(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
5 October 2017 (05.10.2017)

WIPO | PCT

(10) International Publication Number
WO 2017/168167 A1

(51) International Patent Classification:
H01J 49/14 (2006.01) *H01J 49/16* (2006.01)
H01J 49/10 (2006.01) *H01J 27/20* (2006.01)

(21) International Application Number:
PCT/GB2017/050914

(22) International Filing Date:
31 March 2017 (31.03.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
1605613.7 1 April 2016 (01.04.2016) GB

(71) Applicant: **THE UNIVERSITY OF WARWICK** [GB/GB]; University House, Kirby Corner Road, Coventry West Midlands CV4 8UW (GB).

(72) Inventors: **O'CONNOR, Peter**; Dept. of Chemistry, University of Warwick, Coventry West Midlands CV4 7AL (GB). **WOOTTON, Chris**; c/o Dept. of Chemistry, University of Warwick, Coventry West Midlands CV4 7AL (GB). **HUSSEIN, Haytham**; c/o Dept. of Chemistry, University of Warwick, Coventry West Midlands CV4 7AL (GB). **CHIU, Cookson**; c/o Dept. of Chemistry, University of Warwick, Coventry West Midlands CV4 7AL (GB). **WONG, Man Ying**; c/o Dept. of Chemistry, University of Warwick, Coventry West Midlands CV4 7AL (GB).

(74) Agent: **BARKER BRETTELL LLP**; 100 Hagley Road, Edgbaston, Birmingham West Midlands B16 8QQ (GB).

(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, DJ, 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, KH, KN, KP, KR, KW, 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).

Published:

— *with international search report (Art. 21(3))*

(54) Title: IONISING MOLECULES AND ELECTROSPRAY IONISATION APPARATUS

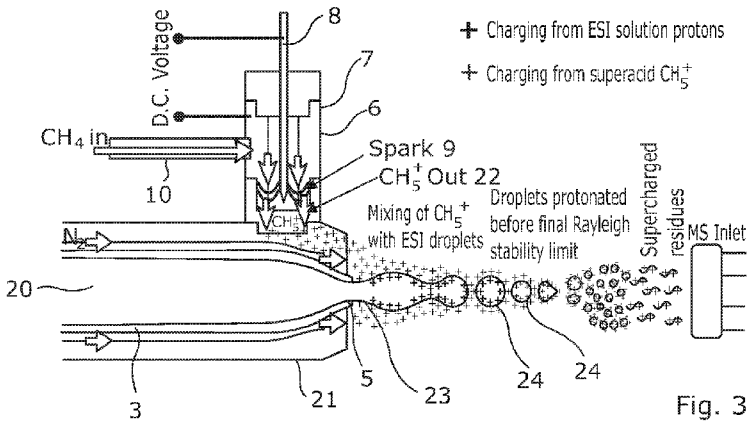

Fig. 3

(57) Abstract: A method of and apparatus for ionising molecules, the method comprising passing a solution (20) containing the molecules through an electrospray emitter which has a potential difference applied thereto, in order to create a jet (23) of solution; and passing a flow of CH_5+ ions (22), or other ions having a lower proton affinity than the solvent of the solution over the jet (23). The method may be carried out in a chamber (2), typically at atmospheric pressure, from which oxygen has been evacuated.

WO 2017/168167 A1

IONISING MOLECULES AND ELECTROSPRAY IONISATION APPARATUS

This invention relates to a method of ionising molecules, and electrospray ionisation apparatus.

Mass spectrometry is a well-known technique, in which molecules can be characterised based upon their mass to charge ratio. However, in order to identify neutral molecules, it is first necessary to ionise them, so that they have a charge and so can be characterised.

Known methods of ionisation include electrospray (ES) ionisation (ESI), whereby a solution of molecules in a solvent is passed through a nozzle which is raised to a potential difference relative to a target. A charge is transferred onto the solution as it exits the nozzle tip and, with judicious choice of solvent, preferentially retained on the molecules (which are of interest) rather than the solvent (which is of little interest). Thus, charge can be transferred onto a molecule.

Electrospray ionisation is referred to as nanoESI (or nano-ESI) when the nozzle has a very fine tip. In nanoESI the nozzle typically has a diameter of less than 10μm and is usually made from pulled glass capillaries.

Chemical ionisation (CI) has been previously used, whereby a proton donor is reacted with the molecules to be analysed. Depending on the proton donor used, the donation of a proton to the molecule can be achieved. CI is carried out at below atmospheric pressure, particularly where the proton donor is CH_5^+ generated by passing CH_4 (methane) over a spark.

The CH_5^+ cation is considered a "super-acid" as it has an extremely low proton affinity, even lower than that of sulfuric acid (531.4 vs. 699.6 kJ/mol, respectively). CH_5^+ was first introduced as an ionization reagent by Munson and Field, 1966, (Munson, M. S. B.; Field, F. H. *J. Am. Chem. Soc.* **1966**, *88*, 2621–2630 and Field, F. H.; Munson, M. S. B. *J. Am. Chem. Soc.* **1965**, *87*, 3289–3294). This method, later referred to as "Chemical ionization" (CI), was capable of producing even-electron $[M+H]^+$ ions from analytes, rather than the fragile and unstable $[M]^{+\cdot}$ ions produced by

the widely used electron ionization (EI). CH_5^+ can be readily produced by reaction with high-energy electrons, as shown in equations 1&2.

$$CH_4 + e^- \rightarrow CH_4^{+\cdot} + 2e^- \quad [Eq.1]$$

$$CH_4 + CH_4^{+\cdot} \rightarrow CH_5^+ + CH_3^\cdot \quad [Eq.2]$$

CH_5^+ ions are the primary, stable reaction products from equations 1&2 and are thus available for further reaction with target analytes. CH_5^+ ions act as extremely strong Brønsted acids (the so called "super-acids") which readily transfer protons to nearby species, producing a protonated analyte species $(M+H)^+$ as shown Equation 3.

$$CH_5^+ + M \rightarrow [MH]^+ + CH_4 \text{ (proton transfer)} \quad [Eq.3]$$

Although chemical ionization greatly assisted in the analysis of many polar and non-polar chemicals, it was not a viable ionization method for the study interrogation of large biomolecules as it involves a gas-phase proton-transfer reaction, meaning CI is of limited use when attempting to ionize non-volatile molecules (such as peptides and proteins).

CI also generally only produced singly charged products which limited its use to smaller species of interest. With the advent of electrospray ionization (ESI) and nanoESI, mass spectrometry analysis of large biomolecules, such as multiply charged protein ions became viable. It was recognized early that the solvent conditions of electrospray ionization influenced the charge states observed. However, in 2001, Iavarone et al (Iavarone, A. T.; Jurchen, J. C.; Williams, E. R., *Analytical Chemistry*, **2001**, *73*, 1455–1460.) discovered that the addition of some molecules such as glycerol and m-Nitrobenzyl alcohol, would increase the average charge state produced. Later, Lomeli et al (Lomeli, S. H.; Peng, I. X.; Yin, S.; Ogorzalek Loo, R. R.; Loo, J. A., *J Am Soc Mass Spectrom*, **2010**, 21 (1), 127.) found other reagents, including sulfolane, that produced a similar effect. This effect was called 'supercharging'.

With the introduction of the supercharged biomolecules concept by Iavarone et al, 2001 (Iavarone, A. T.; Jurchen, J. C.; Williams, E. R., *Analytical Chemistry*, **2001**, *73*,

1455–1460.), enhanced charging of analytes can be achieved, allowing observation of higher charge states for analytes via ESI than previously observed. Addition of supercharging reagents such as glycerol, sulfolane, or m-Nitro benzyl alcohol increases the maximum observed charge states, with increased signal intensity of biomolecules and lower mass-to-charge ratios, allowing for increased achievable resolving powers for Fourier Transform Mass Spectroscopy (FTMS) based instruments (such as Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FT-ICR MS) and Orbitrap platforms). Accessing higher charge states has also been shown to dramatically improve fragmentation efficiency and cleavage coverage during MS/MS experiments, increasing with higher charge-states of selected precursor ions, especially for electron capture dissociation and electron transfer dissociation MS/MS. Enhancing the charge state enables a higher degree of fragmentation, thus more molecular sequence information, which is critical for effective identification of unknown proteins and peptides via MS/MS fragmentation and the analysis of any proteomic-based sample. Studies of post-translational modifications could also be facilitated by this increase in fragmentation efficiency, especially in the ever-growing field of Top-Down MS/MS.

Atmospheric pressure chemical ionisation (APCI) is a similar technique to CI. Here, the analyte in solution in entrained in a flow of gas, typically nitrogen (N_2). The sample in solution is then passed over a spark, which ionises the background air molecules and residual solvent, which then reacts with the analyte to increase the charge thereon.

