer, or

separating using ion mobility spectrometer – isolating using quadrupole - analyzing using a mass spectrometer.

12. The method of claim 10, wherein the normal phase chromatography system comprises supercritical fluid chromatography (SFC) or hydrophilic interaction chromatography (HILIC).

13. The method of claims 10 or 11, wherein the analytes are isolated by stepwise scanning of the quadrupole at a m/z interval of less than about ± 2.5 Daltons.

14. The method of claims 10 or 11, wherein the quadrupole is scanned over a mass range less than about 200 Daltons.

15. The method of claims 10 or 11, wherein analyzing the analytes using a mass spectrometer comprises

- (a) passing ions to a fragmentation means including a collision cell;
- (b) operating the fragmentation means in a first mode wherein at least a portion of the ions are fragmented to produce daughter ions;
- (c) recording a mass spectrum of ions emerging from the fragmentation means operating in the first mode as a high fragmentation mass spectrum;
- (d) switching the fragmentation means to operate in a second mode wherein substantially less ions are fragmented;
- (e) recording a mass spectrum of ions emerging from the fragmentation means operating in the second mode as a low fragmentation mass spectrum; and
- (f) repeating steps (c)-(e) a plurality of times.

FIG. 1A

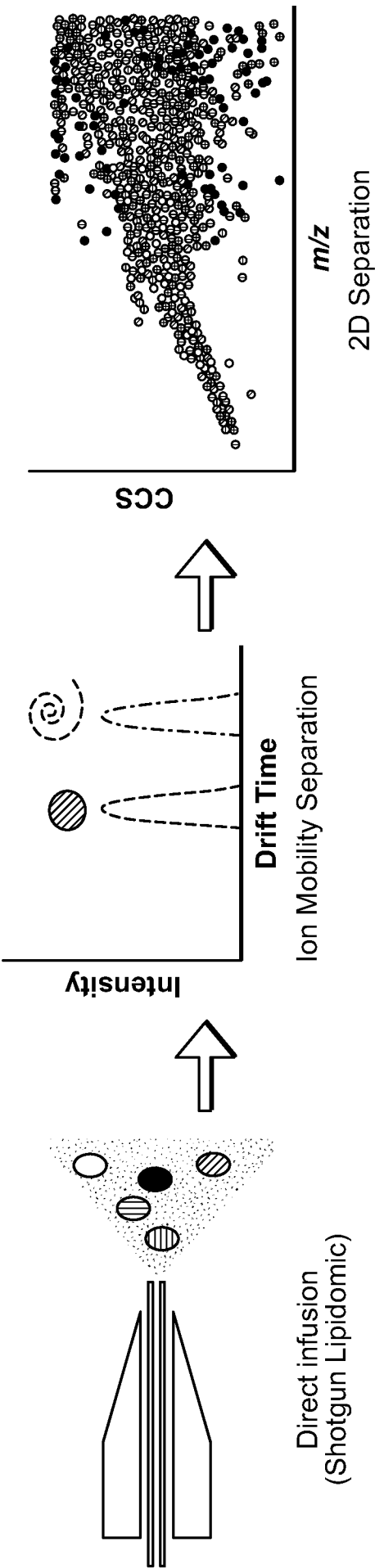
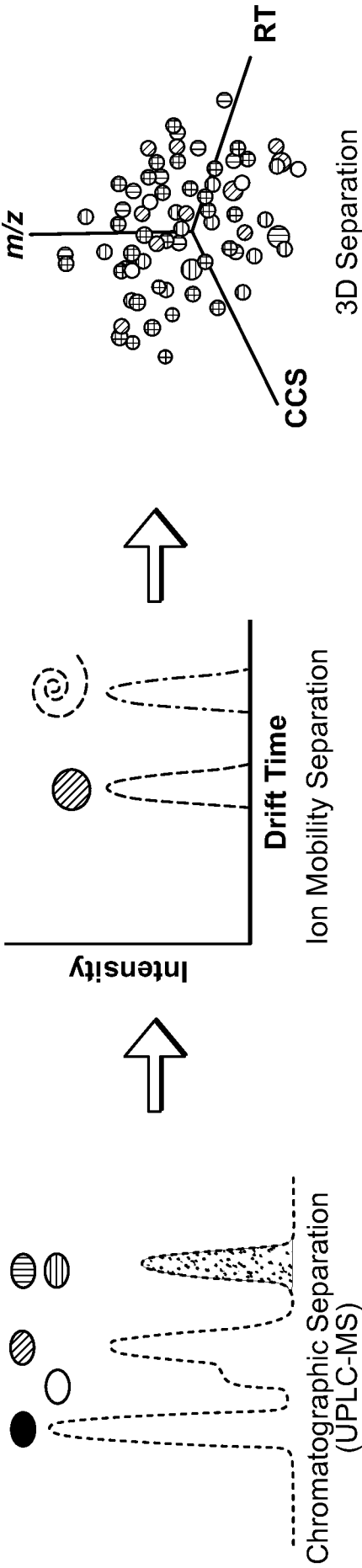


