

Chapter 2

Application of the CIRAD Mass Spectrometry Approach for Lysine Acetylation Site Discovery

Caroline A. Evans, Saw Yen Ow, Duncan L. Smith, Bernard M. Corfe, and Phillip C. Wright

Abstract

Mass spectrometry (MS)-based methods typically assess acetylation by detection of a diagnostic ion at 126.1 m/z , corresponding to the immonium ion of acetyl-lysine $-NH_3$, which is generated by collisionally induced dissociation. A novel implementation of this approach, based on the accurate mass and retention time technique, couples high mass resolution measurement with rapid cycling between low and elevated collision energies to generate intact and fragment high-resolution mass spectra. This allows acetyl lysine diagnostic ions at 126.1 m/z to be monitored and aligned to the precursor m/z based on retention time profile. The technique is termed *Collisionally Induced Release of Acetyl Diagnostic*. Sequence information is also obtained for acetylation site assignment. This technique to identify acetylation species is information independent as it does not require the sequence of the protein/peptides to identify acetylation, and thus complementary to data-dependent methods. It is suitable for analysis of acetylated peptides, or proteins enriched by immunoprecipitation with acetyl lysine-specific antibodies.

Key words: Lysine acetylation, Site-specific analysis, Mass spectrometry, CIRAD

1. Introduction

Protein acetylation of the ϵ side chain of lysine residues occurs on a range of proteins, typically macromolecular complexes—and has emerged a key regulatory modification for many proteins—including histone and nonhistone proteins (1). There are several protein acetylation-specific workflows, which are based on mass spectrometry (2). Lysine acetylation can be analyzed by detection of the acetyl lysine diagnostic ion of m/z 126.1 (3–5). This chapter focuses on a novel MS method, *Collisionally Induced Release of*

Acetyl Diagnostic (CIRAD), for the analysis of lysine acetylation of peptides and proteins using an information-independent approach, which is distinct from, but complementary to, other MS-based methods describing acetylation site analysis in this volume in Chapters 1 and 3.

The key features of CIRAD are the following:

- A method for targeted analysis of lysine acetylation.
- Based on analysis of peptides derived by proteolytic cleavage of the protein of interest.
- A data-independent MS method—It is not essential to know the a priori identity of the target protein or proteolytic fragment m/z values.
- Specificity is achieved by high-resolution and high-accuracy mass measurement of the 126.09 acetyl lysine diagnostic ion and the precursor m/z (better than 2 ppm), high confidence identification, as well as high specificity ion extraction (hrEIC).
- Correlation of the acetyl diagnostic ion to the precursor m/z based on the time-overlap and elution pattern of chromatographic profile; no assumption is made as to the presence (or not) of other PTMs or the sequence in the peptide to enable the detection of acetylation.
- Application to a protein gel “band” obtained by SDS-PAGE, or mixtures of acetylated peptides enriched by acetyl lysine immunoprecipitation and analyzed by LC-MS/MS.

1.1. Principle of CIRAD

Analysis of peptides by MS generates a survey (or MS1) scan with m/z ratios plotted against ion current. Amino acid sequence can be determined by fragmentation along the peptide backbone, promoted by collision with an inert gas at low pressure, and termed collision-induced dissociation (CID). The resulting MS/MS (or MS2) product ion spectrum shows the m/z ratios for different fragments of the selected peptide precursor. Conventionally, MS/MS data for a given precursor ion population present in the survey scan is acquired by sequential selection and fragmentation of (typically) the most intense precursor ions (6). In this data-dependent acquisition (DDA) mode, fragment ions are generated from a single precursor ion population. Peptide sequences and sites of modification can be assigned by matching experimental MS/MS spectra to theoretical fragment masses (generated in silico for any given protein database) via protein database searching (7).

Acetylated lysine residues can generate product ions of 143.1 m/z and 126.1 m/z following peptide fragmentation (CID) (3–5). The 143.1 m/z and 126.1 m/z ions correspond to the acetyl lysine immonium ion and the immonium ion $-\text{NH}_3$, respectively. While the 143.1 m/z is only semi-diagnostic, the release of 126.1 m/z ion is diagnostic since it is acetylation specific (see Notes 1 and 2).