According to a first aspect of the invention, there is provided a method of ionising molecules, the method comprising:

- passing a solution containing the molecules through an electrospray emitter which has a potential difference applied thereto relative to a housing thereof, in order to create a jet of solution; and
- passing a flow of CH_5^+ ions over the jet and droplets.

Thus, we have appreciated that the CH_5^+ ion can be used to supercharge molecules that have been subject to electrospray treatment, in particular electrospray ionisation.

Typically, as the jet leaves the electrospray emitter it will pass through a first region in which the jet is unfragmented and then a second region in which the jet fragments into droplets; as such, the flow may pass over the jet in the first region. This makes it easier for the $CH5^+$ ions to approach the solution as, when the jet fragments, the charge on each droplet due to the electrospray process will make it harder for the CH_5^+ to overcome the Coulomb repulsion inherent in approaching a (typically) identically charged droplet.

Typically, the method would also comprise the creation of the CH_5^+ ions, typically in a source of CH_5^+ ions. In one embodiment, this would comprise passing methane (CH_4) over an electrical arc or another source of electrons such as a hot filament, electron emitter, photoemission of electrons from molecules or surfaces by interaction with light, or a plasma created by heating, including use of an inductively coupled resonance plasma.

The electrospray emitter and the source of CH_5^+ ions may be provided within a chamber. As such, the method may comprise removing oxygen from the chamber, typically before CH_5^+ ions are created. This has the advantage that the method can then be carried out at atmospheric pressure, as there will be no combustion of the methane with the oxygen, which could lead to catastrophic failure of the chamber. The chamber may be sealed against the ingress of oxygen, at least after the oxygen is removed. The oxygen may be removed until at most 5%, 3%, 2% or 1% of the gas within the chamber is oxygen.

The oxygen may be removed by flushing the chamber with a gas which does not comprise oxygen (for example, nitrogen gas). Alternatively or additionally, the method may comprise reacting the oxygen, for example by controlled combustion or using an oxygen scavenger.

The chamber may be at atmospheric pressure, typically 1 atm (101.325 kPa) to within 10%, 5%, 2% or 1%. This has not been possible (or even contemplated) with prior art chemical ionisation techniques using CH_5^+.

In other embodiments, the pressure within the chamber may be higher than atmospheric pressure (e.g. two or three times atmospheric pressure), or lower than atmospheric pressure (e.g. up to or approaching a pure vacuum).

The solution may comprise a solvent, which may be water, methanol, acetonitrile, acetone, hexane, or any other convenient solvent with a proton affinity greater than the chemical ionization reagent gas, typically methane.

The method may comprise flowing a sheath gas over the jet as it exits the electrospray emitter; typically, the sheath gas may be a relative inert gas (e.g. compared to oxygen), such as nitrogen (N_2). The sheath gas may improve the nebulization of the jet (i.e. the fragmentation of the jet into droplets) and/or the evaporation of the solvent.

Alternatively, there may be no sheath gas passed over the jet, and the flow of CH_5^+ ions may pass over the jet without having to pass through a flow of sheath gas.

The flow may be produced substantially orthogonally to the jet; optionally, the flow may be within 15, 10 or 5 degrees of orthogonal, or the flow may be collinear with the jet.

Typically, the molecules are likely to be molecules, such as proteins, DNA, RNA, oligosaccharides, polymers, lipids, or organic and inorganic molecules.

The method may comprise passing the jet (which may have fragmented into droplets) to a mass spectrometry device after the flow has passed over it. The method may comprise carrying out mass spectrometry on the jet.

According to a second aspect of the invention, there is provided an electrospray ionisation apparatus, comprising:

- a housing defining a chamber therewithin;
- an electrospray emitter within the chamber provided with a potential difference source so as to provide a potential difference between the electrospray emitter and a housing of the emitter such that when a solution containing molecules is

introduced to the electrospray emitter with the potential difference applied, a jet of solution is emitted from the electrospray emitter; and

- a source of CH_5^+ ions within the chamber arranged to emit a flow of CH_5^+ ions;

in which the source of CH_5^+ ions is positioned so as to pass the flow over the jet.

Thus, we have appreciated that the CH_5^+ ion can be used to supercharge molecules that have been subject to electrospray treatment, in particular electrospray ionisation.

The electrospray emitter may comprise a tip where the jet is emitted from the electrospray emitter and, typically, as the jet leaves the electrospray emitter it will pass through a first region in which the jet is unfragmented and then a second region in which the jet fragments into droplets; the source of CH_5^+ ions may be positioned sufficiently close to the tip such that the flow passes over the jet in the first region.

It will generally be preferred that the CH_5^+ ions are allowed to react with the jet both before and while it fragments. When the jet fragments into positively charged droplets, the coulombic repulsion between the ions and the droplets will increase causing a decrease in the fraction of ions which have sufficient kinetic energy to approach the droplet.

The source of CH_5^+ ions may comprise an arc creation device (such as a needle raised to a high potential difference relative to a body) over which methane can be passed; the apparatus may then be provided with a source of methane (such as a bottle thereof). Alternatively, the source of CH_5^+ ions could be a reaction of the methane gas with any source of electrons. For example, the source may comprise a hot filament and/or an electron emitter over which methane can be passed. Other sources may include a light source which may result in photoemission of electrons from molecules or surfaces due to interaction with the emitted light.

The apparatus may comprise a means for heating the CH_5^+ ions, such as a heater or plasma generated by photons or inductive coupled heating. This has the effect of increasing the kinetic energy on the CH_5^+ ions, improving their ability to overcome the potential repulsion barrier and donate their proton, thus increasing the charge on the jet and droplets.

The apparatus may comprise means for removing oxygen from the chamber. This has the advantage that the method can then be carried out at atmospheric pressure, as there will be no combustion of the methane with the oxygen, which could lead to catastrophic failure of the chamber. The chamber may be sealed against the ingress of oxygen, at least after the oxygen is removed.

The oxygen may be removed by flushing the chamber with a flushing gas which does not comprise oxygen (for example, nitrogen gas); as such, the means for removing oxygen may comprise a pair of ports for introducing and release the flushing gas, and/or a source of the flushing gas. Alternatively or additionally, the means for removing the oxygen may comprise means for reacting the oxygen, for example a controlled combustion device or an oxygen scavenger. The apparatus may be provided with an oxygen sensor, so as to determine when the oxygen level is sufficiently low as to allow the generation of CH_5^+ ions.

In use, the chamber may be at atmospheric pressure, typically 1 atm (101.325 kPa) to within 10%, 5%, 2% or 1%. This has not been possible (or even contemplated) with prior art chemical ionisation techniques using CH_5^+.

The apparatus may comprise a source for a flow of a sheath gas over the jet as it exits the electrospray emitter; typically, the sheath gas may be a relative inert gas, such as nitrogen (N_2). Alternatively, there may be no sheath gas passed over the jet, and the flow of CH_5^+ ions may pass over the jet without having to pass through a flow of sheath gas.

Typically, the molecules are likely to be large molecules, such as proteins, DNA, RNA, oligosaccharides, polymers, lipids, or organic and inorganic molecules.

The chamber may be provided with a port for allowing the jet to leave the chamber; typically, this would mate with a suitable mass spectrometry apparatus.

The apparatus may be used to carry out the method of the first aspect of the invention.

According to a third aspect of the invention, there is provided a method of ionising molecules, the method comprising:

- passing a solution comprising the molecules dissolved in a solvent through an electrospray emitter which has a potential difference applied thereto relative to a housing of the emitter, in order to create a jet of solution; and
- passing a flow of ions over the jet, the ions having a lower proton affinity than molecules of the solvent.

Thus, we have appreciated that the teachings of the first two aspects of the invention could be extended to other ions that will preferably donate a proton to the solution.

Typical ions for the flow of ions could include protonated ions of methane (e.g. CH_5^+ ions) or ions of other gases such as ethane, acetylene, acetone, or any other ions which have a lower proton affinity than the solvent molecules in the solution.

The electrospray emitter and a source of the flow of ions may be provided within a chamber. The pressure within the chamber may be at atmospheric pressure, typically 1 atm (101.325 kPa) to within 10%, 5%, 2% or 1%.

In other embodiments, the pressure within the chamber may be higher than atmospheric pressure (e.g. two, or three or more times atmospheric pressure), or lower than atmospheric pressure (e.g. up to or approaching a pure vacuum).

The method may have any of the optional features of the first aspect of the invention, in which the reference to the CH_5^+ ions are replaced by a reference to the ions having a lower proton affinity.

According to a fourth aspect of the invention, there is provided an electrospray ionisation apparatus, comprising:

- a housing defining a chamber therewithin;
- an electrospray emitter within the chamber provided with a potential difference source so as to provide a potential difference between the electrospray emitter and a housing of the emitter such that when a solution comprising molecules dissolved in a solvent is introduced to the electrospray emitter with the potential difference applied, a jet of solution is emitted from the electrospray emitter; and
- a source of ions within the chamber arranged to emit a flow of ions;

in which the source of ions is positioned so as to pass the flow over the jet and in which the ions have a lower proton affinity than molecules of the solvent.

