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Structural characterization of acetogenins from *Annona muricata* by supercritical fluid chromatography coupled to high-resolution tandem mass spectrometry

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Abstract

Introduction – Acetogenins are plant polyketides known to be cytotoxic and proposed as antitumor candidates. They are also suspected to be alimentary neurotoxins. Their occurrence as complex mixtures renders their dereplication and structural identification difficult using liquid chromatography coupled to tandem mass spectrometry and efforts are required to improve the methodology.

Objective – To develop a supercritical fluid chromatography – high resolution tandem mass spectrometry method, involving lithium post-column cationization, for the structural characterization of Annonaceous acetogenins in crude extracts.

Methodology – The seeds of *Annona muricata* L. were extracted with methanol. Supercritical fluid chromatography of the extract, using a 2-ethylpyridine stationary phase column, was monitored using a high resolution quadrupole time-of-flight mass spectrometer. Lithium iodide was added post-column in the make-up solvent. For comparison, the same extract was analyzed using high pressure liquid chromatography coupled to the same mass spectrometer, with a column based on solid core particles.

Results – Sensitivity was similar for both HPLC and SFC approaches. Retention behavior and fragmentation pathways of three different isomer groups are described. A previously unknown group of acetogenins was also evidenced for the first time.

Conclusion – The use of post-column lithium-cationization and SFC-MS/MS allows the reduction of the time of analysis, of environmental impact and an increase in the chromatographic resolution, compared to liquid chromatography. This new methodology enlightened a new group of acetogenins, isomers of montanacin-D.

Short abstract for table of contents

A new method for the structural elucidation of Annonaceous acetogenins was based on supercritical fluid chromatography coupled to high resolution tandem mass spectrometry, including lithium post-column cationization. This method appeared as sensitive as classical liquid chromatography coupled to the same mass spectrometer but significantly improved the chromatographic resolution and thus allowed the annotation of a new group of isomers of montanacin-D.

Keywords

Annonaceous acetogenin; annonacin; high-resolution tandem mass spectrometry; lithium cationization ; supercritical fluid chromatography.

INTRODUCTION

Annonaceous acetogenins, which are specifically found in plants of the Annonaceae family, are polyketides constituted of 35 or 37 carbon atoms arranged as a long alkyl chain terminated by a γ -methyl- γ -lactone. Some oxygenated functions, namely hydroxyls, ketones, or heterocycles such as tetrahydrofuran rings (THF) or tetrahydropyran rings (THP), are present along the carbon chain (Bermejo et al., 2005). These compounds show a strong cytotoxicity and are proposed as antitumor candidates (McLaughlin, 2008; Liaw et al., 2010). They are also suspected to be environmental neurotoxins causing atypical Parkinsonian syndromes via chronic consumption of Annonaceae fruits and medicinal products (Höglinger et al., 2005; Lannuzel et al., 2007; Höllerhage et al., 2009), as evidenced by animal feeding studies (Rottscholl et al., 2016). High-Performance Liquid Chromatography (HPLC) coupled to high resolution tandem mass spectrometry (MS/HRMS) was used for the dereplication of acetogenins in Annonaceae derived products (Allegrand et al., 2010; Le Ven et al., 2012), allowing the identification of more than 80 acetogenins in an *Annona cherimolia* Mill. alcoholic beverage (Le Ven et al., 2014). Nevertheless, limited chromatographic resolution was observed, limiting the number of annotated peaks.

With the launch of modern commercial systems SFC has experienced a revival since 2011 due to the development of user-friendly instruments (Lesellier and West., 2015). By mixing supercritical carbon dioxide with a low amount (3 to 20 %) of organic solvent (such as ethanol, methanol, isopropan-2-ol or acetonitrile) as an eluent, a large range of molecules can be separated. In comparison to HPLC, SFC generally leads to improved chromatographic resolution and decreased analysis time. Moreover, as SFC consumes low volumes of organic solvent, even if the typical flow rate is comprised between 1 and 3 mL/min, the wastes produced by an analysis remaining limited (Ghosh, 2012). It is thus often considered that SFC is a more environment-friendly technique than HPLC.

The aim of this present work is to investigate the capabilities of supercritical fluid chromatography (SFC) – high resolution tandem mass spectrometry (MS/HRMS) for the structural characterization of acetogenins within a complex mixture.

First, a SFC-MS/HRMS method development including lithium post-column cationization for the separation and structural characterization of acetogenins from a methanol extract of the seeds of *Annona muricata* will be described. A comparison between the results SFC and LC coupled to the same tandem mass spectrometer will be discussed in terms of analysis time, chromatographic resolution, and quality of structural information. Moreover, linearity, limits of detection (LOD) and limits of quantification (LOQ) will be estimated for both SFC-MS and LC-MS analytical systems.

EXPERIMENTAL

Chemical reagents

All solvents were LC-MS grade. Methanol and acetonitrile were purchased from J.T. Baker (Center Valley, USA), ethanol was purchased from Fisher Scientific (Illkirch, France). Lithium iodide was purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). MilliQ water was prepared in house. Carbon dioxide (purity > 99.7 %) was purchased from Air Liquide (Grigny, France).

Standards

Annonacin (**1**) ($C_{35}H_{64}O_7$; monoisotopic mass: 596.4652 Da) and squamocin (**2**) ($C_{37}H_{66}O_7$; monoisotopic mass: 622.4808 Da) (Fig. 1) were obtained from several batches of the seeds of *Annona muricata* L. and *Annona squamosa* L. respectively, using previously described procedures (Bermejo et al., 2005). Stock solutions were prepared in methanol at a concentration of 1.5 mM and a working solution containing both acetogenins was prepared at a final concentration of 10 μ M in methanol. Isoannonacin was obtained, as diastereoisomeric pair, by refluxing 10 mg of **1** in 10 mL methanol basified with NaOH 1M, overnight (yield 75 %) (Duret et al., 1994). Structures of the standards were established using usual spectroscopic techniques (1D, 2D NMR, UV, IR, polarimetry, HRMS/MS), in comparison with authentic standards. Their purity (>99%) was assessed by HPLC-UV-MS.

Plant material

Annona muricata L. fruit was purchased in Colombia. Seeds (640 g) were manually pulled from the pulp, ground and then extracted with methanol (3 overnight extractions with 4 L of solvent at room temperature (about 20°C)). After solvent evaporation under reduced pressure, an oily pale-orange material was obtained (yield: 10.9 %). The extract was dissolved in methanol at 1 mg/mL and filtered using an Acrosdisc CR 4 mm syringe filter (PTFE membrane, 0.45 μ m) before chromatographic analysis.

Supercritical Fluid Chromatography

Supercritical fluid chromatography experiments were performed using a 1260 Infinity Analytical SFC system (Agilent Technologies, Waldbronn, Germany). This system consists of two main parts: the Aurora module and a “LC-like” system. The Aurora module has two functions. First it pumps and pre-compresses gaseous carbon dioxide, and second it contains the back pressure regulator (BPR) fixing the pressure in the system. The “LC-like” system includes six modules: a vacuum degasser, a SFC binary pump with a solvent mixer, a solvent selection valve, a thermostated autosampler with a 1.2 μL injection loop and two thermostated column compartments which can contain up to eight columns.

Seven different analytical columns were tested. Five columns were purchased from Agilent Technologies (Massy, France): Zorbax Eclipse plus C18 RRHT (150 \times 2.1 mm, 1.8 μm), Poroshell 120 SB-C18 (100 \times 2.1 mm, 2.1 μm), Pursuit 3 PentaFluoroPhenyl (PFP) (150 \times 2.0 mm, 3 μm), Zorbax SB-Cyano (CN) RRHT (100 \times 2.1 mm, 1.8 μm), Zorbax RX-Silica (Si) RRHT (100 \times 2.1 mm, 1.8 μm). The Luna Amino (NH_2) (150 \times 2.0 mm, 3 μm) column was purchased from Phenomenex (Le Pecq, France). The Acquity UPC² BEH 2-Ethylpyridine (2-EP) (100 \times 2.1 mm, 1.7 μm) column was purchased from Waters (Guyancourt, France).

Injection volume was fixed at 1 μL . Mobile phase was composed of carbon dioxide/ethanol. Isocratic mode (5 % ethanol) was employed for column screening whereas a gradient was optimized for MS and MS/MS coupling, as follows: 3 to 11.5 % ethanol (1 to 11 min), 11.5 to 20 % ethanol (11 to 12 min), 20 to 3 % ethanol (13 to 14 min). Different values of column oven temperature (20, 30 and 40 $^{\circ}\text{C}$), of back pressure (130, 150 and 170 bar), and of mobile phase flow rate (1.3, 1.5, 1.7 mL/min) were investigated. Final values chosen for MS and MS/MS experiments were 30 $^{\circ}\text{C}$, 150 bar and 1.5 mL/min, respectively.