1.2. CIRAD: Application to Analysis of Acetylated Peptides

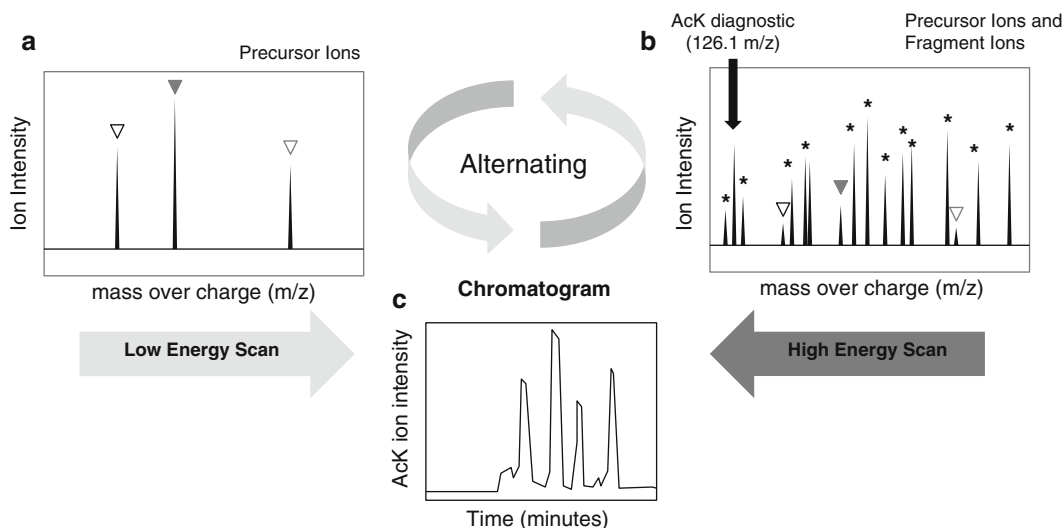
The CIRAD technique utilizes the Broad Band CID (bbCID) functionality of the MS instrumentation to simultaneously fragment all precursors observed in the survey scan. Alternating between MS survey (low energy) scans and bbCID (high collision energy) at rapid intervals enables generation of MS scans for all precursor ions and their fragments. Data then can be correlated to link individual co-eluting precursors and their corresponding product ions without the need for specific m/z -based precursor selection (see Note 3). The method essentially performs data-independent acquisition and as such is distinct from conventional DDA analysis; the first quadrupole of the MS simply functions in RF mode only to transfer ions of all m/z values (“broadband” ion transmission) to the collision cell where low and high alternating collision energies are applied. The presence of acetylated peptides is assessed based on detection of the acetyl lysine diagnostic immonium ion (minus NH_3) at m/z 126.092, analyzed by high-resolution extracted ion chromatogram (hrEIC) to “search” for the potential acetylated precursors. The use of an ultra high-resolution time-of-flight instrument generates high-resolution, high-mass-accuracy data, which allows both the use of providing unambiguous precursor and product m/z values for peptide identification.

Coupling online to high-performance liquid chromatography (HPLC) peptide fractionation simplifies the mixture of peptides for analysis. Peptides are resolved by HPLC, using reversed-phase chromatography at nanolitre per minute flow rates (200–300 nL/min “nanoflow”). The column eluent is directly coupled to the MS and gas-phase ions are generated and introduced to the MS via nano electrospray ionization (nano ESI). Alternating low and high energy data is acquired at a rate of 1 Hz (1 scan recorded per second) over the course of the RP-HPLC chromatography run, which typically lasts for 90–120 min. Processing of the LC/MS raw data with a specific extraction algorithm (the Dissect algorithm) links precursors and product ions of similar chromatographic behavior, traced across the LC elution peak using both the high and low energy data. A mascot generic format file (with “.mgf” extension) is generated for database searching and sequence assignment. The workflow schematic for CIRAD is shown in Fig. 1.