Similarly, the apparatus may have any of the optional features of the second aspect of the invention, in which the reference to the CH_5^+ ions are replaced by a reference to the ions having a lower proton affinity than the molecules of the solvent.

Typical ions for the flow of ions could include protonated ions of methane (e.g. CH_5^+ ions) or ions of other gases such as ethane, acetylene, acetone, or any other ions which have a lower proton affinity than the solvent molecules in the solution.

There now follows, by way of example only, description of embodiments of the invention, described with reference to the accompanying drawings, in which:

Figure 1 shows a cross section through an electrospray ionisation apparatus in accordance with an embodiment of the invention;

Figure 2 shows a perspective exploded view of the apparatus of Figure 1;

Figure 3 shows a schematic view of the emission of a jet of fluid within the apparatus of Figure 1;

Figure 4 shows an enlarged view of Figure 4; and

Figures 5 to 8 each show a pair of mass spectrometry spectra showing the effect of using the apparatus of the embodiment of Figure 1.

The embodiments described below utilize the possibility of enhancing the charging, i.e. supercharging, through a direct reaction between methane super-acid, i.e. CH_5^+ ions (Brønsted acid), with the sprayed solution droplets of an analyte at atmospheric pressure during the electrospray ionisation (ESI) process. Due to their inherent strong protonation capability, and extremely low proton affinity, CH_5^+ "super-acid ions" are ideal species for exploring the combination of ESI and gas-phase supercharging via APCI generated reagent ions. Enhanced droplet charge translates to increased protonation of charge sites in proteins and increased intensity and/or higher observed

charge states of analyte species. Potentially, supercharging MS analytes with superacid CH_5^+ ions will aid the analysis of slightly polar compounds, large and small biomolecules (such as peptides and proteins) and enable increased cleavage coverage during subsequent MS/MS experiments which can benefit Top-down protein analysis and the entire Bottom-Up proteomics field as, in theory, it is possible to hyphenate this concept to liquid chromatography readily.

An electrospray ionisation apparatus in accordance with an embodiment of the invention is shown in Figures 1 and 2 of the accompanying drawings. The apparatus comprises a chamber 2 into which a copper/brass sheath 3 extends. In other examples, a brass sheath 3 may be used. The sheath has a passage 4 therewithin through which a solution comprising a solvent and molecules of an analyte can pass. The passage 4 terminates at a tip 5 of the sheath 3 where the passage 4 is open into the chamber. A potential difference can be applied between the sheath 3 and the frame of the instrument. The sheath 3 therefore forms an electrospray ionisation (ESI) needle.

The chamber is also provided with a source of CH_5^+ ions 6. This comprises a glow discharge chamber which comprises a cylindrical wall 7 and a central needle 8. Placing a DC voltage across the wall 6 and needle 8 will generate a spark 9. The source 6 is also provided with a port 10 which allows the introduction of methane (CH_4) from outside of the chamber 2 into the source 6 so that it passes over the spark 9.

The chamber is also provided with a port 11 for the introduction of a sheath gas (e.g. Nitrogen (N_2)) into the chamber. It also includes a one-way pressure relief valve 13, which will actuate to relieve the pressure in the chamber if it is more than 5 psi (34 kPa) above local atmospheric pressure.

There is furthermore a port 12 for connection to a mass spectrometry device.

Typically, the oxygen is purged from the chamber by flushing the chamber with the methane or sheath gas (e.g. Nitrogen) before the DC voltage is turned on. The methane or sheath gas may be allowed to flow through the chamber for at least one minute before the DC voltage is turned on.

In use, the super-acid CH_5^+ is produced by ionizing a stream of methane gas flowing through a potential difference region between the needle 8 and the walls 7. The source 6 comprises a tailor-made threaded brass cylinder 7 isolated from both the sheath 3 and the needle 8 via insulating Teflon caps. It has a tapered shape along the inside of the chamber; the position of the needle 8 ensures the flow of methane is focused into the exit of the source 6, where the electrical discharge (spark 9) occurs, improving the efficiency of CH_5^+ production, which occurs nearby the ESI needle tip 5.

The distance between the tip 5 of the ESI needle and the mass spectrometer port 12 is around 1.7 cm. Nebulizing gas (Nitrogen) was introduced via the sheath gas port 11 surrounding the ESI needle 3, enabling effective ESI plume generation.

Analyte solution can be delivered to the ESI needle 3 via a 250 μL (microlitre) syringe connected to a transfer line and driven via a syringe pump at a rate of 100-200 μL/hour. The ESI needle was biased from +4000 to +6000 V to achieve stable ESI. A voltage difference of +3500 V was applied to the glow discharge system via an external high-voltage power supply.

A cylindrical mesh (not shown) surrounded the ESI assembly and was held at positive potential to focus the ions, allowing the improved interaction between the generated super-acid CH_5^+ and the sample solution in the gas phase and preventing charge build up on the source walls. Finally, an aluminium flange connects the apparatus 1 to the inlet of the (in this embodiment) Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FT-ICR) mass spectrometer.

The effect of super-acid CH_5^+ as a supercharging reagent depends on geometry, pressure, flow rate, and the potential difference between the ESI needle and mass spectrometer inlet. Different pressures/flow rates of methane did not show appreciable differences on the supercharging effect past 0.3 bar. The distance between the tip of the ESI needle and origin of super-acid CH_5^+ generation has been found to play a role in the supercharging process.

The operation of the apparatus 1 can be demonstrated with respect to Figures 3 and 4 of the accompanying drawings. The analyte 20 is introduced through the sheath 3 and exits the tip 5 surrounded by sheath gas (nitrogen) 21 to form a jet 23. The flow 22 of

CH_5^+ ions is introduced orthogonally to the jet 23, but is carried along the jet 23 by sheath gas 21.

Due to the effects of the electrospray process, the solution is emitted from the tip 5 with a positive charge. This means that, as the solvent evaporates, the jet will tend to break up into finer and finer droplets 24. The flow 22 is arranged to flow over the jet 23 before it disperses into droplets 24.

The effects of the flow can be seen in Figure 4. The CH_5^+ ions are shown surrounding a droplet 24. They approach the droplet (the Coulombic repulsion not yet being insurmountable) and donate protons to the droplet, leaving as methane (CH_4) molecules 26. The droplets are therefore supercharged; as the solvent evaporates, the charge donated by the CH_5^+ ions will preferentially be retained on the analyte molecules.

Examples

All experiments were conducted on a 12 Tesla SolariX Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FT-ICR MS) (Bruker Daltonik GmbH, Bremen, Germany), all mass spectra were collected in positive ion mode. Supercharging experiments were conducted using the apparatus 1 with the external high voltage off (for normal ESI control experiments) and on with methane flowing (for super-acid supercharging experiments). Control spectra conducted with methane flowing through the discharge chamber, without the external high voltage glow discharge on yielded the same mass spectral results as the normal ESI control. The FT-ICR instrument was tuned to scan 147 to 3000 m/z and 10-20 spectra were signal-averaged per run. Ions were externally accumulated in a hexapole collision cell for 0.1-0.2 s before being transferred to an infinity cell (e.g. an Ion Cyclotron Resonance trap) for excitation and detection. An example of an infinity cell is disclosed in Caravatti, P., Allemann, M., RF Shim by Trap Segmentation, *Org. Mass Spectrom*, **1991**, 26, 514-518.

Bovine heart Cytochrome C, chicken egg-white Lysozyme, and Equine heart Myoglobin were obtained from Sigma-Aldrich, UK. The samples were prepared by dissolving the proteins in solvent system that consists of either purified water 18 MΩ

cm^{-1} only or 50/50 %, V/V water/methanol. Methane reagent gas was purchased from CK special gases (UK). L-(+)-ascorbic acid ($C_6H_8O_6$), was purchased from Alfa Aesar, Heysham, Lancashire, UK. High purity grade N_2 (99.99 % BOC, UK) was used as the nebulizer gas for ESI and APCI-ESI experiments. Ultrapure water (18.2 MΩ cm^{-1}) was obtained from a Direct-Q water purification unit (Millipore, Watford, UK) and LC-MS grade methanol (VWR Chemicals, UK) were used in the sample preparation. All reagents were used as received.

Figure 5a shows the measured MS spectrum of 1μM Lysozyme dissolved in water, a very narrow range of charge states was observed (8^+, 9^+, and 10^+).