FIG. 1B



Chemical Complexity of Biological Samples

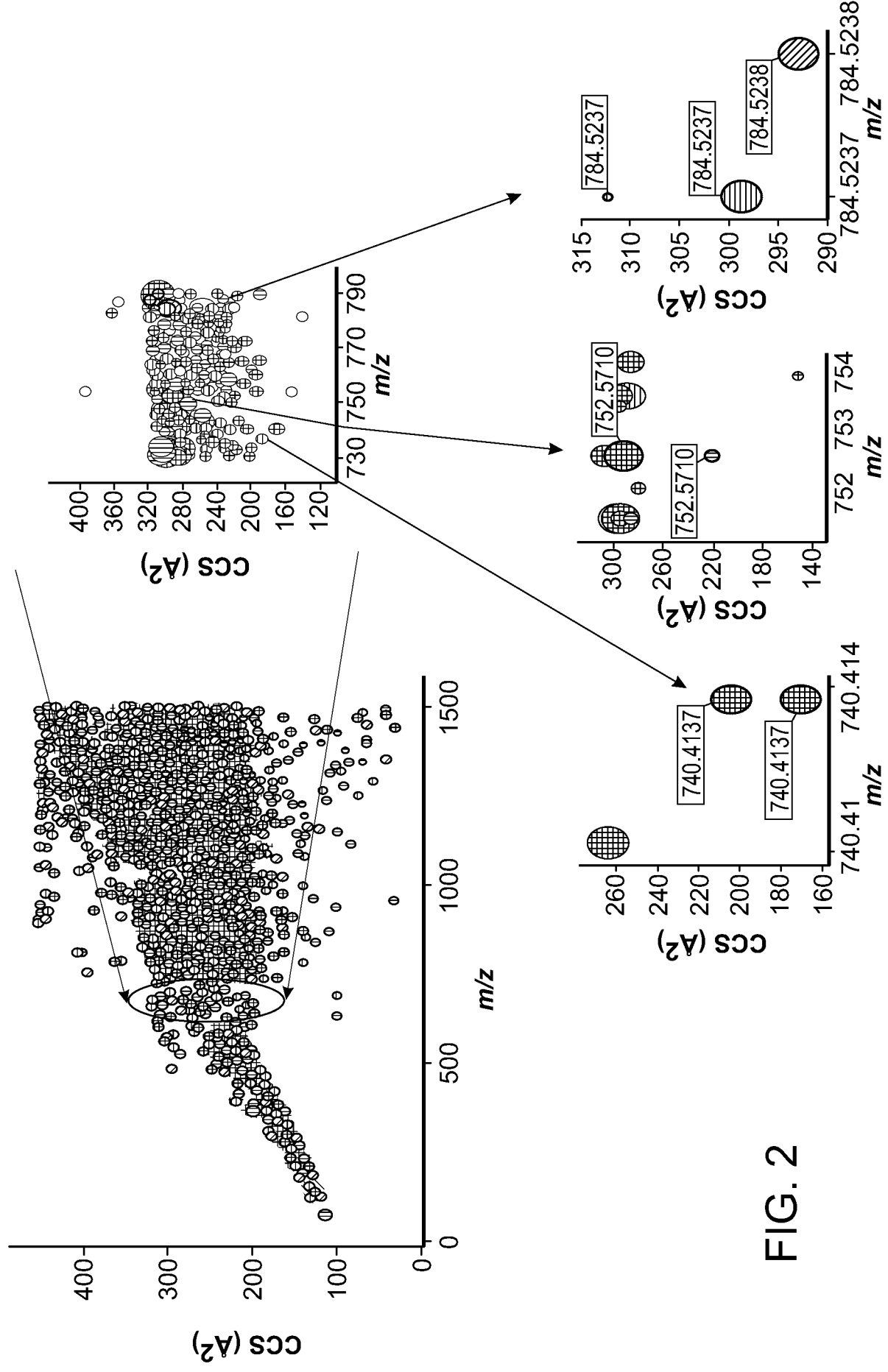
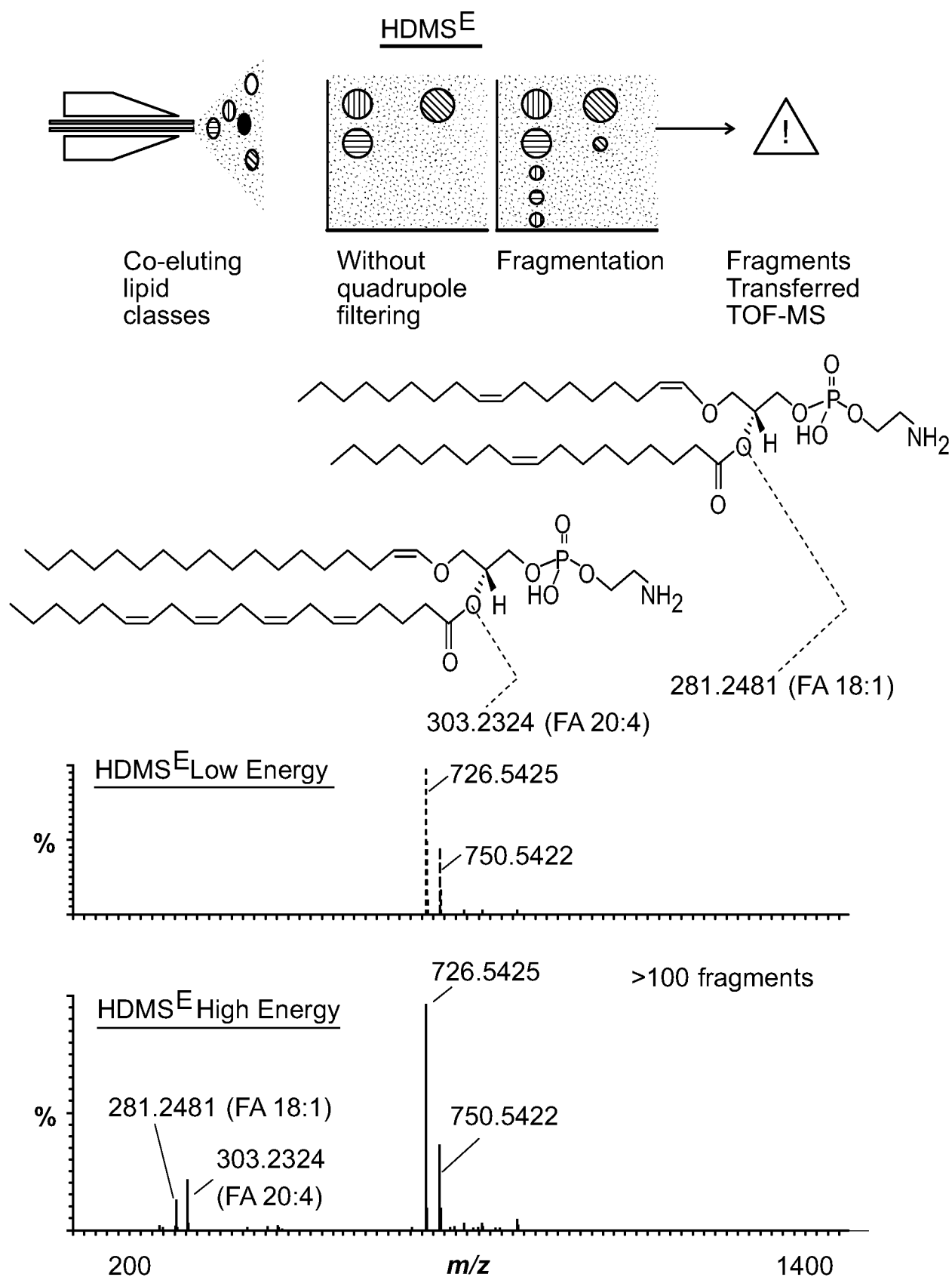