2. Materials

1. Standard proteins for method setup: Acetylated protein standard, for example acetylated bovine serum albumin (BSA) (Invitrogen, Paisley, UK, or Sigma, Poole, UK).
2. Trypsin: Proteomics grade. Perform solution digest with a ratio of trypsin:acetylated BSA of 1:50 in 25 mM ammonium bicarbonate buffer.

Collisionally Induced Release of Acetyl Diagnostic (CIRAD)



High resolution mass measurement for assignment of sequence and acetyl lysine diagnostic ions

Fig. 1. CIRAD workflow for acetylation site analysis. The CIRAD technique utilizes the Broad Band CID (bbCID) functionality of the MS instrumentation to simultaneously fragment all precursors observed in the survey scan. Alternating between MS survey (low energy) scans and bbCID (high collision energy) at rapid intervals enables generation of MS scans for all precursor ions and their fragments. (a) Three different precursors are shown (*triangles*) together with their precursors, including an acetylated precursor (*black triangle*) in the lower energy scan. (b) Alternating to high energy promotes collisionally induced dissociation to fragment ions (*asterisk*), which for an acetylated precursor generates the acetyl lysine diagnostic ion at 126.1 m/z . Sequence information is provided by other m/z fragments. (c) Precursor and product ion connectivity are determined based on common chromatographic elution (retention time) profiles. The presence of acetylated peptides is indicated by use of high-resolution extracted ion chromatograms (hrEIC) for 126.09.

3. Reversed phase-HPLC column for nanoLC and micro precolumn C18 for online sample loading and desalting at flow rates of 300 nL/min and 30 μ L/min, respectively.
4. HPLC solvents: Acetonitrile and water (HPLC grade), formic acid.
5. Glass sample vials with lids, 300 μ L glass insert, fused into a 2 mL crimp top vial (Chromacol (ThermoFisher), Welwyn Garden City, UK). For sample pick up prior to RP-HPLC analysis.
6. Mass spectrometer, maXis™ UHR-Qq-ToF ToF (Bruker), fitted with electrospray source and EasyNano electrospray needle. Data Analysis software 4.1, with the Dissect Algorithm (see Note 3).

7. Electrospray tunemix ESI-L (Agilent, West Lothian, UK), directly infused at 300 nL/min using a Hamilton syringe matching pump (KD Scientific, MA, USA).
8. NanoLC system: Ultimate U3000 (Thermo Fisher, Hemel Hempstead, UK) modular system (solvent degasser, micro and nanoflow pumps, flow control module and autosampler) or equivalent.

3. Methods

3.1. CIRAD Workflow

The method requires both detection of the 126.1 m/z acetyl lysine diagnostic ion and higher mass fragment ions (up to 1,200 m/z) to obtain sequence information and this assigns the site of acetylation. Representative CIRAD data for an acetylated BSA peptide (MS survey and associated bbCID CID spectra from sequential scans) is shown in Fig. 2.

3.1.1. MS Method Setup for *maXis™* UHR ToF

In micrOToF control software, open the default.m method, under the “Method” tab, and then save the method to another filename so that this method can be edited, leaving the default.m method for day-to-day instrument calibration and tuning.