Figure 5b shows the same protein-containing solution ionized via the apparatus 1. The supercharged lysozyme spectrum shows the 11+ charge state of the protein appearing in good intensity and a decrease of lower charge states (8^+ and 9^+), focusing ion populations into the higher charge states. The weighted average charge state for each spectrum was calculated via equation 5 (below) where N is the number of observed charge states i^{th}, q_i is the charge value and W_i is the signal intensity. The calculations show a distinct increase in average charge from 9.41 (without the apparatus functioning) to 10.02 (with the apparatus functioning). The incorporation of apparatus derived CH_5^+ ions has clearly enhanced charging of the protein analyte species.

$$q_{average} = \sum_i^N qi\,Wi \,/ \sum_i^N Wi \qquad \text{[Eq. 5]}$$

Figure 5 insets (a.1-3 and b.1-3) show the protonated lysozyme species from both the apparatus 1 non-functioning and the apparatus functioning spectra, along with the protonated species sodium adducts are observed. The comparison of the two spectra shows that no change/modification of the analyte species has been observed (e.g. via methylation, CH_5^+ adduction, or reaction with any other intermediates from equations 1-5 (above)), the ionization method of this embodiment is shown to produce a similar distribution of protonated and sodium-adduct peaks, only with the benefit of enhanced charge states and with increased signal intensity.

Figure 6a and 6b show the apparatus-not-functioning and apparatus-functioning mass spectra respectively of denatured myoglobin (a 16.9 kDa oxygen transport protein) electrosprayed from a 50:50 v/v water:methanol solution. Denatured Myoglobin shows

a range of charge states upon ESI-MS ranging from 10^+ to 17^+ (Figure 6a). The denatured myoglobin ionized via the apparatus 1 attained a much greater increase in charge states (see inset), previously unobtainable without supercharging.

Denaturation of Myoglobin causes loss of the non-covalently-bound Heme group, however ESI-MS does not show a detectable Heme species released. Using the apparatus 1 shows increased signal of the $[Heme]^+$ ion released upon Myoglobin denaturation, allowing it to be detected. The normal spectrum of denatured Myoglobin showed a weighted average charge of 14.5, a base charge state of 15^+, and a max charge state of 18^+. The spectrum of Myoglobin using the apparatus showed an average charge of 16.7, a base charge of 17^+, and a maximum charge of 25^+.

The ability to access these increased charge states for protein analytes can have a drastic effect on the quality of MS/MS spectra and sequence information produced. McLafferty *et al* (Horn, D. M.; Breuker, K.; Frank, A. J.; Mclafferty, F. W.; V, C. U.; York, N.; August, R. V. **2001**, 9792–9799.) showed that each individual charge states of proteins can produce different MS/MS fragments during electron based dissociation (ECD or ETD, for example), and that higher charge states provided much more sequence information than lower ones, this effect has also been observed for the more commonly used collision based dissociation (CAD/CID).

Though other chemical-based supercharging methods have been developed, and successfully implemented, in many studies, this embodiment represents a new method of achieving these charge states without the addition of chemical additives to the sample solution, and thus avoiding unwanted side reactions/changes in chemical equilibria which can occur upon addition of new species to solution chemical mixtures. In addition, use of this apparatus 1 requires no further sample preparation steps, unlike chemical additive-supercharging, as each sample is simply introduced in the same way as a normal ESI-MS sample.

There is an increasing interest in attempting to retain protein structure during ionization and mass spectrometry analysis, the so-called Native-MS method. Usually involving the use of stabilizing buffers and sometime salt-containing solutions, Native MS attempts to keep proteins analytes folded in their solution conformation as much as possible during analysis, thus charge state distributions, surface labelling strategies

(such as Hydrogen-Deuterium Exchange (HDX; see Katta, V.; Chait, B. T., Hydrogen-Deuterium Exchange Electrospray-Ionization Mass-Spectrometry - a Method for Probing Protein Conformational-Changes in Solution. *Journal of the American Chemical Society* **1993,** *115* (14), 6317-6321) and Fast Photochemical Oxidation of Proteins (FPOP; see Chen, J.; Rempel, D. L.; Gau, B. C.; Gross, M. L., Fast photochemical oxidation of proteins and mass spectrometry follow submillisecond protein folding at the amino-acid level. *J Am Chem Soc* **2012,** *134* (45), 18724-31)), and careful MS/MS experiments can then inform and interrogate tertiary protein structure via mass spectrometry.

Unfortunately the use of high-concentration buffer solutions and/or salt solutions are detrimental to effective ESI, desolvation, and protein MS analysis as a whole. As a result Native MS usually produces low signal to noise spectra (due to ineffective desolvation), with protein species observed with many adducted salt/buffer ions (decreasing signal to noise further as analyte ions are distributed into many signal channels), and very low charge states compared to water/denatured conditions. The latter of these disadvantages decreases observed intensity of ions (as many MS techniques are inherently more sensitive for higher charge state ions), and dramatically impedes MS/MS fragmentation characterization and subsequent sequence coverage. Therefore effective characterization of proteins in their native state is particularly challenging, and would benefit greatly from the enhanced charging effects supercharging MS can offer.

As a result Native MS of Lysozyme was conducted using the Supercharging ESI-APCI source. The results, shown in Figure 7, show that Native MS (without using the apparatus 1) of the Lysozyme protein presents a narrow charge state distribution, at lower charge states than the water solution above (Figure 7a). Lysozyme in the 7^+, 8^+, and 9^+ charge states were observed at relatively low signal to noise ratio.

Using the apparatus 1 on the same solution of Lysozyme (aqueous 200mM ammonium acetate buffer) as shown in Figure 7b, shows the effect of enhanced supercharging on the sample; the observed intensity of the Lysozyme peaks has increased by on average 285%, a dramatic increase over ESI-MS. The same charge states are with and without use of the apparatus 1, despite the obvious enhanced charging, suggesting that, in its

native conformation, the available protonation sites of lysozyme have been fully protonated, with multiple charge states being observed due to different conformations.

The large increase in both intensity and signal to noise for the native lysozyme species is of great benefit for both detection and accurate characterization of the protein species by MS, an should be readily applicable for other protein species in the future, and aid in subsequent Top-Down MS/MS analysis.

In addition to biomolecule supercharging, small molecule ionization can vary greatly depending on the chemical nature of the groups within the analyte, with some (particularly non-polar) species being particularly difficult to ionize effectively via ESI. As a result, the effect of the apparatus 1 on small molecule ionization was investigated using ascorbic acid. Ascorbic acid ($C_6H_8O_6$) is a small (176.032088Da) molecule, a form of vitamin C, believed to provide anti-oxidant activity in the body. Figure 8a and 8b show the spectra without and with the apparatus 1 of ascorbic acid. The main species observed in the spectra of Ascorbic acid without using the apparatus 1 were the protonated and Sodiated forms of ascorbic acid ($[C_6H_8O_6+H]^+$ and $[C_6H_8O_6+Na]^+$), along with a high intensity peaks for the protonated and Sodiated Ascorbic acid dimer ($[C_6H_8O_6\ C_6H_8O_6+H]^+$ and $[C_6H_8O_6\ C_6H_8O_6+Na]^+$).

The spectrum using the apparatus 1 is shown in figure 8b; though no peak corresponding to doubly charged Ascorbic acid $[C_6H_8O_6+2H]^{2+}$ was observed, the singly protonated ascorbic acid species was shown to increase in intensity by 22%, with respect to ESI only (Figure 8b, inset a.1 and b.1), this increase is believed to be a result from the increased proton transfer from the CH_5^+ reagent gas and neutral ascorbic acid species. Figure 8b (inset a.2 and b.2) also shows a much larger increase in observed intensity for the ascorbic acid dimer species of 291%, which is a substantial increase over traditional electrospray.

It appears that not only can CH_5^+ assist in attaining higher charge states for biomolecules, but also increase the ionization efficiency of small molecules, on average peak intensities increased by 103% (and up to 291% for some species), this could lead to increased limits of detection (LOD) for small molecule species and enable more sensitive MS detection of key compounds during analysis where low

LOD's are crucial, e.g. trace organic analysis, forensic testing, trace heavy metal quantitation etc.

Whilst the examples above have been given with respect to CH_5^+ as the supercharging ions, we appreciate that it may be possible to make use of any molecules that have a low proton affinity, particularly less than water (697 kJ/mol) or the solvent used for the electrospray solution (i.e. the molecules of solvent). For example, other ions such as ethane, hexane, acetylene, acetone could be used, provided that the proton affinity of the supercharging reagent gas is lower than the proton affinity of the electrospray solution solvent molecules.

CLAIMS

1. A method of ionising molecules, the method comprising:
   - passing a solution containing the molecules through an electrospray emitter which has a potential difference applied thereto, in order to create a jet of solution; and
   - passing a flow of CH_5^+ ions over the jet.

2. The method of claim 1, in which, as the jet leaves the electrospray emitter it will pass through a first region in which the jet is unfragmented and then a second region in which the jet fragments into droplets; as such, the flow may pass over the jet in the first region.

3. The method of claim 1 or claim 2, comprising the creation of the CH_5^+ ions, typically in a source of CH_5^+ ions.

4. The method of claim 3, in which the electrospray emitter and the source of CH_5^+ ions are provided within a chamber, the method comprising removing oxygen from the chamber, typically before CH_5^+ ions are created.