For the coupling with the mass spectrometer, an isocratic pump was added to inject a make-up solvent (methanol or methanol + 12 μM lithium iodide (post-column cationisation

experiment), 200 $\mu\text{L}/\text{min}$) between the column and the BPR. By working on these conditions, one SFC separation uses less than 5 mL of solvent (ethanol: < 2 mL, methanol: 3 mL).

High pressure liquid chromatography

The HPLC experiments were performed with a HPLC Ultimate 3000 system (Dionex, Voisins-le-Bretonneux, France) consisting of four modules: a degasser, a quaternary pump, a thermostated autosampler and a column oven. The HPLC separation method was previously described (Allegrand et al., 2010). In order to improve the chromatographic resolution and to be as close as possible to SFC conditions, the method was improved by using a column based on solid core particles (Accucore RP-MS, 100 x 2.1 mm, 2.6 μm , Thermo Scientific, Courtaboeuf, France) equipped with a guard column. The column oven temperature was set at 20°C. The mobile phase was composed of water + 0.1 % formic acid (A) and acetonitrile + 0.1 % formic acid (B), following the gradient on B 40 % to 100 % (25 min) and 100 % during 7 min at a flow rate of 0.3 mL/min. Injection volume was fixed at 1 μL for the analysis of the methanol extract of *Annona muricata* and at 5 μL for the evaluation of the linearity, limits of detection and quantification. By working on these conditions, one HPLC separation uses about 12 mL of solvent (twice more than SFC separation).

Post-column lithium cationization

Lithium salt was added post-column for the MS/MS experiments. As previously described (Allegrand et al., 2010), lithium iodide (2 mM in methanol) was infused by using a syringe pump at a flow rate of 5 $\mu\text{L}/\text{min}$ for HPLC-MS/MS.

For the SFC-MS/MS experiments, lithium iodide was directly added to the make-up solvent. Three different concentrations (2, 20 and 200 μM) were tested, and a final concentration of 12 μM was chosen. Without post-column salt addition both protonated species and sodium adducts (due to the systematic presence of sodium in methanol) are detected.

Mass spectrometer

An Agilent 6540 Q-ToF (Agilent Technologies, Waldbronn, Germany) was used in its high resolution mode ($M/\Delta M > 40\,000$ [FHHM] at m/z 922). An electrospray ionization source was used in positive ion mode. Source parameters were set as follows: gas temperature set at 350°C, drying gas at 10 L/min, nebulizer pressure at 25 psi, capillary voltage at 3 500 V. An internal calibration was used (m/z 121.0509 and m/z 922.0098) to enable mass accuracies below 2 ppm.

For the MS mode, accessible mass ranged between m/z 100 and m/z 950 at an acquisition rate of 2 spectrum/s whereas, for MS/MS mode, the m/z range was optimized for each precursor ion and the acquisition rate was fixed at 1 spectrum/s. Collision energy was optimized for the lithium adducts of annonacin (**1**) and two values (60 and 80 eV with two successive injections) were selected in order to detect the major fragment ions which are structurally relevant but of low abundance. Each extracted ion chromatogram shown is obtained using an extraction of the theoretical m/z with a symmetric window width of ± 5 ppm.

Estimation of limits of detection/quantification

Linearity and limits of detection (LOD) and quantification (LOQ) were evaluated with equimolar solutions of both standards at concentrations between 7.5×10^{-7} to 7.5×10^{-4} mol/L in methanol (13 different values). Solutions were injected in triplicate to determine the linearity range and LODs and LOQs were calculated using the statistic method ($\text{LOD} = 3.3 \times \text{standard error of the predicted y-value/slope}$; $\text{LOQ} = 10 \times \text{standard error of the predicted y-value/slope}$).

RESULTS AND DISCUSSIONS

Determination of SFC conditions

Screening of stationary phases

Even if some previous studies offer classifications of stationary phases in SFC (Lesellier 2015; West, 2008a, b; Galea et al., 2015) and retention models (Leško et al., 2013; Tyteca et al., 2015), screening the columns remains mandatory as a first step of method development. This can be performed by using either an isocratic mode (Méjean et al., 2013) or a gradient mode (Yan et al., 2015). The first option was chosen using 5 % of ethanol in the mobile phase and a mixture of the two acetogenins standards, annonacin (**1**) and squamocin (**2**). Post-column addition of lithium salt was not required for the development of the chromatographic step. Because of the structural differences between these two molecules (*e.g.* the number of THF rings and hydroxyl groups) a high chromatographic resolution is expected. Extracted ion chromatograms of the sodium adducts of (**1**) and (**2**) at m/z 619.4550 and m/z 645.4707, respectively, were obtained for various columns (Fig. 2).

The results confirm the importance of the stationary phase chemistry regarding the separation (West et al., 2008a). Indeed, for the C18 phase columns, no separation was observed for the fully porous one (Fig. 2a) whereas a strong co-elution was noted for the Poroshell column (Fig. 2b). This discrepancy may be explained by a difference in the end-capping of each column. For the PFP (Fig. 2c) and CN (Fig. 2d) stationary phase columns, the resolutions look similar but the order of retention times is opposite. As tens of acetogenins, including isobaric ones, are expected in the seeds extract, a higher resolution is mandatory to avoid significant co-elutions. This requirement was achieved when using the three last stationary phases (NH₂, Si and 2-EP). The final choice fell on the 2-EP column (Fig. 2g) according to a better peak symmetry and shorter retention times of both standards.

Optimization of the SFC separation

Ethanol was chosen as co-solvent since it provided symmetric peaks, reasonable retention times and it is also significantly less toxic than methanol or acetonitrile. First, a generic gradient (1 to 21 min: 3 to 20 %, 23 to 24 min: 20 to 3 % ethanol) was tested on the standard mixture (Fig. 3a). As expected, peak width and resolution were significantly improved. The generic gradient was then tested on a seeds extract of *Annona muricata*. Ten extracted ion chromatograms of acetogenins already described in this plant species (Le Ven et al., 2012) were displayed (Fig. 3b) at m/z_{calc} 571.4697 ($[\text{C}_{35}\text{H}_{64}\text{O}_4+\text{Na}]^+$), m/z_{calc} 599.4282 ($[\text{C}_{35}\text{H}_{60}\text{O}_6+\text{Na}]^+$), m/z_{calc} 601.4438 ($[\text{C}_{35}\text{H}_{62}\text{O}_6+\text{Na}]^+$), m/z_{calc} 603.4595 ($[\text{C}_{35}\text{H}_{64}\text{O}_6+\text{Na}]^+$), m/z_{calc} 617.4387 ($[\text{C}_{35}\text{H}_{62}\text{O}_7+\text{Na}]^+$), m/z_{calc} 619.4544 ($[\text{C}_{35}\text{H}_{64}\text{O}_7+\text{Na}]^+$), m/z_{calc} 627.4595 ($[\text{C}_{37}\text{H}_{64}\text{O}_6+\text{Na}]^+$), m/z_{calc} 631.4908 ($[\text{C}_{37}\text{H}_{68}\text{O}_6+\text{Na}]^+$), m/z_{calc} 641.4387 ($[\text{C}_{37}\text{H}_{62}\text{O}_7+\text{Na}]^+$), and m/z_{calc} 645.4700 ($[\text{C}_{37}\text{H}_{66}\text{O}_7+\text{Na}]^+$). As the elution of these selected species occurs between 1 and 10 min, the generic gradient (dashed line in Fig. 3b) was shortened (solid line in Fig. 3b) by a factor of two, without sacrificing the chromatographic resolution.

The last step of SFC method optimization was to adjust the flow rate of the mobile phase, the temperature of the column oven, and the pressure of the BPR. A higher chromatographic resolution was obtained for BPR at 150 bar, an oven temperature set at 30°C and a flow rate of 1.5 mL/min.