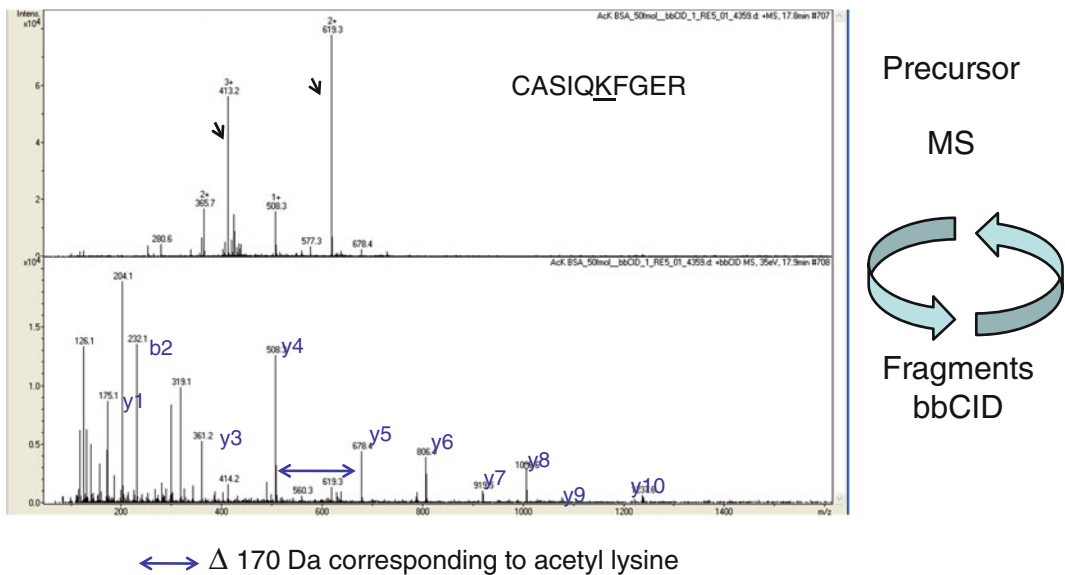


Fig. 2. A representative MS/MS spectrum for an acetylated bovine serum albumin peptide. The MS survey data and product ion spectrum obtained by switching between low and high collision energies are shown for an acetylated peptide. The precursor present as the doubly and triply charged forms. The high energy spectrum is annotated for the y ion series of peptide fragments. The mass difference of 170 Da between the y4 and y5 observed fragments is confirmatory of acetylation and corresponds to the mass of lysine plus acetylation: 128 + 42 Da, respectively. The 126.1 acetyl lysine immonium is present in this spectrum.

3.1.2. General Instrument Settings

1. The maXis is run in the positive mode, with the following settings (as viewed and set in the “Source” tab of micrOTOF control software v3.0): As a guide, our settings are shown, as listed from top to bottom on the left-hand side in the “Source” area of the window, EndPlate Offset -500 V, capillary voltage $5,000$ V, nebulizer gas 0.4 bar, dry gas 6.0 L/min, and dry temperature 150°C .

3.1.3. Optimization of Ion Transmission for CIRAD

Optimization can be simply performed using a low concentration (100-fold dilution) of an electrospray tunemix containing ten defined compounds that ionize to cover the 118 – $2,722$ m/z range. This is convenient, as this is typically used for mass calibration of the maXisTM on a regular basis. This approach is described in detail by Ow et al. (8), in this case for the use of iTRAQ reagents, where similar considerations apply to optimization for acetylation analysis, i.e., improved transmission and sensitivity towards low m/z ions (in this case, 126.09 m/z acetylation diagnostic ion), but retaining a sufficient population of higher mass sequence ions.

In order to improve detection of the acetylation derived ions, guide voltages and storage times from the collision cell down to the ion cooler are adjusted to give the most appropriate balance between improved sensitivity of the 126.09 m/z diagnostic ion versus the stability of other larger fragment ions. Four key parameters as noted by Ow et al. (8) can be adjusted here for the transmission of the collision cell and the ion cooler cell (RF guide voltages CC_{RF} and IC_{RF} , transfer time IC_{TT} , and pre-pulse time IC_{PP}).

In short, varying the guide voltage of the collision cell, CC_{RF} (affecting fragment ion stability), and ion cooler, IC_{RF} (cooling of ion beam), will adjust the net balance of ion population transferred. More generally, decreasing the guide voltage value for the collision cell and ion cooler allows lower m/z ions to be effectively transmitted, thus improving CIRAD sensitivity.

Likewise, the ion cooler voltage (IC_{RF}), together with the co-balancing of ion cooler transfer (IC_{TT}) and of pre-pulse times (IC_{PP}), can be co-adjusted to enable improved transmission and sensitivity towards low m/z ions.

3.1.4. Implementation of Collision Sweeping

This allows linked operation of different IC_{RF} transmission parameters (either those which focus squarely on low mass or those that transmit high mass) and is achieved by evenly scheduling different transmission parameters across the entire accumulation time. This is commonly termed as “collision sweeping.” This implementation allows up to five steps of IC_{RF} to be made during the course of the scan allowing different IC_{RF} adjustments that are optimized for different mass ranges.