5. The method of claim 4, in which the chamber is at atmospheric pressure, typically 1 atm (101.325 kPa) to within 10%, 5%, 2% or 1%.

6. The method of any preceding claim, comprising flowing a sheath gas over the jet as it exits the electrospray emitter; typically, the sheath gas may be a relative inert gas such as nitrogen (N_2).

7. The method of any preceding claim, in which the flow is produced substantially orthogonally to the jet.

8. An electrospray ionisation apparatus, comprising:
   - a housing defining a chamber therewithin;
   - an electrospray emitter within the chamber provided with a potential difference source so as to provide a potential difference between the electrospray emitter and a housing of the emitter such that when a solution containing molecules is

introduced to the electrospray emitter with the potential difference applied, a jet of solution is emitted from the electrospray emitter; and

- a source of CH_5^+ ions within the chamber arranged to emit a flow of CH_5^+ ions;

in which the source of CH_5^+ ions is positioned so as to pass the flow over the jet.

9. The apparatus of claim 8, in which the electrospray emitter comprises a tip where the jet is emitted from the electrospray emitter and in which, as the jet leaves the electrospray emitter it will pass through a first region in which the jet is unfragmented and then a second region in which the jet fragments into droplets; the source of CH_5^+ ions being positioned sufficiently close to the tip such that the flow passes over the jet in the first region.

10. The apparatus of claim 8 or claim 9, in which the source of CH_5^+ ions comprises an arc creation device over which methane can be passed.

11. The apparatus of any of claims 8 to 10, comprising a means for heating the CH_5^+ ions.

12. The apparatus of any of claims 8 to 11, comprising means for removing oxygen from the chamber.

13. The apparatus of any of claims 8 to 12, provided with an oxygen sensor.

14. The apparatus of any of claims 8 to 13, comprising a source for a flow of a sheath gas over the jet as it exits the electrospray emitter.

15. A method of ionising molecules, the method comprising:

- passing a solution comprising the molecules dissolved in a solvent through an electrospray emitter which has a potential difference applied a housing of the emitter in order to create a jet of solution; and
- passing a flow of ions over the jet, the ions having a lower proton affinity than molecules of the solvent.

16. The method of claim 15, in which the electrospray emitter and a source of the flow of ions may be provided within a chamber, the pressure within the chamber being at atmospheric pressure, typically 1 atm (101.325 kPa) to within 10%, 5%, 2% or 1%.

17. The method of claim 15 or claim 16, wherein the ions are selected from the group comprising ethane, hexane, acetylene and acetone.

18. An electrospray ionisation apparatus, comprising:

- a housing defining a chamber therewithin;
- an electrospray emitter within the chamber provided with a potential difference source so as to provide a potential difference between the electrospray emitter and a housing of the emitter such that when a solution comprising molecules dissolved in a solvent is introduced to the electrospray emitter with the potential difference applied, a jet of solution is emitted from the electrospray emitter; and
- a source of ions within the chamber arranged to emit a flow of ions;

in which the source of ions is positioned so as to pass the flow over the jet and in which the ions have a lower proton affinity than molecules of the solvent.

19. The apparatus of claim 18, wherein the ions are ethane, hexane, acetylene, or acetone.

20. The apparatus of claim 18 or claim 19, wherein the ions have a proton affinity of less than 697 kJ/mol.

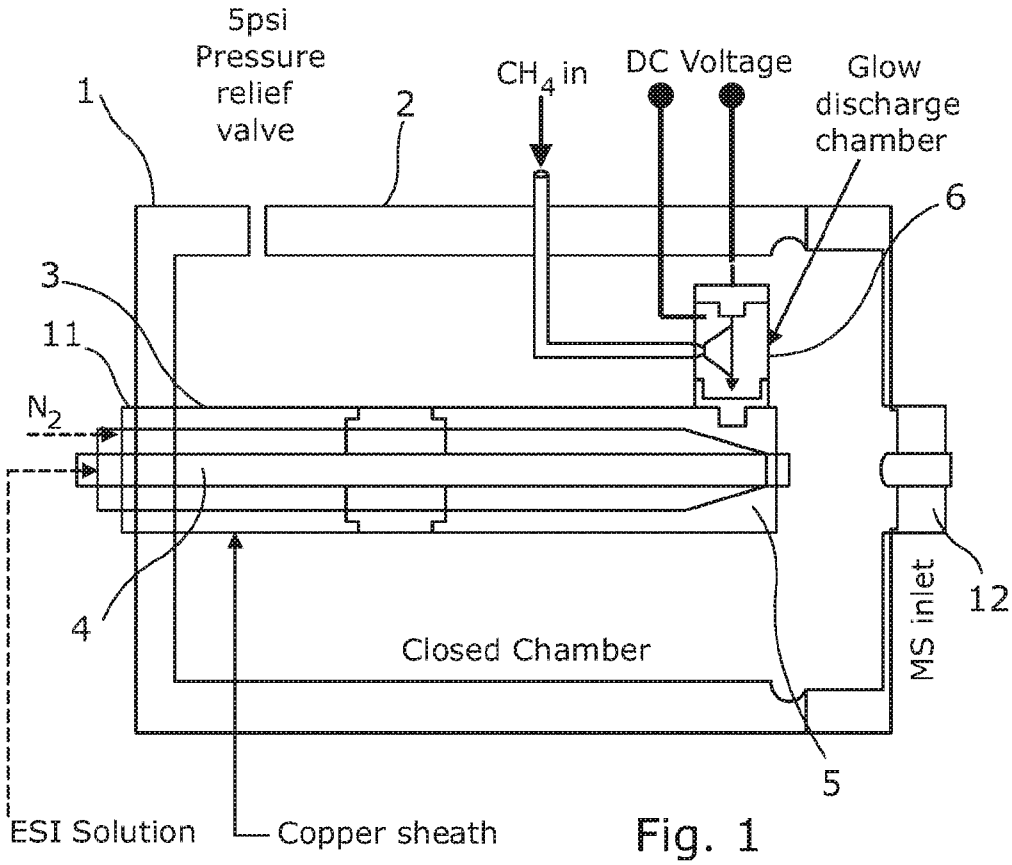
5psi
Pressure
relief
valve
1
2
CH_4 in
DC Voltage
Glow
discharge
chamber
6
3
11
N_2
4
Closed Chamber
MS inlet
12
5
ESI Solution
Copper sheath

Fig. 1

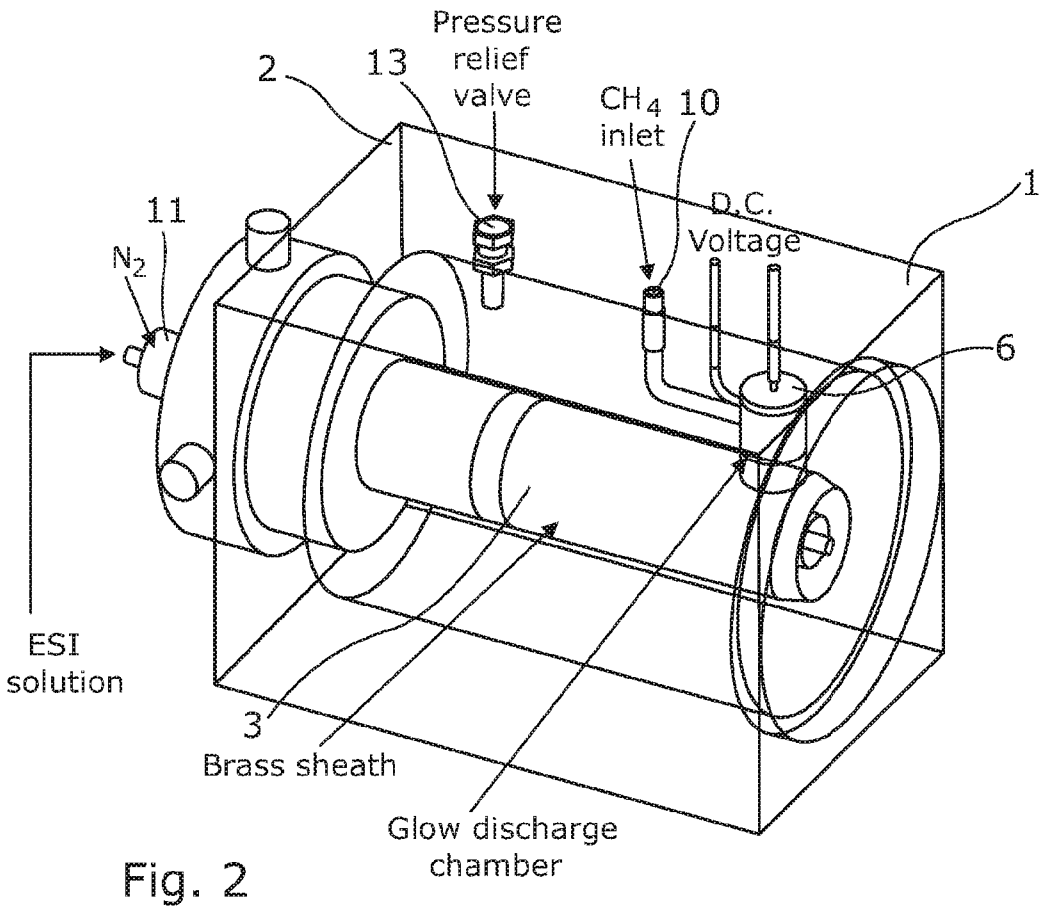
Pressure
relief
valve
2
13
CH_4
inlet
10
D.C.
Voltage
1
11
N_2
6
ESI
solution
3
Brass sheath
Glow discharge
chamber