Development of the MS and MS/MS conditions

MS experiments

As previously described, acetogenins can be detected as protonated $[\text{M}+\text{H}]^+$ and sodium adducts $[\text{M}+\text{Na}]^+$ (Allegrand et al., 2010). Potassium-adducts $[\text{M}+\text{K}]^+$ are also generated at a lower yield. Acetogenins can be identified by their experimental exact mass ($\Delta m < 2$ ppm) giving access to their molecular formula combined with the excellent match between experimental and theoretical isotopic patterns, and the presence of both $[\text{M}+\text{H}]^+$ and $[\text{M}+\text{Na}]^+$

species. An example of a mass spectrum, highlighting these three criteria of identification, is shown in supplementary material (Fig. S1).

The calculated LODs and LOQs (Tables 1, 2) indicate that SFC-HRMS and LC-HRMS exhibit similar sensitivities, in the low picomolar range of injected material.

MS/MS experiments

MS/MS experiments are mandatory because one single molecular formula generated by a single mass measurement corresponds to many acetogenin isomers (Bermejo et al., 2005; Le Ven et al., 2012, 2014). The fragmentation pathway of acetogenins has been extensively described before (Laprévotte and Das, 1994; Allegrand et al., 2010; Le Ven et al., 2012;). Lithium-adducts were favored to gain access to a large piece of structural information, as the protonated or sodium-adducts only lead to the losses of one or several molecules of water (Allegrand et al., 2010).

To produce lithium-adducts, lithium salt must be added into the system before the ion source (without this adding protonated species and sodium-adducts are obtained). For HPLC-MS/MS experiments, Li^+ was introduced by using a syringe pump filled by a solution of lithium iodide in methanol and located between the column and the entrance of the mass spectrometer. The situation is rather different for the SFC-MS system. The syringe pump was firstly placed between the BPR and the mass spectrometer. Unfortunately, the residual pressure of carbon dioxide, which was about 10 bar, created an opposite flow of carbon dioxide back into the syringe. The alternative way was to add the lithium iodide salt in the make-up solvent during the MS/MS experiments. As this set-up was never described before, the concentration of lithium iodide had to be optimized in order to get a rather complete conversion rate between the protonated/sodium adducts and the lithium adducts without any significant loss of sensitivity due to ion suppression effect.

Extracted ion chromatograms were displayed (Fig. 4) for sodium and lithium adducts of (1) and its isomers, with different concentrations of lithium iodide in the make-up solvent. At

2 μM , the signal of $[\text{M}+\text{Li}]^+$ (m/z_{calc} 603.4806) is maximum but the $[\text{M}+\text{Na}]^+$ (m/z_{calc} 619.4544) signals remain high. When the concentration of Li^+ increases, the intensity of sodium adducts decreases because of a better conversion rate into lithium adducts. However the $[\text{M}+\text{Li}]^+$ signals decrease also due to a suppression ion effect. At 200 μM of lithium iodide in methanol, sodium adducts are no longer detected but the signals of lithium adducts are much less intense than with the lower lithium iodide concentrations. Hence a concentration of 12 μM of lithium iodide in the make-up solvent was chosen for our SFC-MS/MS study. It must be noted that it was necessary to daily rinse the isocratic pump during a few minutes with pure methanol to eliminate any residue of lithium iodide salt.

The $[\text{M}+\text{Li}]^+$ ion species of (**1**) was selected by the quadrupole and fragmented in the collision cell with a collision energy varying by steps of 5 eV between 0 and 100 eV. The relative signal intensities of each expected fragment ion were plotted as a function of the collision energy (Fig. S2). The X fragments (Fig. 1) give access to the position of free hydroxyls while the positions of the THF and THP cycles are given by the Y and Z fragments (Lapr v te and Das, 1994). The signal of the X_4 fragment ion reaches its maximum at 60 eV whereas those of the X_1 , X_2 and X_3 fragment ions are the highest between 75 and 85 eV but the intensities are low especially for X_1 which is difficult to discriminate from the noise signal. Only the Z_1 fragment was detected with its highest intensity around 85 eV. The A, B and C fragments are not detected. Two values of collision energy, in two successive injections, were finally chosen: the first one at 60 eV to get the maximum of X_4 signal, typical of a hydroxyl in beta position to the lactonic ring, and the second at 80 eV to detect the other structurally relevant fragments (Fig. 5).

Examples of structural elucidation: LC-MS/MS *versus* SFC-MS/MS

In order to compare the relative performances of LC-MS/MS and SFC-MS/MS for the chemical characterization of the acetogenins from a crude seeds extract, three examples are described in detail.

C₃₅H₆₂O₇

Extracted ion chromatograms (Fig. 6a and 6b) for m/z 601.4656 (± 5 ppm window), correspond to the isomers of annonacinone (**3**). Chromatograms show two main peaks (RT_{LC} : 21.0 and 21.2 min; RT_{SFC} : 6.0 and 6.3 min). Since for this comparison the same sample and injection volumes were used, it must be noted that the two chromatographic methods led to similar sensitivity and resolution, while a dramatic reduction of the analysis time by a factor of 4 was observed for SFC. The MS/MS information is independent from the separative methods (Fig. S3). For the most intense peak, the position of two hydroxyl groups (X_2 at m/z 305.2661, $\Delta m = 0.46$ ppm; X_3 at m/z 489.4126, $\Delta m = 0.08$ ppm), of the ketone (m/z 417.3548, $\Delta m = 0.6$ ppm), and of the THF ring (Y_1 at m/z 233.2449, $\Delta m = 0.9$ ppm) were unambiguously determined, excluding bis-THF analogues. However, no fragment ion led to determine the position of the last hydroxyl, which lies between C-20 and C-32. Nevertheless, as there is no known Annonaceous acetogenin without hydroxyl in the THF α' -position, the structure of (**3**) is proposed (Fig. 1).

C₃₇H₆₆O₇

The extracted ion chromatograms for m/z 629.4963 (± 5 ppm window) (Fig. 6c and 6d) correspond to the isomers of gonionenin (**4**). The compounds are not resolved well using HPLC (Fig. 6c). By using SFC (Fig. 6d), the peaks fill the complete chromatographic time with an improved chromatographic resolution. Moreover, only 13 individual peaks can be distinguished in the LC chromatogram, against 30 in the SFC one. The two major peaks (labeled A [RT_{SFC} : 6.8 min] and B [RT_{SFC} : 4.2 min] in Fig. 6) of the SFC chromatogram were also pinpointed on the LC chromatogram (RT_{LC} : 22.2 and 23.5 min, respectively). Due to the co-elution with other isomers, the MS/MS spectral purity is insufficient in LC because the MS/MS spectra contain fragments from several isomers. Indeed, the A peak of SFC chromatogram gives a MS/MS spectrum which allowed identification of the position of 2 hydroxyl groups on C-4 and C-12 (X_2 at m/z 375.3435, $\Delta m = 4.00$ ppm; X_4 at m/z 517.4444,

$\Delta m = 1.74$ ppm). A ketone is placed on C-10 (m/z 445.3862, $\Delta m = 1.57$ ppm). No fragment ion led to solve the position of the last hydroxyl and THF ring which could be positioned between C-12 and C-34 (**6**). For the B peak, there is no hydroxyl group on C-4, as previously observed (Allegrand et al., 2010). A hydroxyl group position is identified on C-17 (X_2 at m/z 305.2657, $\Delta m = 3.60$ ppm). The ketone is placed on C-14 (m/z 389.3238, $\Delta m = 0.00$ ppm). Since the first fragment ion observed corresponds to a loss of $C_{14}H_{22}O_3$ from the $[M+Li]^+$ species, we propose the presence of an hydroxyl group on the lactonic ring, possibly at C-36 (type-D lactone), as described for several AAGs isolated from *Annona* spp. (Bermejo et al., 2005). Modification of the unsaturated lactone to a ketolactone, which is typical of artifactual acetogenins formed in protic solvents by lactone opening then closure on OH at C-4 (Duret et al., 1994) appears poorly probable. Indeed, such AAGs are present as diastereoisomeric pairs which should be separated under the present SFC chromatographic conditions (tested with semisynthetic isoannonacin, the artifactual counterpart of (**1**); not shown) (Bermejo et al., 2005). Nevertheless, no artifact arising from annonacin (**1**) could be detected in the extract. Moreover, a typical loss of CO from the lactone can be observed for B peak (m/z 601.5011, $\Delta m = -0.50$ ppm), while it isn't for isoannonacin (not shown). The THF ring position can be assigned between C-18 and C-21 (Y1 at m/z 233.2455, $\Delta m = 0.43$ ppm). Ambiguities can be also highlighted with the position of the last hydroxyl, which can be placed between C-22 and C-34. Interestingly, no acetogenin bearing these structural characteristics has previously been described. To fully characterize these new compounds by NMR, preparative SFC should be implemented for isolation.