FIG. 2

3/11

Stepwise Quad Isolation of Precursors (SQIP) followed by IMS separation and fragmentation (in the transfer region)**FIG. 3A**

4/11

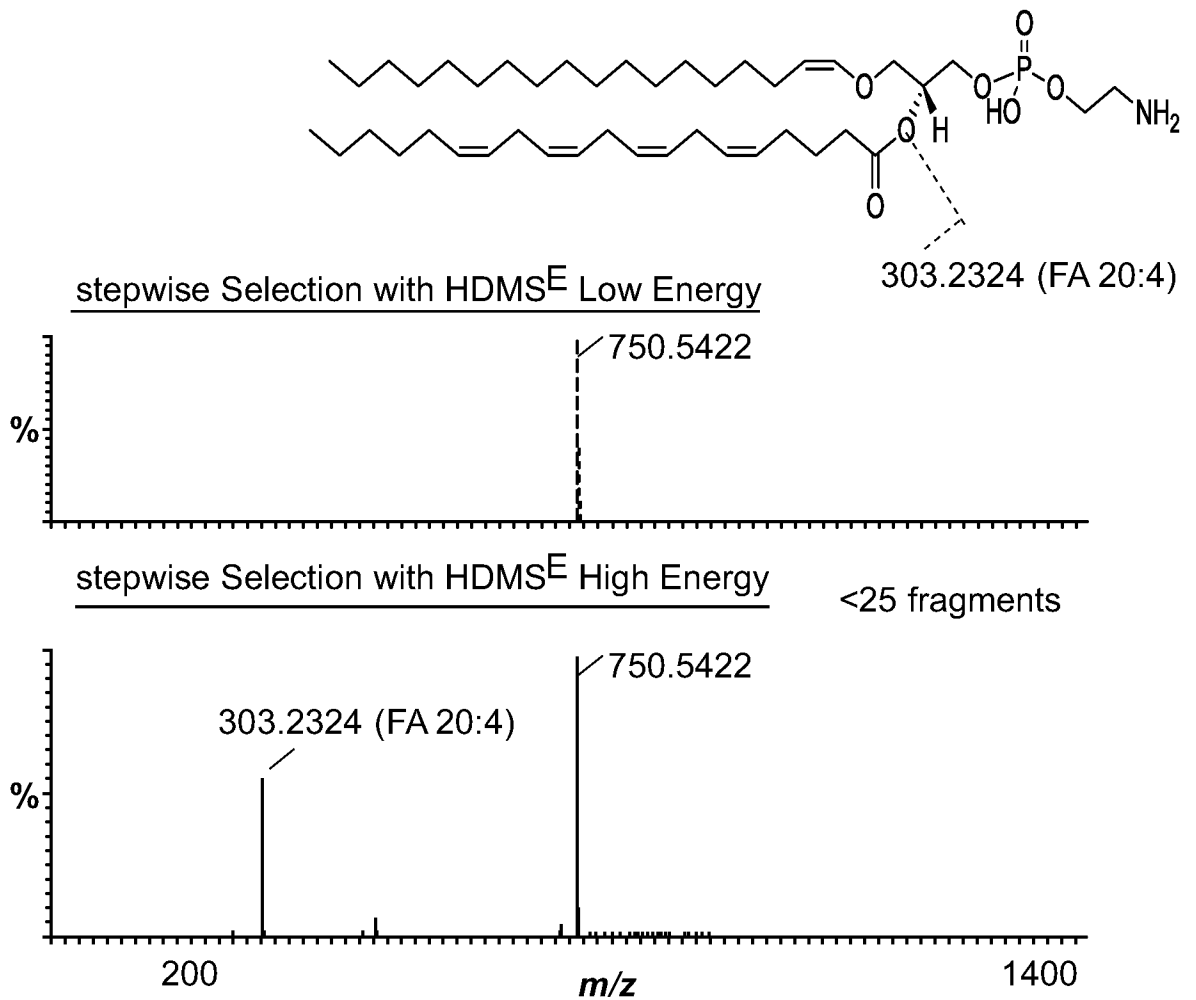
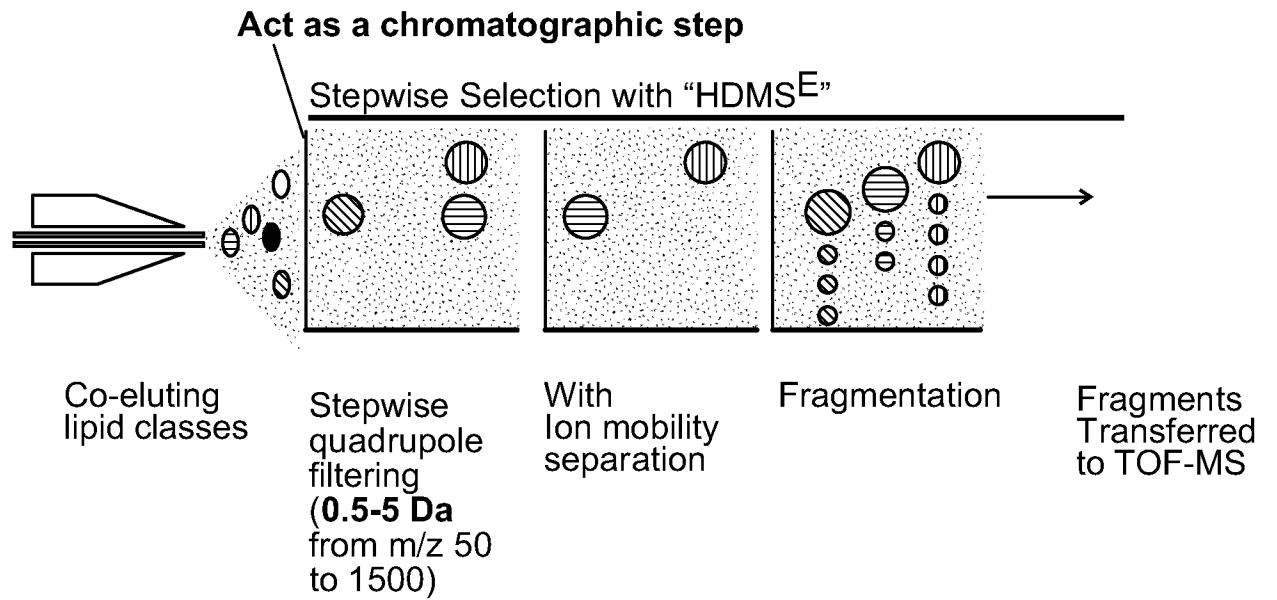
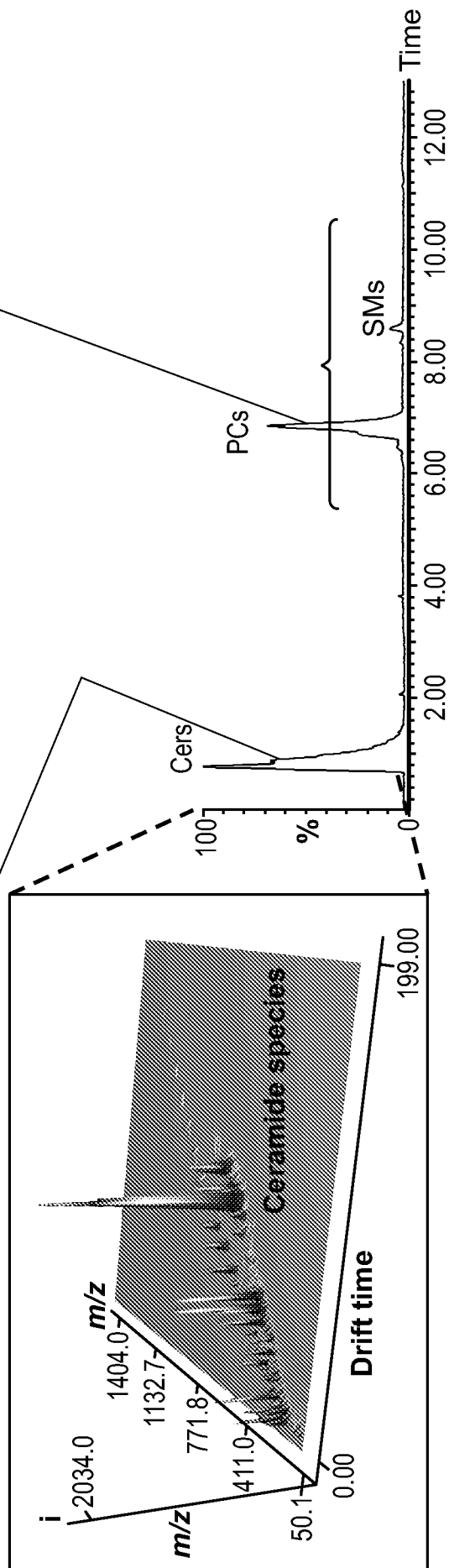
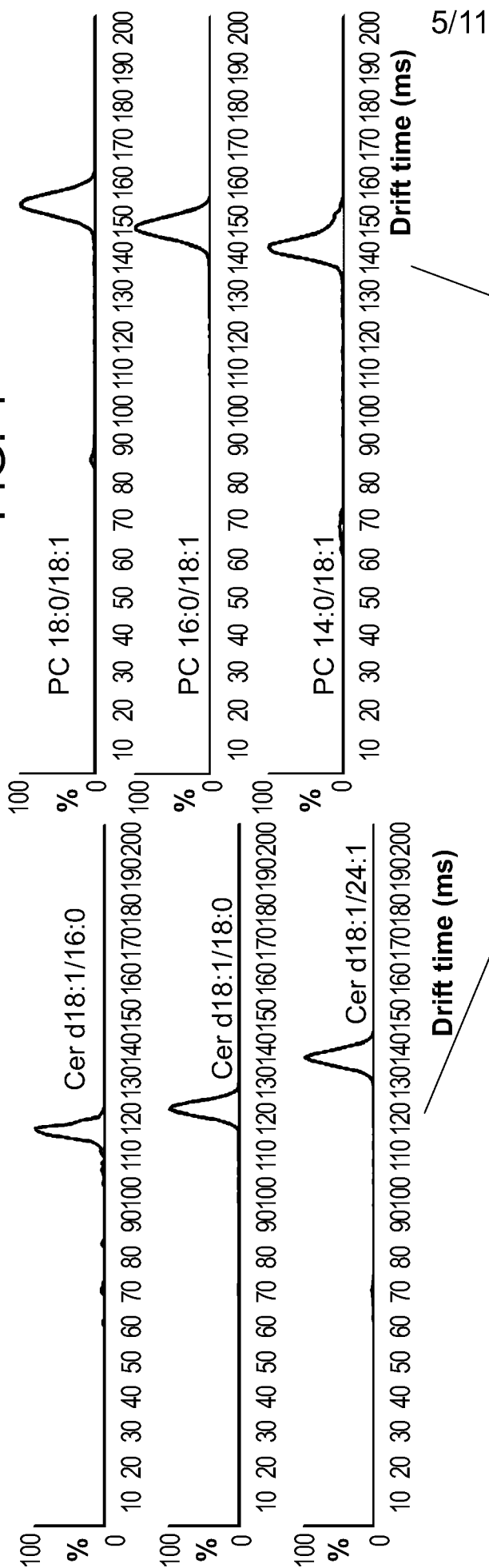