Fig. 2

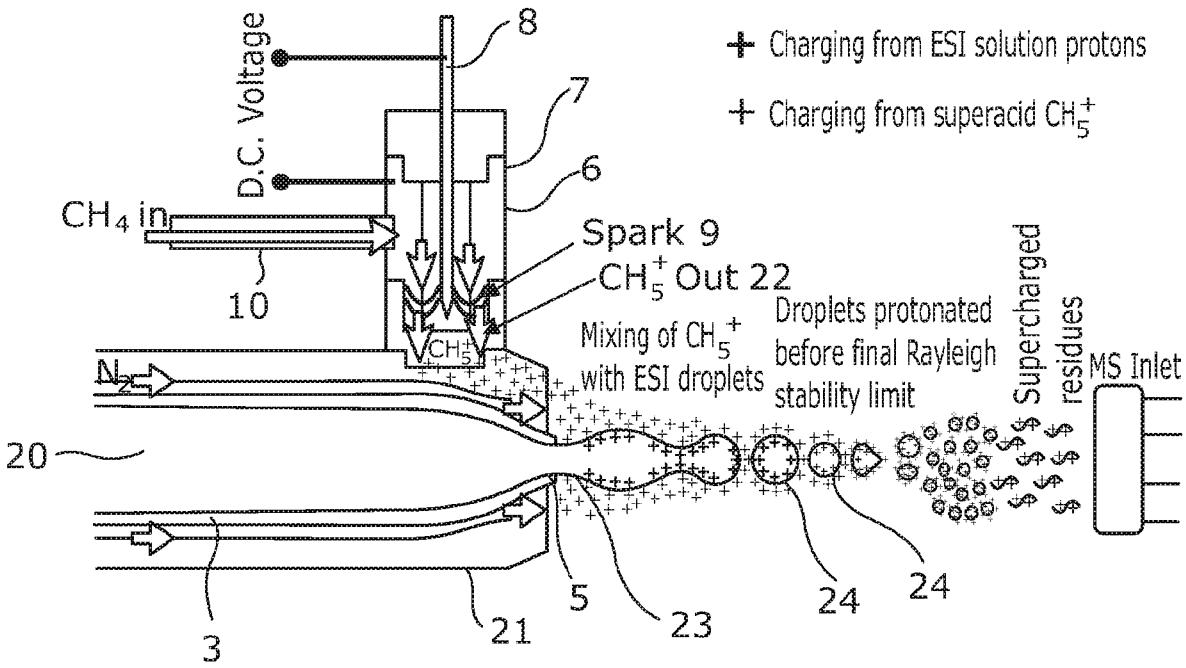
D.C. Voltage
8
7
6
Charging from ESI solution protons
Charging from superacid CH_5^+
CH_4 in
10
Spark 9
CH_5^+ Out 22
Mixing of CH_5^+ with ESI droplets
Droplets protonated before final Rayleigh stability limit
Supercharged residues
MS Inlet
N_2
20
3
21
5
23
24
24

Fig. 3

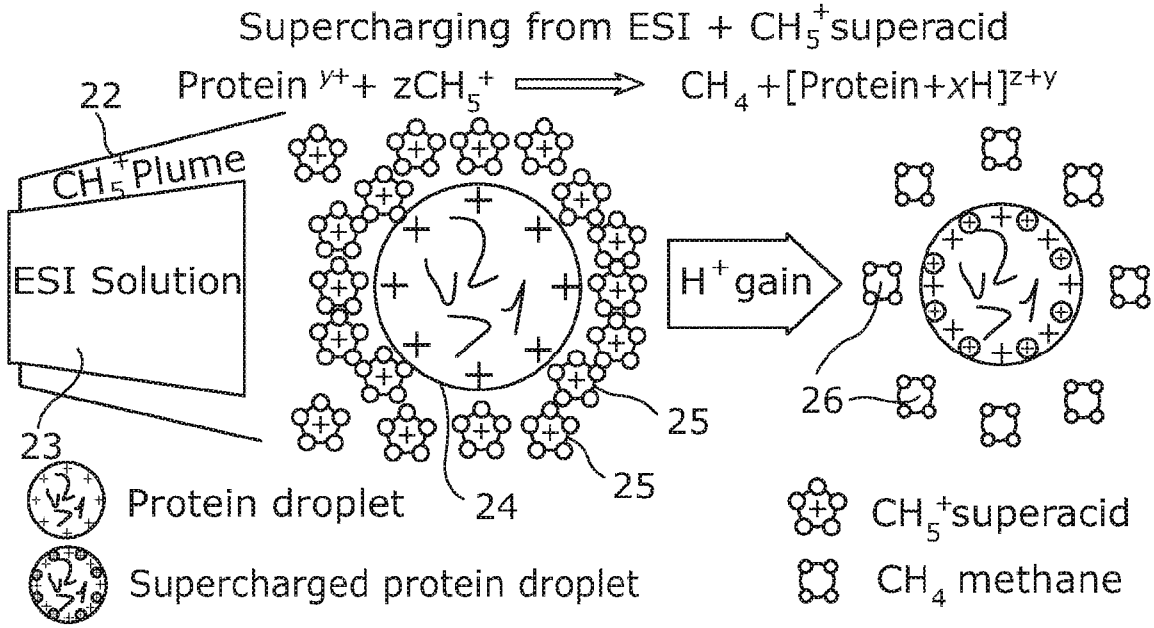
Supercharging from ESI + CH_5^+superacid
Protein $^{y+}$ + zCH_5^+ ⟹ CH_4 + [Protein+xH]$^{z+y}$
22
CH_5^+Plume
ESI Solution
23
H^+gain
25
25
24
26
Protein droplet
Supercharged protein droplet
CH_5^+superacid
CH_4 methane

Fig. 4

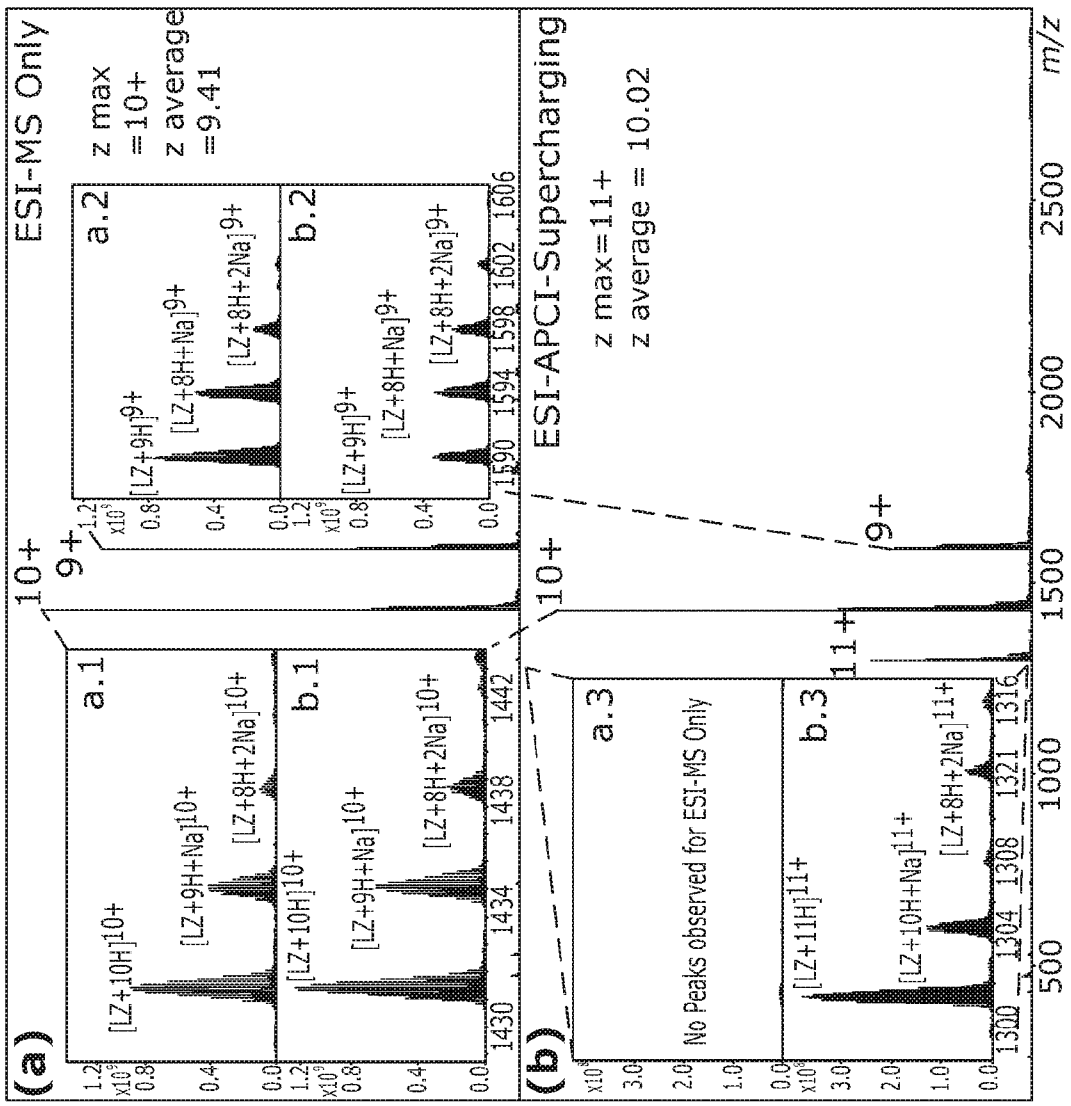
(a)
ESI-MS Only
z max
=10+
z average
=9.41
10+
9+
a.1
b.1
a.2
b.2
[LZ+10H]10+
[LZ+9H+Na]10+
[LZ+8H+2Na]10+
[LZ+9H]9+
[LZ+8H+Na]9+
[LZ+8H+2Na]9+
(b)
ESI-APCI-Supercharging
z max=11+
z average = 10.02
10+
9+
11+
a.3
b.3
No Peaks observed for ESI-MS Only
[LZ+11H]11+
[LZ+10H+Na]11+
[LZ+8H+2Na]11+
m/z