It must also be noted that the order of elution is inverted between SFC and LC. This could be explained by the fact that SFC offers different types of interaction between the solvent, the analytes and the stationary phase compared to LC. When using 2-EP stationary phase, the analyte is interacting with the pyridine group linked to the stationary phase and with the free silanols by forming hydrogen bonds or a Π - Π interaction. Moreover, these interactions are

modulated by three retention mechanisms occurring simultaneously. First, the nitrogen on the pyridine could form hydrogen bonds with the free silanols of the stationary phase leading to a deactivated surface. Secondly, the pyridine aromatic ring could hide active sites on the stationary phase by steric effect. Thirdly, a pyridine group could be protonated by the mobile phase (Zheng et al., 2006).

C₃₅H₆₀O₇

The extracted ion chromatograms for m/z 599.4500 (\pm 5 ppm window width) (Fig. 7) correspond to the isomers of montanacin-D (**5**). SFC evidences the presence of two groups which can be clearly separated based on different fragment ions. The first group, with retention times between 2.0 and 4.4 min, presents a specific fragment ion at m/z 431.3713 which is typically related to the presence of a THP ring between C-4 and C-8. The second group, bearing retention times between 4 and 6 min, exhibits two particular fragment ions at m/z 415.3340 and m/z 487.3975, corresponding to 2 hydroxyls on C-4 and C-8. On the contrary, this distinction is not obvious when using LC-MS/MS, especially for the chromatographic peaks detected between 22 and 23.5 min. This could be explained by the different types of interaction between the solvent, the analytes and the stationary phase offered by SFC in comparison with LC, which prove of strong interest for the dereplication of complex mixtures of Annonaceous acetogenins.

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References

- Allegrand J, Touboul D, Schmitz-Afonso I, Guérineau V, Giuliani A, Le Ven J, Champy P, Laprévote O. 2010. Structural study of acetogenins by tandem mass spectrometry under high and low collision energy. *Rapid Commun Mass Spectrom* **24**: 3602–3608.
- Bermejo A, Figadère B, Zafra-Polo MC, Barrachina I, Estornell E, Cortes D. 2005. Acetogenins from Annonaceae: recent progress in isolation, synthesis and mechanisms of action. *Nat Prod Rep* **22**: 269–303.
- Duret P, Laurens A, Hocquemiller R, Cortes D, Cavé A. 1994. Isoacetogenins, artifacts issued from translactonization from Annonaceous acetogenins. *Heterocycles* **39**: 741–749.
- Galea C, Mangelings D, Heyden YV. 2015. Method development for impurity profiling in SFC: The selection of a dissimilar set of stationary phases. *J Pharm Biomed Anal* **111**: 333–343.
- Ghosh C. 2012. Green bioanalysis: some innovative ideas towards green analytical techniques. *Bioanalysis* **4**: 1377–1391.
- Grand-Guillaume Perrenoud A, Veuthey JL, Guillarme D. 2014. Coupling state-of-the-art supercritical fluid chromatography and mass spectrometry: From hyphenation interface optimization to high-sensitivity analysis of pharmaceutical compounds. *J Chromatogr A* **1339**: 174–184.
- Hartmann A, Ganzera M. 2015. Supercritical Fluid Chromatography – Theoretical background and applications on natural products. *Planta Med* **17**: 1570–1581.

Höglinger GU, Michel PP, Champy P, Féger J, Hirsch EC, Ruberg M, Lannuzel A. 2005.

Experimental evidence for a toxic etiology of tropical Parkinsonism. *Mov Disord* **20**: 118–119.

Höllerhage M, Matusch A, Champy P, Lombès A, Ruberg M, Oertel WH, Höglinger GU.

2009. Natural lipophilic inhibitors of mitochondrial complex I are candidate toxins for sporadic neurodegenerative tau pathologies. *Exp Neurol* **220**: 133–142.

Lannuzel A, Höglinger GU, Verhaeghe S, Gire L, Belson S, Escobar-Khondiker M, Poullain

P, Oertel WH, Hirsch EC, Dubois B, Ruberg M. 2007. Atypical parkinsonism in Guadeloupe: a common risk factor for two closely related phenotypes? *Brain* **130**: 816–827.

Laprévote O, Das B. 1994. Structural elucidation of acetogenins from Annonaceae by Fast

Atom Bombardment Mass Spectrometry. *Tetrahedron* **50**: 8479–8490.

Le Ven J, Schmitz-Afonso I, Lewin G, Brunelle A, Touboul D, Champy P. 2014.

Identification of the environmental neurotoxins Annonaceous acetogenins in an *Annona cherimolia* Mill. alcoholic beverage using HPLC-ESI-LTQ-Orbitrap. *J Agric Food Chem* **62**: 8696–8704.

Le Ven J, Schmitz-Afonso I, Lewin G, Laprévote O, Brunelle A, Touboul D, Champy P.

2012. Comprehensive characterization of Annonaceous acetogenins within a complex extract by HPLC-ESI-LTQ-Orbitrap® using post-column lithium infusion. *J Mass Spectrom* **47**: 1500–1509.

Lesellier E, West C. 2015. The many faces of packed column supercritical fluid

chromatography – A critical review. *J Chromatogr A* **1382**: 2–46.

- Leśko M, Poe DP, Kaczmarek K. 2013. Modelling of retention in analytical supercritical fluid chromatography for CO₂–Methanol mobile phase. *J Chromatogr A* **1305**: 285–292.
- Liaw CC, Wu TY, Chang FR, Wu YC. 2010. Historic perspectives on Annonaceous acetogenins from the chemical bench to preclinical trials. *Planta Med* **13**: 1390–1404.
- McLaughlin JL. 2008. Paw paw and cancer: Annonaceous acetogenins from discovery to commercial products. *J Nat Prod* **71**: 1311–1321.
- Méjean M, Vollmer M, Brunelle A, Touboul D. 2013. Quantification of retinoid compounds by Supercritical Fluid Chromatography coupled to Ultraviolet Diode Array Detection. *Chromatogr* **76**: 1097–1105.
- Rottscholl R, Haegel M, Jainsch B, Xu H, Respondek G, Höllerhage M, Rösler TW, Bony E, Le Ven J, Guérineau V, Schmitz-Afonso I, Champy P, Oertel WH, Yamada ES, Höglinger GU. 2016. Chronic consumption of *Annona muricata* juice triggers and aggravates cerebral tau phosphorylation in wild-type and MAPT transgenic mice. *J Neurochem* **139**: 624–639..
- Tyteca E, Desfontaine V, Desmet G, Guillarme D. 2015. Possibilities of retention modeling and computer assisted method development in supercritical fluid chromatography. *J Chromatogr A* **1381**: 219–228.
- West C, Lesellier E. 2008. A unified classification of stationary phases for packed column supercritical fluid chromatography. *J Chromatogr A* **1191**: 21–39.
- West C, Lesellier E. 2008. Orthogonal screening system of columns for supercritical fluid chromatography. *J Chromatogr A* **1203**: 105–113.

Yan TQ, Riley F, Philippe L, Davoren J, Cox L, Orozco C, Rai B, Hardink M. 2015.

Chromatographic resolution of atropisomers for toxicity and biotransformation studies in pharmaceutical research. *J Chromatogr A* **1398**: 108–120.

Zheng J, Taylor LT, Pinkston JD. 2006. Elution of cationic species with/without ion pair reagents from polar stationary phases via SFC. *Chromatographia* **63**: 267–276.

Figure captions

Figure 1: Structures and fragmentation patterns for the $[M+Li]^+$ ions of 8 Annonaceous acetogenins. Annonacin (**1**) and Squamocin (**2**) are standards used for method development. Annonacinone (**3**), Gonionenin (**4**) and Montanacin-D (**5**) were previously described in *Annona muricata*. (**6**) and (**7**) are hypothetic structures proposed as isomers of (**4**).

Figure 2: Extracted Ion Chromatograms (EIC) of (**1**) and (**2**) obtained for different columns (**a** – C18, **b** – Poroshell C18, **c** – PFP, **d** – CN, **e** – NH₂, **f** – Si, **g** – 2-EP). Injected volume: 1 μ L, flow rate: 1.5 mL/min, mobile phase: 5 % EtOH (isocratic mode), back pressure: 150 bar, column temperature: 30°C.