3.2. CIRAD Method Execution

1. For method setup using acetylated BSA digest, analyze at least 50 fmol on column, for initial evaluation. Recommended RP-HPLC buffers are 2% acetonitrile (v/v): 0.1% formic acid (buffer A) and 80% acetonitrile (v/v): 0.1% formic acid (buffer B), with peptide separation achieved using a gradient of 5% buffer B

to 40% buffer B over 40 min at a flow rate of 300 nL/min. Perform sample desalting by in-line micro-precolumn with loading solvent (0.1% formic acid) delivered at 30 μ L/min for 4 min. The LC program (Chromeleon, LC Packings, The Netherlands) and MS operating parameters are integrated via HyStar software.

2. The maXis™ MS parameters are specified in the micrOTOF control software (v 3.0) (Bruker), run in the positive mode, and use settings optimized for ion transmission as detailed above in Subheading 3.1.2 and accessible under the “Source” tab. Set the mass range (right-hand panel) to 50–1500 m/z .

The “Transfer” settings are as follows:

Funnel RF, 400.0 Vpp; ISCD energy, 0.0 eV; multiple RF 400 Vpp.

Quadrupole ion energy, 5.0 eV; low mass, 100.00 m/z .

Collision cell settings: collision energy, 10.0 eV; collision RF, 600 Vpp.

The “Ion Cooler” settings are as follows:

Ion cooler RF, 400.0 Vpp, transfer time, 65 μ s; pre-pulse storage, 5.0 μ s (see Note 4). Data acquisition rate is 1 Hz (see Note 5).

3. Click on “MS/MS” tab, selecting MRM option in the left-hand section. Check the boxes for both MS/MS (MRM) and Alternate Collision Energies in the Scan Mode window. This enables the run to be performed with alternating low and high collision energies in bbCID mode. Set the collision energy parameters to 10 and 35 eV in the first and second lines of the table, to specify low and high collision, respectively. Set the Acquisition Factor to 2 for high collision energy; this is set to 1 for the low collision energy as the default (non-editable) value.
4. In the Hystar Acquisition table, specify the CIRAD method as the MS method option and select an LC gradient when using online RP-HPLC. A data file name and storage location will also need to be specified.

3.3. Data Analysis

1. Within Compass Data Analysis v. 4.1 software (Bruker Daltonics), open the data file via the File menu. The file will be visible in the Analysis list, and click on the + symbol to the left of the file to view the chromatograms of total ion current (TIC); these are TIC +All MS and TIC +bbCID. These can be visualized in the Chromatogram window by checking the selection box for each chromatogram. The data in the TIC can be viewed for the duration of the MS, on a spectra-by-spectra basis by highlighting the “arrow” in the tool bar and then clicking on a particular point of interest on the TIC (Fig. 2). The TIC+ All MS shows sequential low and high energy scans and TIC +bbCID shows the high energy scans.

2. If the MS acquisition were performed using a calibrant mass, recalibration can be performed, for fine adjustment of mass calibration. For example, the calibrant mass of 1221.9906 (Molecular composition $C_{24}H_{19}F_{36}N_3O_6P_3$) can be used for recalibration. Calibration in Data Analysis v 4.1 can be performed by clicking on the “Calibrate” tab and selecting “Apply lock mass” or “Automatic internal” to recalibrate either with lock mass calibration (which performs a scan-to-scan correction) or automatic internal algorithms (by also specifying a window of which calibrant signals are averaged and used). If using, the 1221 calibrant mass, this can be selected for the reference list as a component of the “Tuning Mix ESI-ToF (pos)” which is accessed via the software query “Use of specify reference list.”
3. To visualize data containing the acetyl lysine diagnostic ion, perform an hrEIC: Check the TIC+bbCID data, right click in this window, and select “edit chromatograms.” From the drop-down menu, select the following options for Type and Filter: Extracted Ion Chromatogram and All MS, respectively. Type in the mass 126.09, with a mass selection window of 0.05 Da. Select polarity as positive and background to none and edit color of chromatogram if required. Click Add, and then Apply.
4. To link precursor m/z to associated product ions based on LC elution profile: Go to “Find” and select Compounds-Dissect option. Precursors and products are linked by elution time. Creation of the Dissect.mgf allows data to be processed for sequence information by submitting the mgf peak lists generated via “Dissect” to a search tool such as MASCOT (9). The mgf is created by exporting the compounds to the mgf file, via File and then Export Compound.