Fig. 5

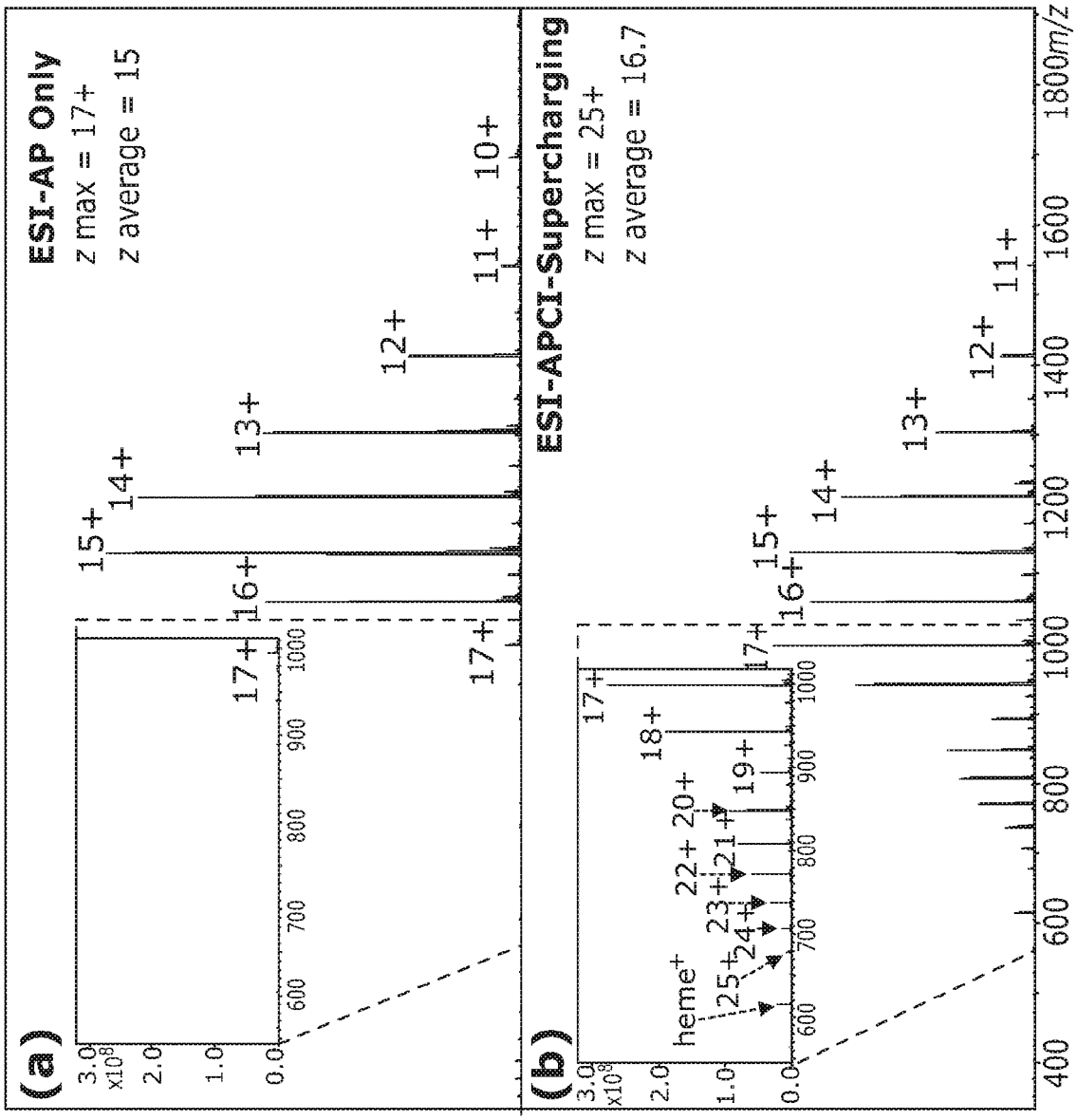
(a)
ESI-AP Only
z max = 17+
z average = 15
17+
16+
15+
14+
13+
12+
11+
10+
(b)
ESI-APCI-Supercharging
z max = 25+
z average = 16.7
heme+
17+
18+
19+
20+
21+
22+
23+
24+
25+
m/z

Fig. 6

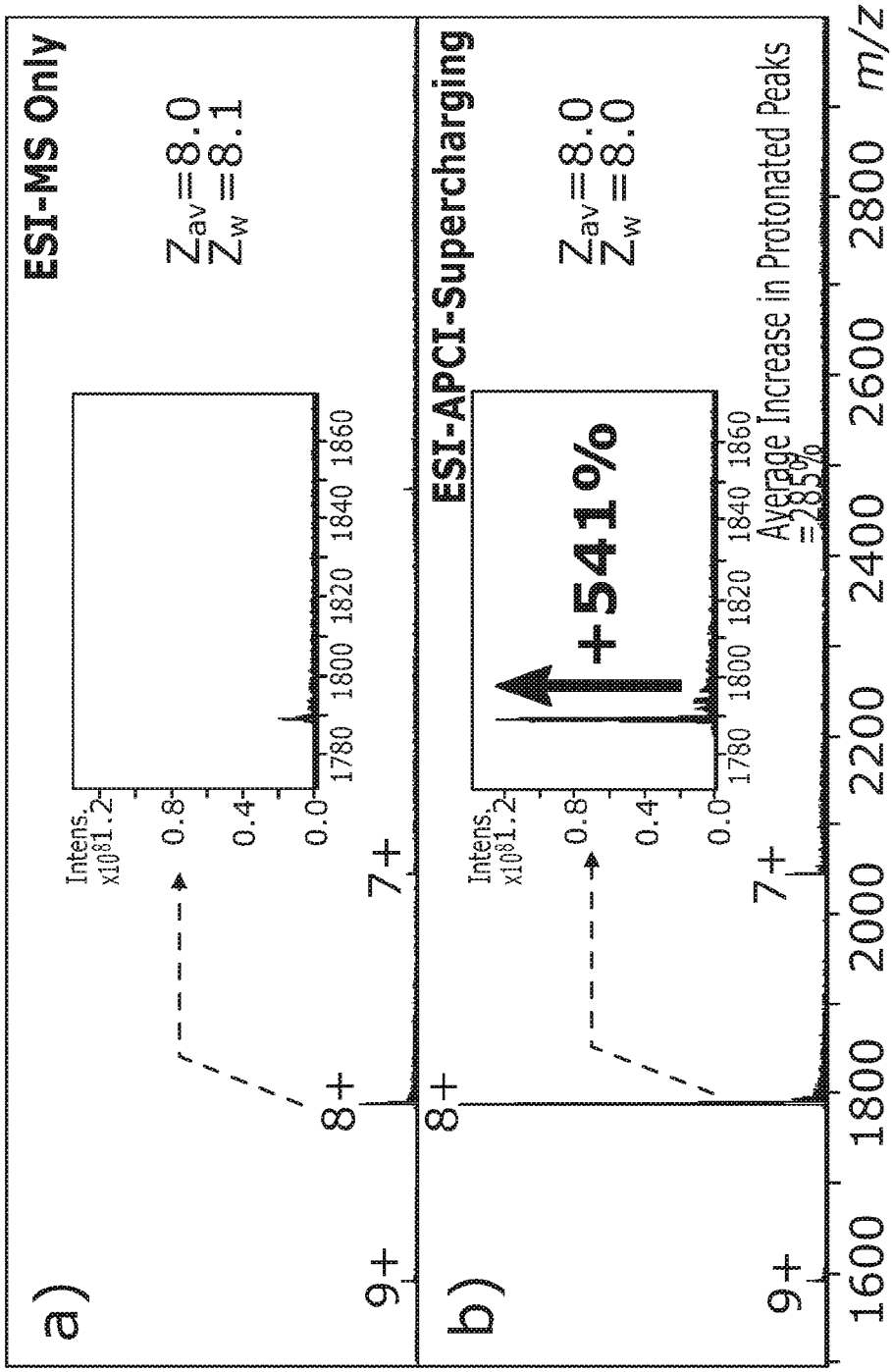
a)
ESI-MS Only
Z_{av}=8.0
Z_w=8.1
Intens.
x10^8
1.2
0.8
0.4
0.0
1780 1800 1820 1840 1860
8+
7+
9+
b)
ESI-APCI-Supercharging
Z_{av}=8.0
Z_w=8.0
+541%
Average Increase in Protonated Peaks
=285%
1600 1800 2000 2200 2400 2600 2800 m/z