Figure 3: Determination of the elution gradient. (**a**) generic gradient on mixture of (**1**) and (**2**). (**b**) generic (dashed line) and final (solid line) gradients. Chromatograms show the sum of 10 extracted ion chromatograms (EICs) at m/z 571.4697 ($[C_{35}H_{64}O_4+Na]^+$), m/z 599.4282 ($[C_{35}H_{60}O_6+Na]^+$), m/z 601.4438 ($[C_{35}H_{62}O_6+Na]^+$), m/z 603.4595 ($[C_{35}H_{64}O_6+Na]^+$), m/z 617.4387 ($[C_{35}H_{62}O_7+Na]^+$), m/z 619.4544 ($[C_{35}H_{64}O_7+Na]^+$), m/z 627.4595 ($[C_{37}H_{64}O_6+Na]^+$), m/z 631.4908 ($[C_{37}H_{68}O_6+Na]^+$), m/z 641.4387 ($[C_{37}H_{62}O_7+Na]^+$) and m/z 645.4700 ($[C_{37}H_{66}O_7+Na]^+$) from the seeds extract of *Annona muricata*.

Figure 4: Extracted ion chromatograms of $[M+Na]^+$ (m/z_{calc} 619.4544) (left column) and $[M+Li]^+$ (m/z_{calc} 603.4806) ions (right column) of annonacin (**1**) isomers from the seeds extract of *Annona muricata*, for Li⁺ concentrations in the make-up solvent of 2, 20 and 200 μ M.

Figure 5: MS/MS spectra of annonacin (**1**) from the seeds extract of *Annona muricata* for two collision energies: **a** – 60 eV, **b** – 80 eV.

Figure 6: (**a**) and (**b**) – EIC of $[M+Li]^+$ adducts of (**3**) and its isomers (m/z 601.4656) from the seed extract of *Annona muricata* using LC-MS/MS and SFC-MS/MS respectively. (**c**) and (**d**)

–EIC of $[M+Li]^+$ adducts of (**4**) and its isomers (m/z 629.4969) from the seeds extract using LC-MS/MS and SFC-MS/MS respectively. See text for peaks labeled A and B.

Figure 7: (**a**) and (**b**) EIC of $[M+Li]^+$ ions of (**5**) and its isomers (m/z 599.4500) from the seeds extract using SFC (left column) and LC (right column), respectively. EIC of fragments from $[M+Li]^+$ at m/z 487.3969, m/z 415.3394 and m/z 431.3707.

Figure S1: Mass spectrum of annonacin (**1**): experimental values of precision and resolution (**a**), comparison between experimental and theoretical isotopic pattern (**b**).

Figure S2: Relative intensities of annonacin $[M+Li]^+$ adduct and its fragments as a function of the collision energy (by steps of 5 eV). Solid lines are to guide the eye.

Figure S3: MS/MS spectrum at 60 eV collision energy from the major peak of the EIC of annonacinone (**3**) (m/z 601.4656) using SFC (**a**) or LC (**b**).

Annonacin	[M+H] ⁺		[M+Na] ⁺		[M+K] ⁺	
	SFC	LC	SFC	LC	SFC	LC
Linearity range (mol/L)	7.5x10 ⁻⁷ to 9.75x10 ⁻⁵	7.5x10 ⁻⁷ to 9.9x10 ⁻⁶	7.5x10 ⁻⁷ to 9.9x10 ⁻⁶	7.5x10 ⁻⁷ to 3.0x10 ⁻⁵	7.5x10 ⁻⁶ to 7.5x10 ⁻⁵	7.5x10 ⁻⁷ to 3.0x10 ⁻⁶
r ²	0.999	0.998	0.988	1.000	0.974	0.993
LOD (pmol _{inj})	3.24	2.91	1.54	1.43	20.66	1.70
LOQ (pmol _{inj})	9.80	8.82	4.68	4.33	62.61	5.15

Table 1. Linearity range, coefficient of determination, LOD and LOQ of annonacin species using LC-HRMS and SFC-HRMS.

Squamocin	[M+H] ⁺		[M+Na] ⁺		[M+K] ⁺	
	SFC	LC	SFC	LC	SFC	LC
Linearity range (mol/L)	9.9x10 ⁻⁷ to 9.9x10 ⁻⁶	7.5x10 ⁻⁷ to 3.0x10 ⁻⁵	7.5x10 ⁻⁷ to 9.9x10 ⁻⁶	7.5x10 ⁻⁷ to 7.5x10 ⁻⁶	7.5x10 ⁻⁷ to 9.9x10 ⁻⁶	7.5x10 ⁻⁷ to 3.0x10 ⁻⁶
r ²	0.979	1.000	0.979	0.993	0.975	0.988
LOD (pmol _{inj})	2.09	1.43	2.09	4.47	2.29	2.25
LOQ (pmol _{inj})	6.34	4.33	6.34	13.56	6.94	6.81

Table 2. Linearity range, coefficient of determination, LOD and LOQ of squamocin species using LC-HRMS and SFC-HRMS.