3.4. Sample Analysis: Beyond Protein Standards

A key technical consideration is the low stoichiometry of PTMs in general, including acetylation, which results in acetylated peptides being difficult to detect without targeted MS or specific enrichment strategies. For lysine acetylation, enrichment is typically achieved by affinity capture of protein or peptides by use of acetyl lysine-specific antibodies (1, 10). In terms of acetylation analysis using CIRAD, a preparation of either acetylated peptides enriched by immunoaffinity capture or immunoaffinity-enriched proteins is recommended. Alternatively, enrichment of specific proteins based on subcellular localization can be performed (11). In a typical experiment, to analyze specific proteins of interest, the protein complex is purified and subsequently resolved by SDS-PAGE followed by Coomassie blue staining to visualize the protein. In order to generate peptides, the gel band needs to be excised from the gel, destained to remove Coomassie blue, and then be subjected to tryptic digestion. Typically this would provide sufficient material for several CIRAD runs.

3.5. Acetylation Site Assignment

When assigning acetylation to a particular peptide sequence, the presence of the 126.1 m/z acetyl lysine ion is diagnostic, and where present, the 143.1 m/z provides additional confirmation. The site of acetylation can be assigned on y ions and b ions a little distant from the modification site; a full sequence is not essential to infer sequence or site of assignment in a database search. A mass difference between two product ions of 170 Da corresponding to an acetylated lysine residue confirms acetyl lysine site assignment (Fig. 2) and in an ideal scenario these ions would be detected.

CIRAD is of particular value for proteins that are co-modified by acetylation and, e.g., phosphorylated or unknown modification, where targeted analysis (assuming the presence of acetylation only) would fail to identify since it is based on a precursor m/z that is not theoretically present in this scenario. As research into PTM continues apace, it has become evident that different PTMs enable cross talk (12) and that different PTMs can occur within the same amino acid sequence, making this feature valuable to acetylation (see Note 6). This approach also provides precursor–fragment pair information for targeted analysis via selected reaction monitoring (SRM) for further PTM analysis, e.g., by MIDAS (13–15) or quantification (16), and thus is complementary to other MS-based techniques. The CIRAD technique is potentially applicable to other modifications, e.g., acylation by propionylation which generates a diagnostic m/z at m/z 140.1063 on fragmentation (17).

4. Notes

1. However, the 143 Da is not of itself truly diagnostic, since it can also be associated with mono-methylated arginine, and thus lacks specificity for acetyl lysine. However, the presence of 143.1 Da can be confirmatory of lysine acetylation when 126.1 Da is also present in the product ion spectrum.
2. Lysine acetylation can be difficult to distinguish from isobaric lysine trimethylation at the MS level, since they both result in an addition of 42 Da to the precursor m/z relative to the unmodified counterpart (monoisotopic masses of 42.0105 and 42.0469 Da, respectively). The specificity of the 126.1 ion for lysine acetylation enables discrimination between acetylation and trimethylation of lysine (5).
3. This protocol can also be performed using MS instrumentation other than the maXis™ that has bbCID functionality and is essentially an MS^E time approach, as used for “label-free” protein quantification (18).

4. These parameters are maXis™ specific. MS instruments can vary and we have found this to be the case for the maXis as well (8). The guidelines thus provide a starting point for setting up CIRAD, for maXis™ instrumentation, with the rationale on how to optimize parameters in Subheading 3.1.
5. This rate can be faster. Essentially the faster the rate the more data will be sampled across the elution peak; the signal intensity will be lower in this case, and the settings given are assuming an elution peak width of 30 s.
6. PhosphoSitePlus® is an online database (www.phosphosite.org), listing manually curated PTMs, providing information on sites of phosphorylation and other commonly studied PTMs including acetylation, glycosylation which can be useful in assessing the current information available for a specific protein, in terms of known PTMs.

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