FIG. 3B

HILIC and SFC separate lipid classes based on their polar head groups



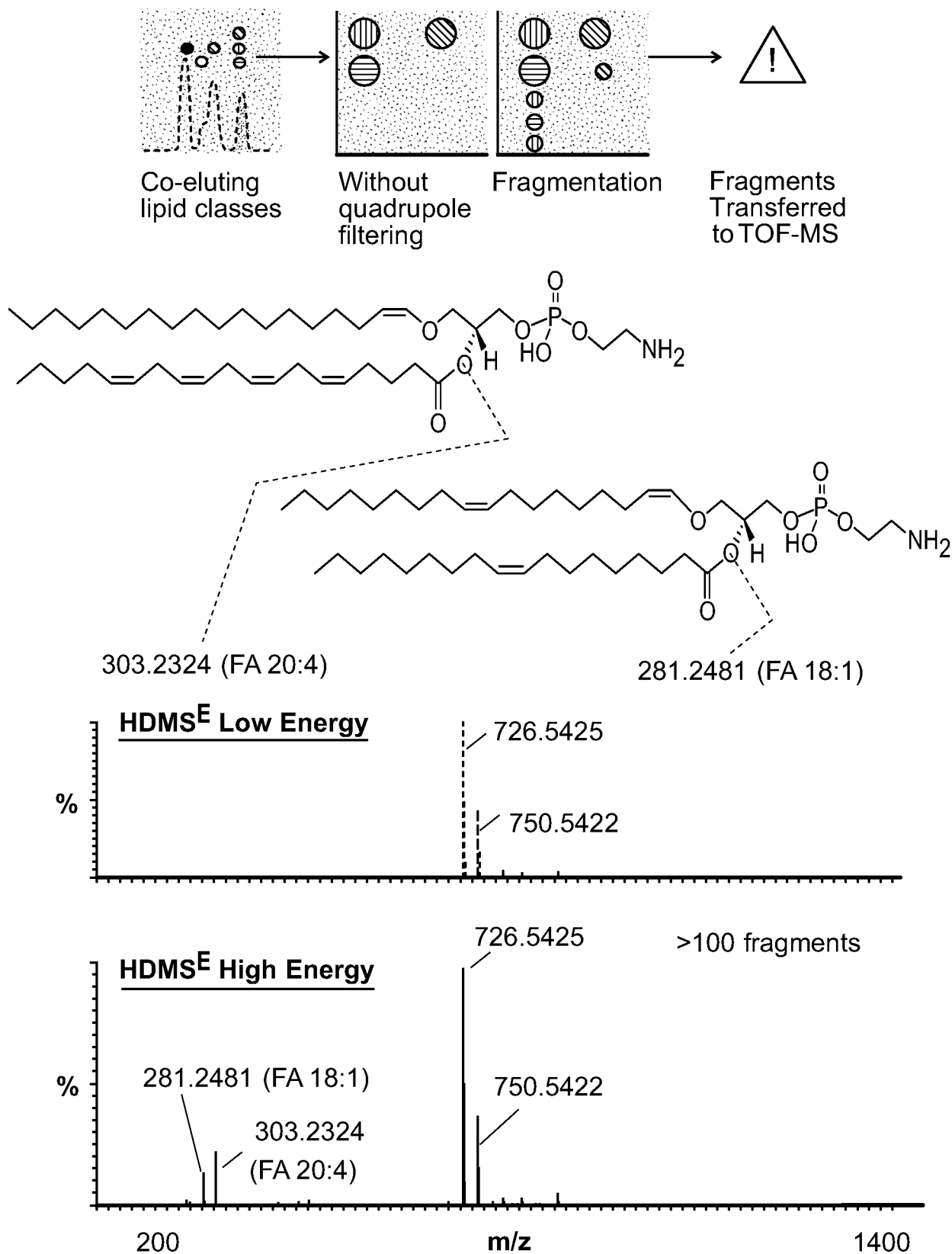
6/11

Separated lipid classes

Lipid Class	ES Polarity	RT window	m/z window	stepwise
FA	Neg	0-1	200 -400	
Cer	Pos	0-2	500 -800	
HexCer	Neg	0-2	...	
ST	Neg	0-2	...	
DiHexCer	Neg	2-4		
PG	Neg	1-3		
PE	Neg/Pos	5-7		
PI	Neg	3-5		
PS	Neg	4-6		
PC	Pos	5-7		
LPE	Neg/Pos	6-8		
SM	Pos	7-9		
LPC	Pos	8-10		

FIG. 5

7/11

Stepwise Quadrupole Isolation of Precursors (SQIP) followed by IMS separation and fragmentation (in the transfer region)**HDMSE****FIG. 6A**