Figure 1

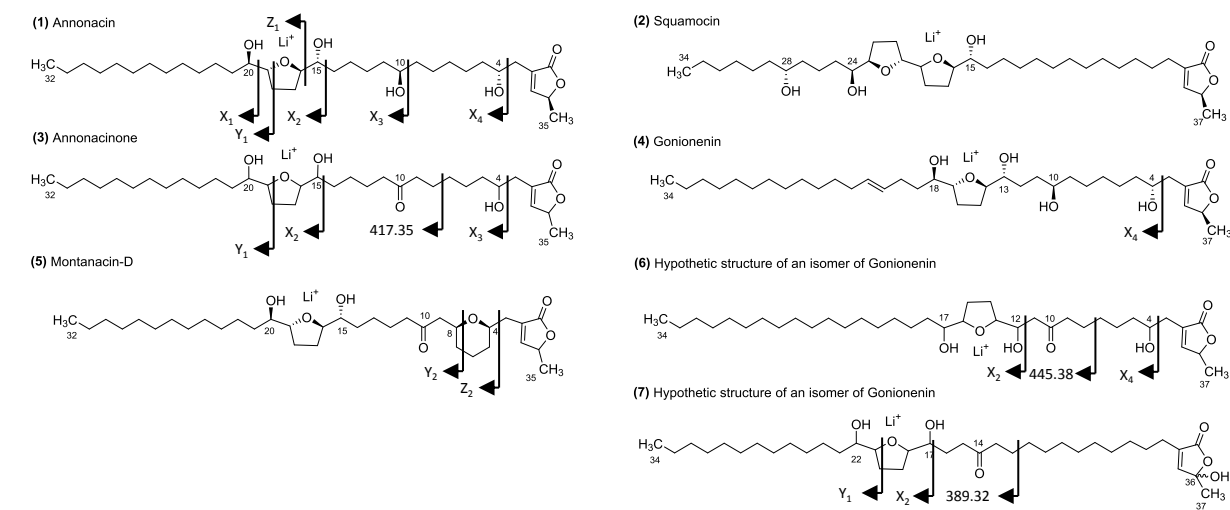


Figure 2

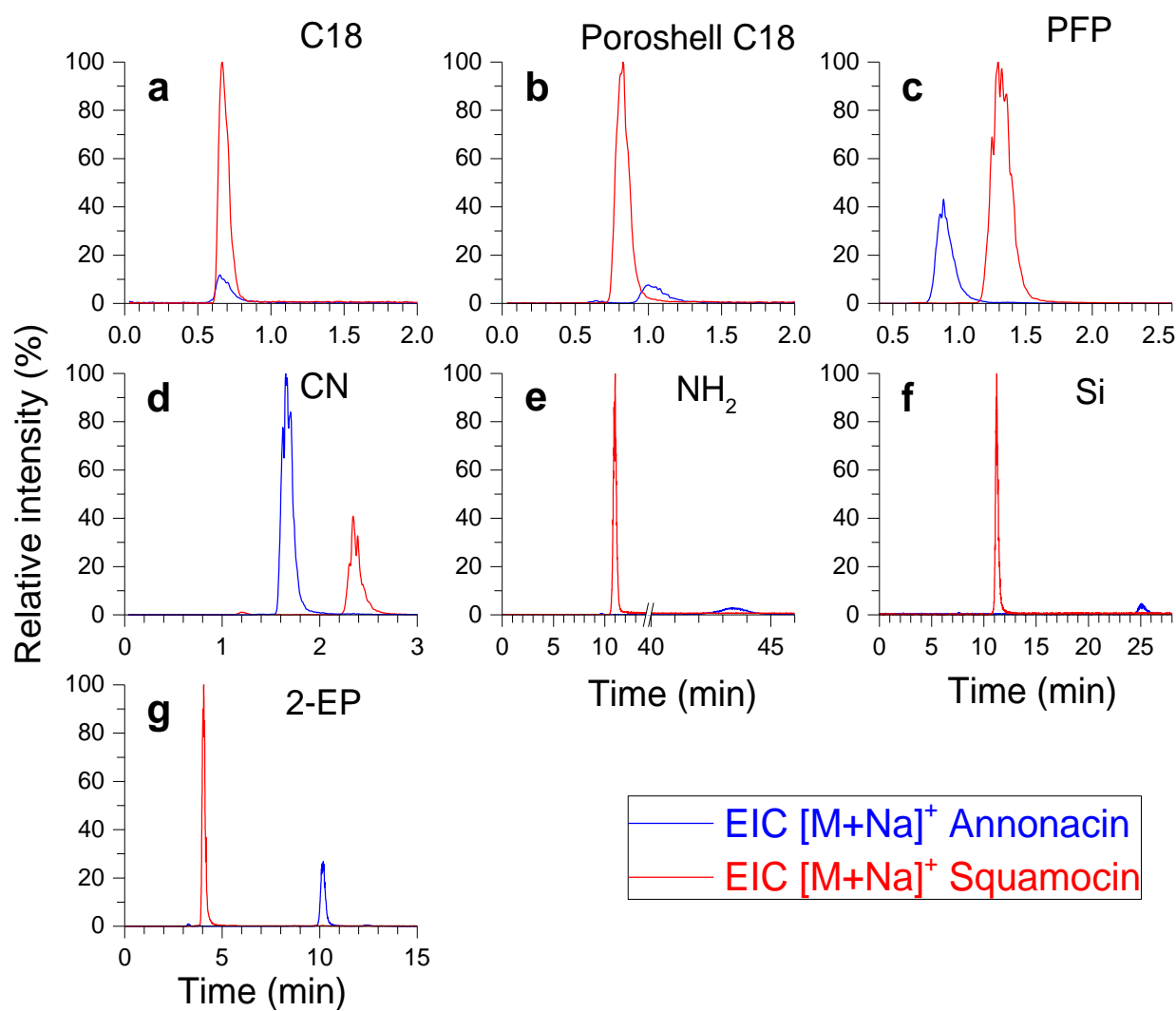


Figure 3

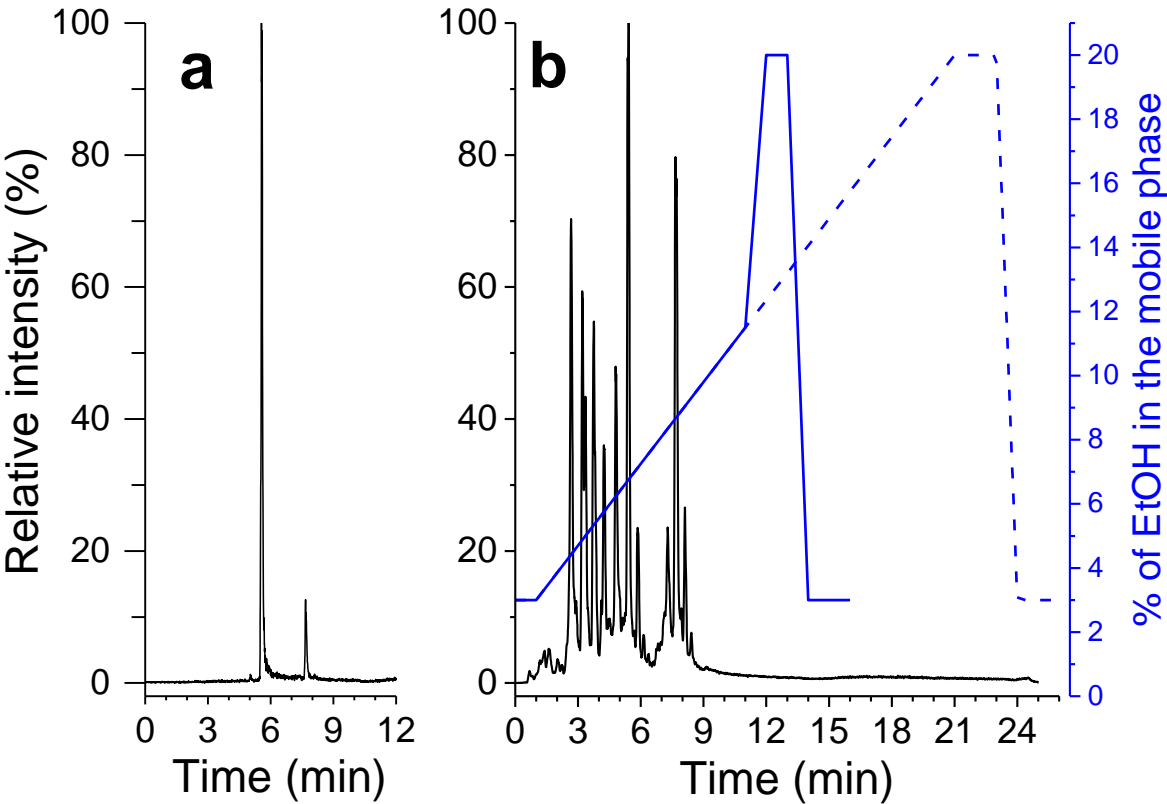


Figure 4