Fig. 7

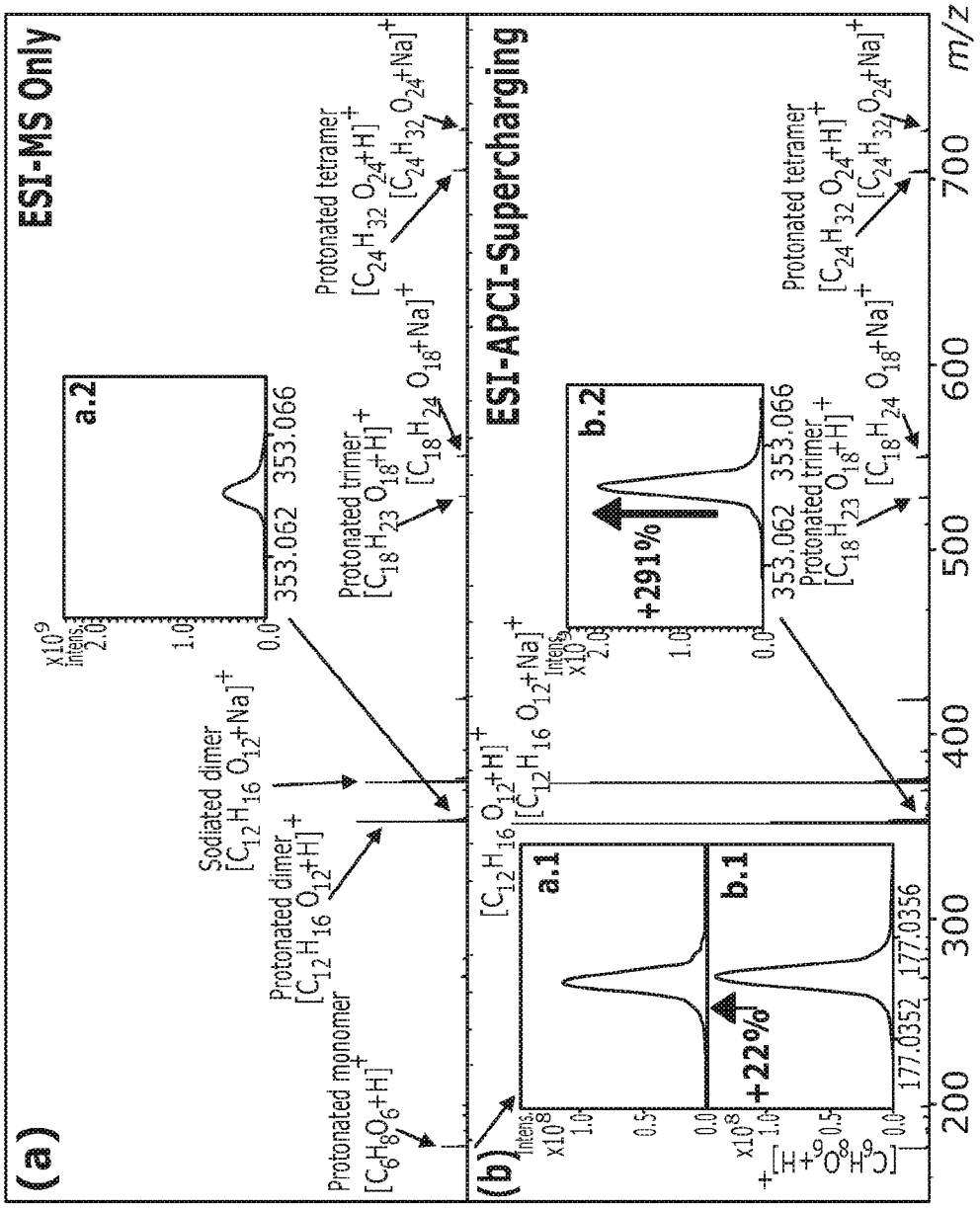
(a)
ESI-MS Only
Protonated monomer
[C6H8O6+H]+
Protonated dimer
[C12H16O12+H]+
Sodiated dimer
[C12H16O12+Na]+
Protonated trimer
[C18H23O18+H]+
[C18H24O18+Na]+
Protonated tetramer
[C24H32O24+H]+
[C24H32O24+Na]+
a.2
353.062
353.066
(b)
ESI-APCI-Supercharging
[C12H16O12+H]+
[C12H16O12+Na]+
a.1
b.1
+22%
177.0352
177.0356
b.2
+291%
353.062
353.066
Protonated trimer
[C18H23O18+H]+
[C18H24O18+Na]+
Protonated tetramer
[C24H32O24+H]+
[C24H32O24+Na]+
m/z
200
300
400
500
600
700

Fig. 8

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2017/050914

A. CLASSIFICATION OF SUBJECT MATTER
INV. H01J49/14 H01J49/10 H01J49/16 H01J27/20
ADD.

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)
H01J

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SASA M. MILADINOVIC ET AL: "In-Spray Supercharging of Peptides and Proteins in Electrospray Ionization Mass Spectrometry", ANALYTICAL CHEMISTRY, vol. 84, no. 11, 5 June 2012 (2012-06-05), pages 4647-4651, XP055140099, ISSN: 0003-2700, DOI: 10.1021/ac300845n	15,16, 18,20
A	section "Methods" on page 4648 figure accompanying the abstract	1-14,17, 19

-/--

[X] Further documents are listed in the continuation of Box C.

[X] 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	Date of mailing of the international search report
13 June 2017	21/06/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Cornelussen, Ronald

2

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2017/050914

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	M. S. B. MUNSON ET AL: "Chemical Ionization Mass Spectrometry. I. General Introduction", JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, vol. 88, no. 12, 1 June 1966 (1966-06-01), pages 2621-2630, XP055194093, ISSN: 0002-7863, DOI: 10.1021/ja00964a001 cited in the application the whole document	1-20
A	US 2015/097114 A1 (GREEN MARTIN RAYMOND [GB] ET AL) 9 April 2015 (2015-04-09) paragraph [0176] figure 1	1-20
A	CHEN A. TEO ET AL: "Solution Additives for Supercharging Proteins beyond the Theoretical Maximum Proton-Transfer Limit in Electrospray Ionization Mass Spectrometry", ANALYTICAL CHEMISTRY, vol. 86, no. 9, 6 May 2014 (2014-05-06), pages 4455-4462, XP055378832, US ISSN: 0003-2700, DOI: 10.1021/ac500304r the whole document	1-20
T	Christopher A Wootton: "Chapter 7: Super-acid supercharging using CH5 +", Exploring biomolecules, metallodrugs, and their interactions via the use of UHR-FT-ICR Mass Spectrometry, 1 April 2016 (2016-04-01), pages 327-366, XP055378239, Retrieved from the Internet: URL:http://wrap.warwick.ac.uk/85417/1/WRAP_Theses_Wootton_2016.pdf [retrieved on 2017-06-02] the whole document	

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2017/050914

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2015097114 A1	09-04-2015	CA 2873613 A1	21-11-2013
		EP 2850639 A2	25-03-2015
		EP 3048635 A1	27-07-2016
		GB 2506466 A	02-04-2014
		GB 2518048 A	11-03-2015
		JP 2015519706 A	09-07-2015
		US 2015097114 A1	09-04-2015
		WO 2013171495 A2	21-11-2013