8/11

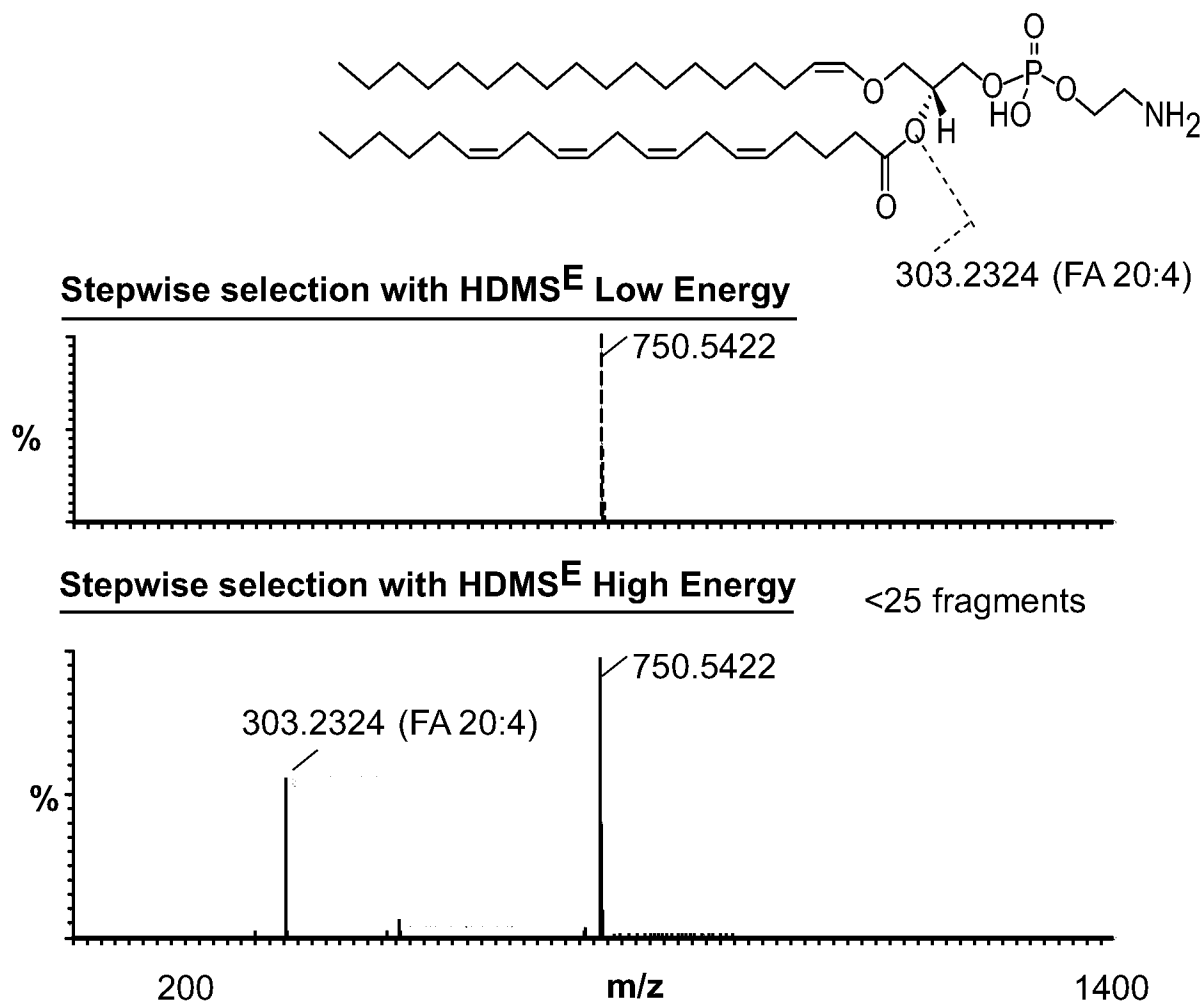
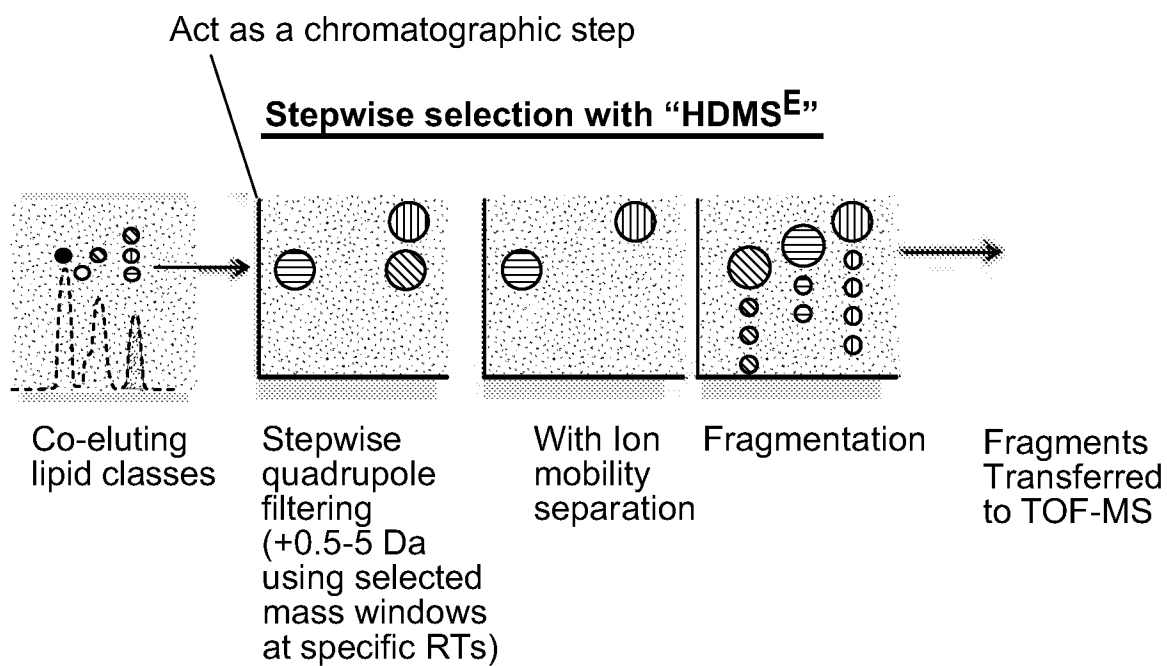