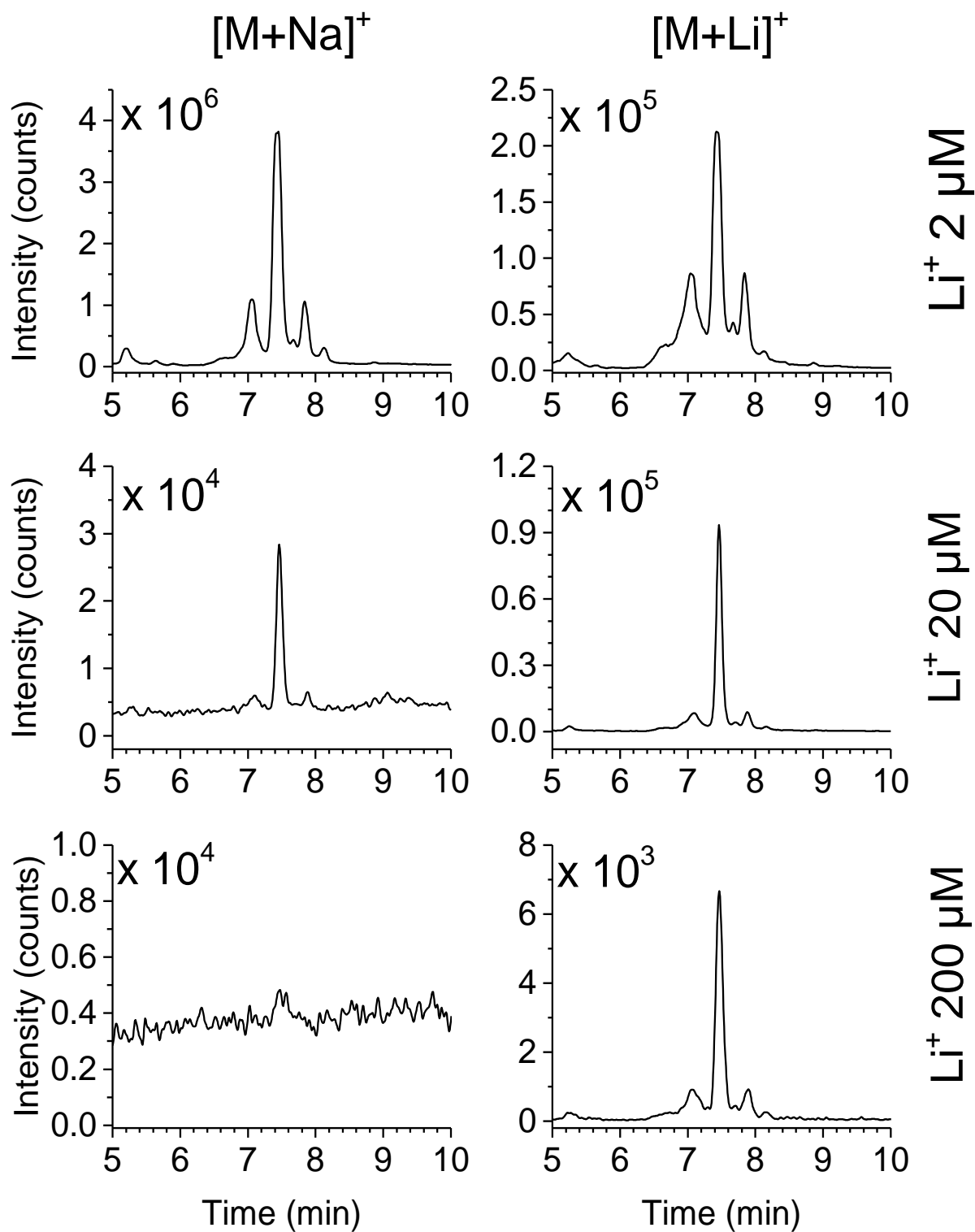


Figure 5

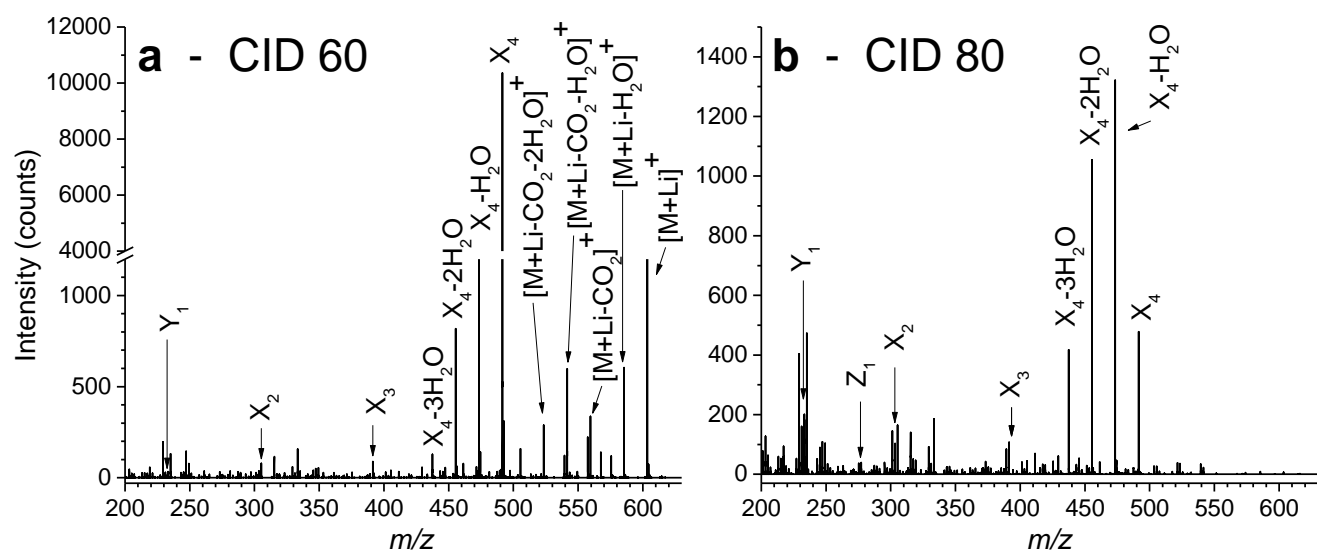


Figure 6

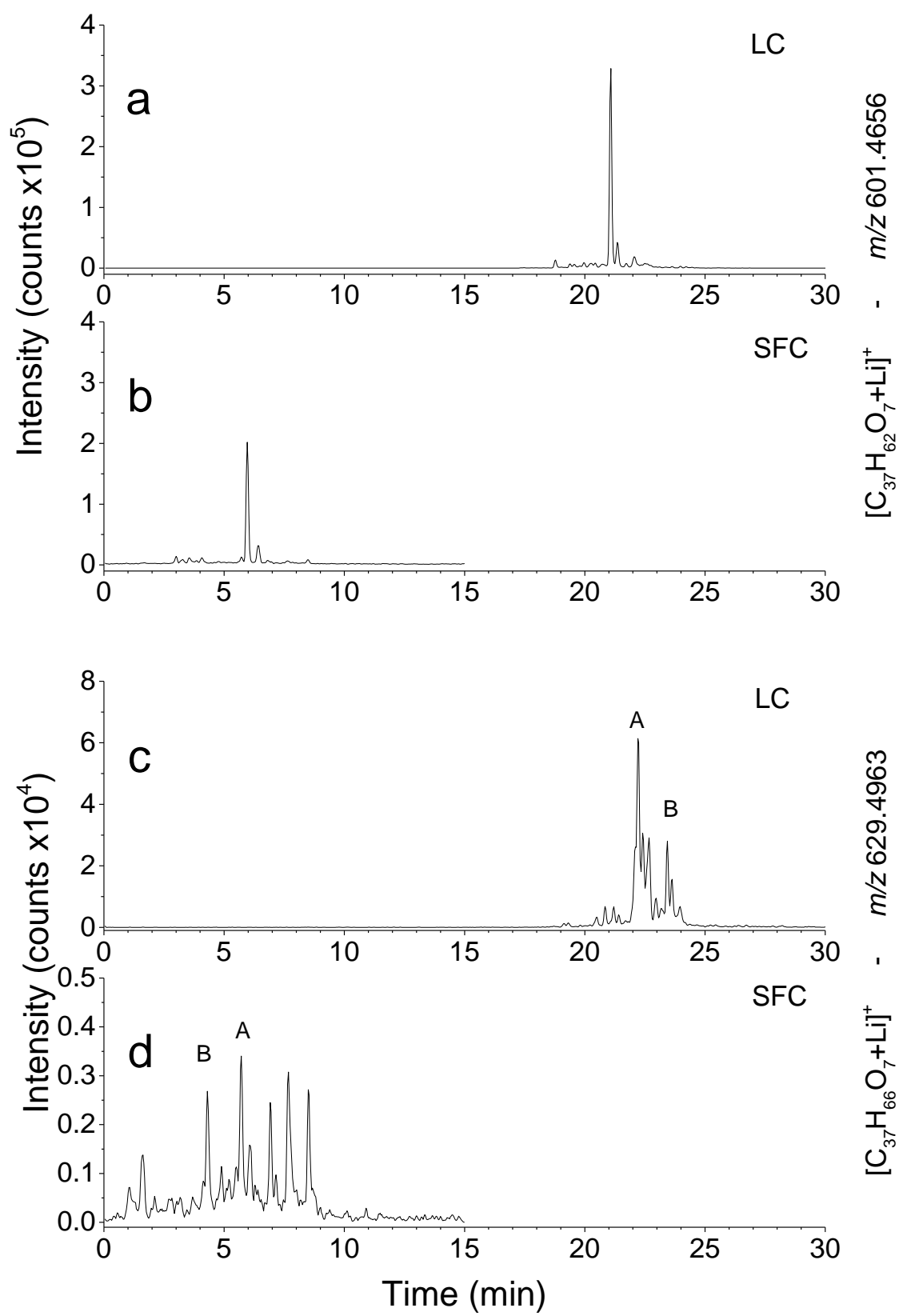
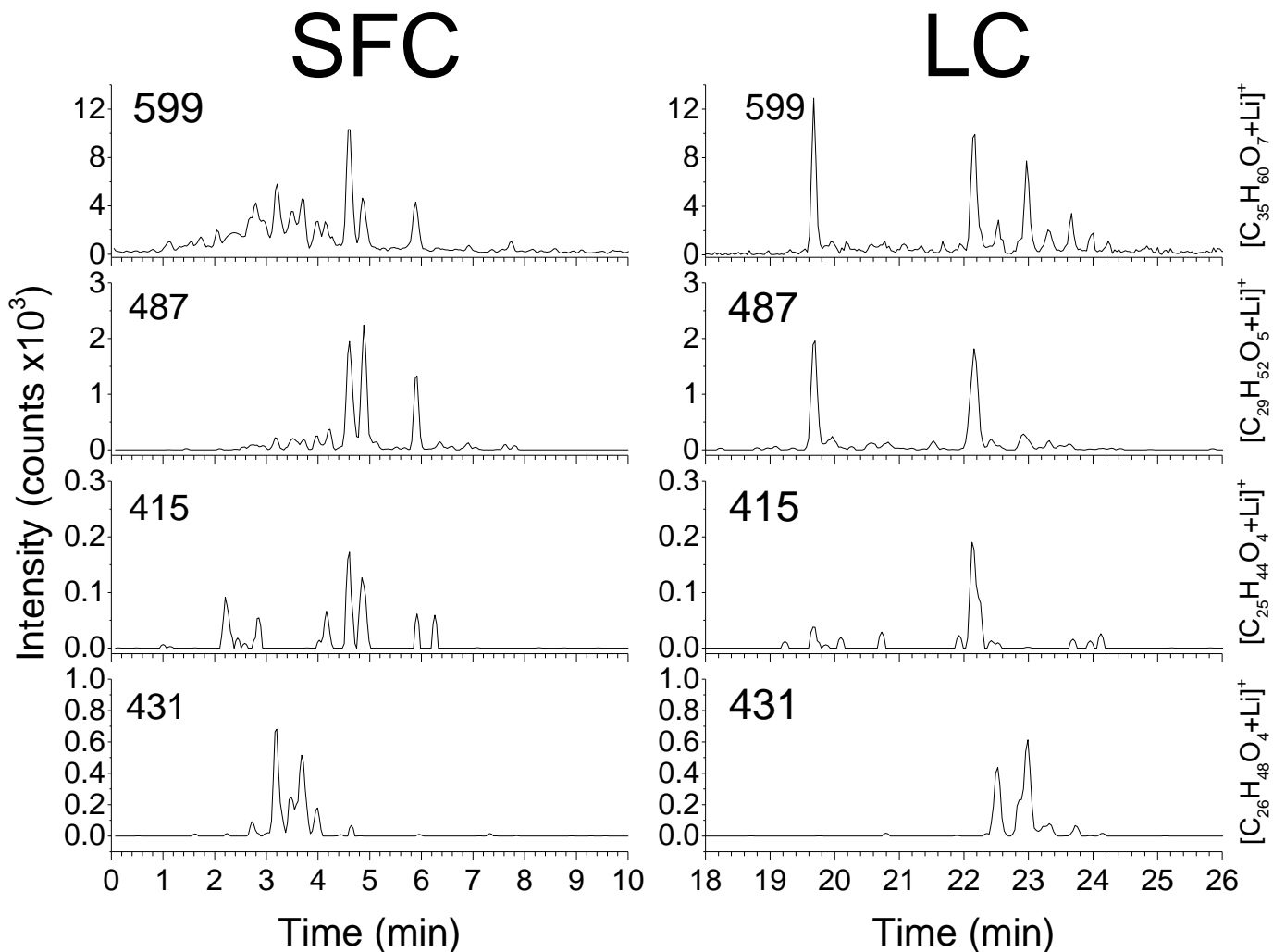


Figure 7



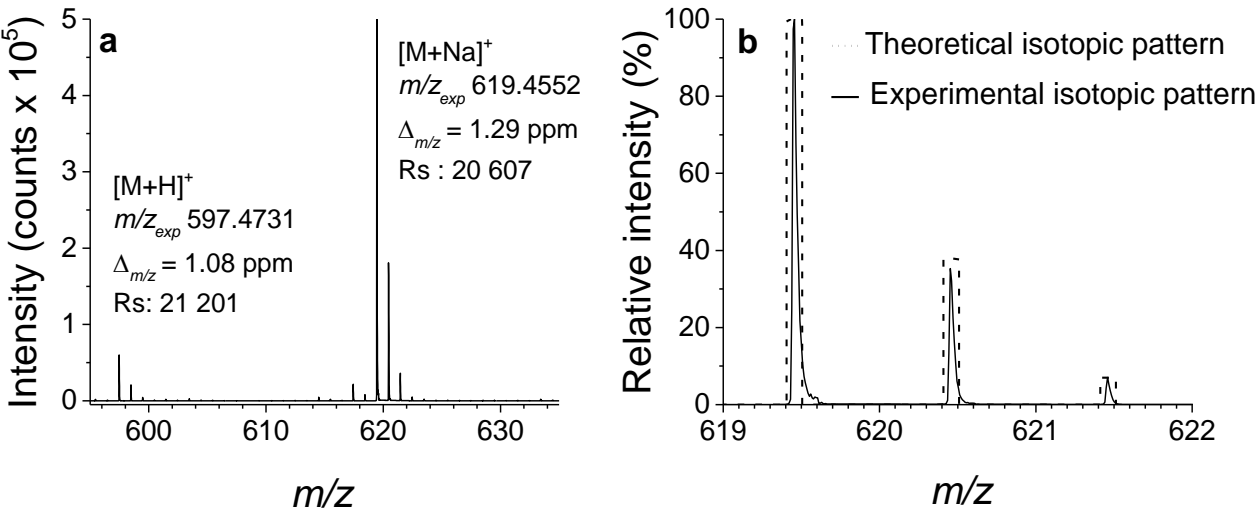


Figure S2

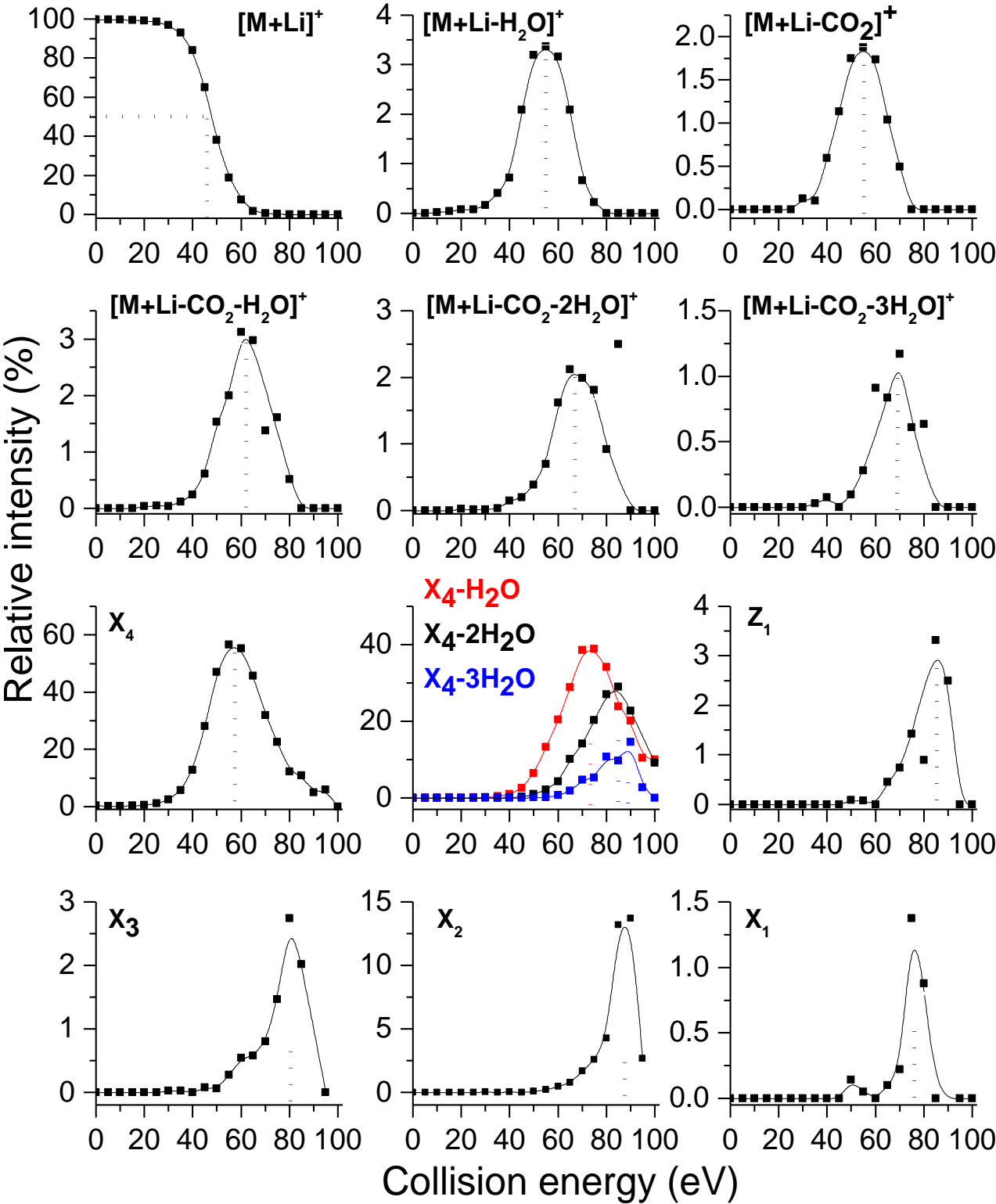


Figure S3