FIG. 6B

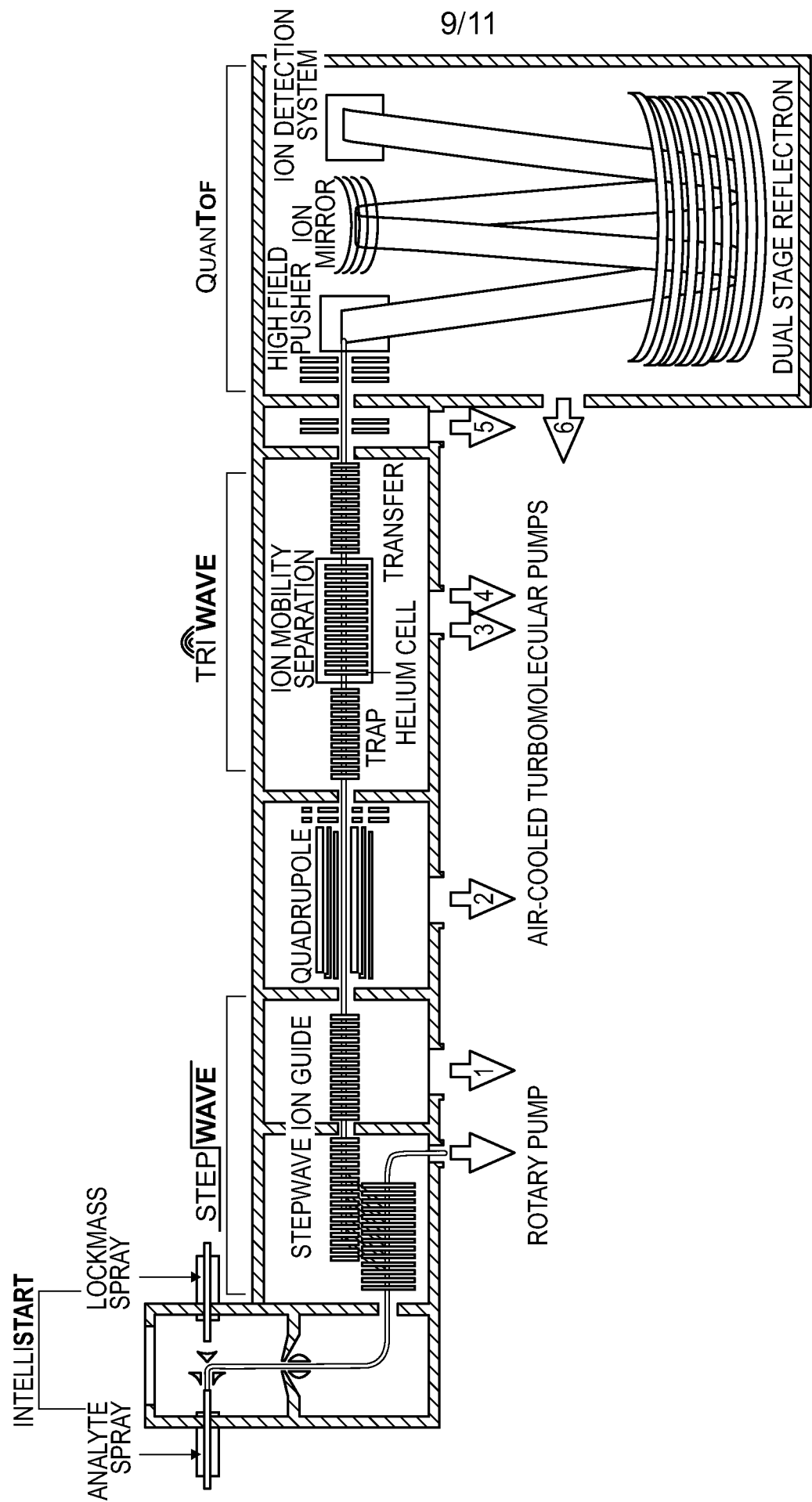


FIG. 7

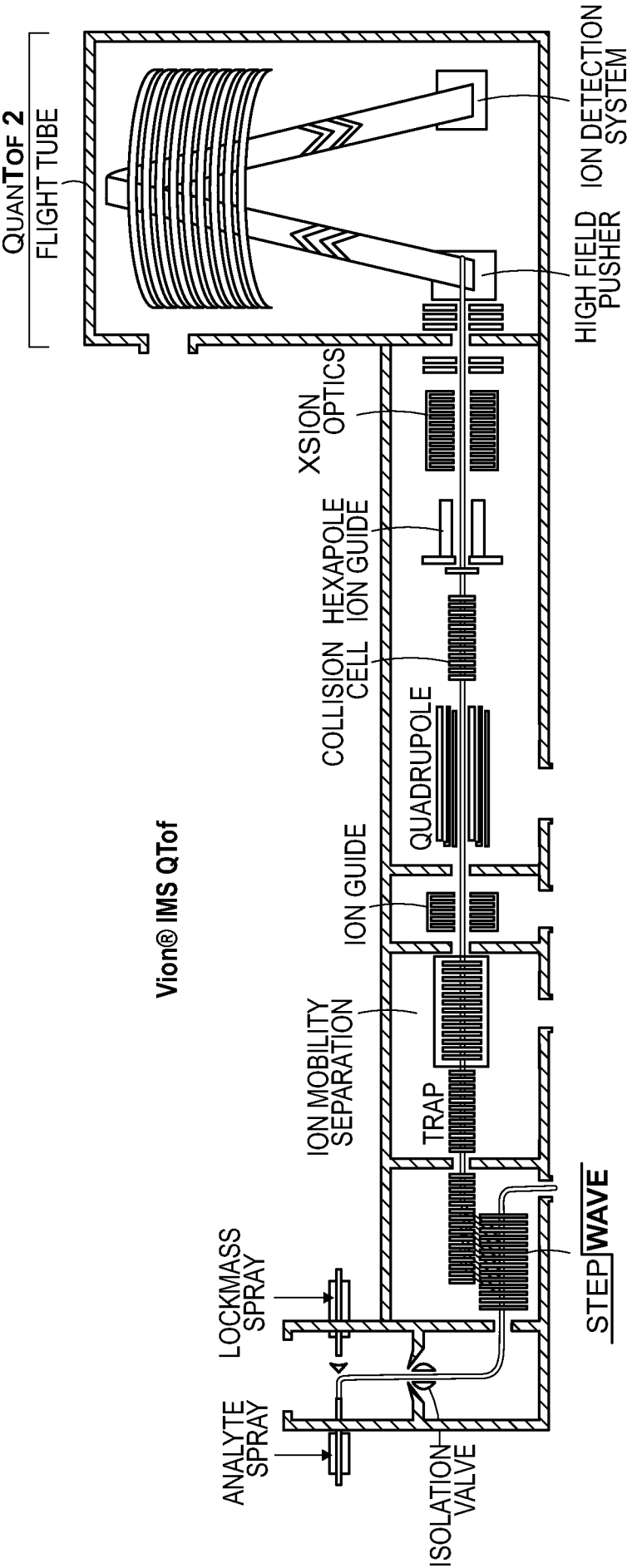


FIG. 8

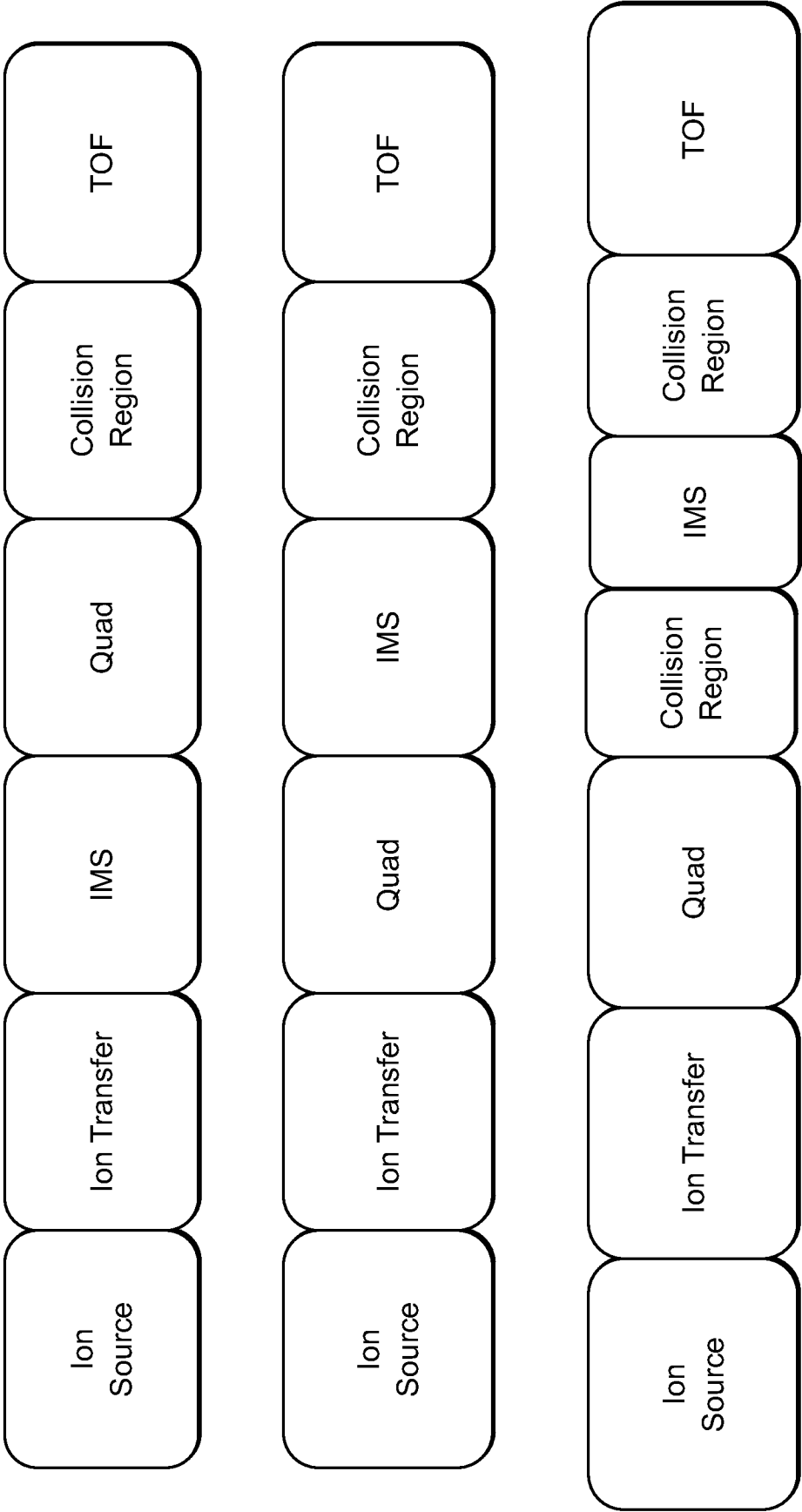


FIG. 9

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2016/034293

A. CLASSIFICATION OF SUBJECT MATTER IPC(8) - H01J 49/00; B01D 15/08; B01D 59/44; G01N 31/00 (2016.01) CPC - H01J 49/02; B01D 15/08; G01N 27/622; H01J 49/025 (2016.05) According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC - H01J 49/00; B01D 15/08; B01D 59/44; G01N 31/00 CPC - H01J 49/02; B01D 15/08; G01N 27/622; H01J 49/025 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched USPC- 250/281, 282, 283, 290, 304; 210/198.2 (keyword delimited) Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) Orbit, Google Patents, ProQuest Search terms used: quadrupole, ion mobility spectrometer, mass spectrometry, spectrometer, normal phase chromatography,		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2010/0108879 A1 (BATEMAN et al) 06 May 2010 (06.05.2010) entire document	2, 6-9, 11

Y		1, 3-5, 10, 12-15
Y	US 2014/0047906 A1 (COHESIVE TECHNOLOGIES INC) 20 February 2014 (20.02.2014) entire document	1, 3, 10, 12
Y	US 2011/0215235 A1 (SCHOEN et al) 08 September 2011 (08.09.2011) entire document	4, 13
Y	US 2014/0131569 A1 (DH TECHNOLOGIES DEVELOPMENT PTE, LTD) 15 May 2014 (15.05.2014) entire document	5, 14
Y	US 2001/0052569 A1 (BATEMAN et al) 20 December 2001 (20.12.2001) entire document	15
A	US 2006/0186028 A1 (HUGHES) 24 August 2006 (24.08.2006) entire document	1-15
A	US 7,529,629 B2 (WANG) 05 May 2009 (05.05.2009) entire document	1-15
☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 25 July 2016		Date of mailing of the international search report 01 SEP 2016
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300		Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774