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Short communication

Accelerating photofragmentation UV Spectroscopy–Mass spectrometry fingerprinting for quantification of isomeric peptides

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ABSTRACT

Identification of isomeric biomolecules remains a challenging analytical problem. A recently developed spectroscopic method that combines UV photofragmentation and mass spectrometry for fingerprinting of cold ions (2D UV-MS), has already demonstrated its high performance in the library-based identification and quantification of different types of biomolecular isomers. The practical use of the method has been hindered by a slow rate of data acquisition, which makes the fingerprinting incompatible with high-throughput analysis and online liquid chromatography (LC) separation. Herein we demonstrate how the use of a few pre-selected wavelengths can accelerate the method by two orders of magnitude without a significant loss of accuracy. As a proof of principle, 2D UV-MS fingerprinting was coupled to online LC separation and tested for quantification of isomeric peptides containing either Asp or isoAsp residues. The relative concentrations of the peptides mixed in solution have been determined, on average, with better than 4% and 6% accuracy for resolving and non-resolving gradients of LC separation, respectively.

1. Introduction

Although absolutely identical in chemical composition, isomeric biomolecules may drastically differ in their functionality, which makes identification of their isoforms indispensable in many biological applications but remains challenging [1–4]. Tandem mass spectrometry (MS), which is the core technique for the bottom-up proteomics analysis, typically cannot distinguish isomeric biomolecules [5]. Occasionally, they can be identified by MS using specific dissociation techniques. For instance, electron-capture/transfer dissociation enables distinguishing isomeric peptides with asparagine, aspartic acid and iso-aspartic acid residues by monitoring the unique diagnostic fragment ions of the peptides [6–8]. The technique of ^{18}O-labeling [9,10] and the use of liquid chromatography (LC) or ion mobility further increase the capabilities of MS in distinguishing the isomers [11,12]. Despite all the advances of these techniques, the accurate quantification of isomeric peptides by LC-MS remains challenging [13–16].

Recently, the method of 2D UV-MS fingerprinting of cryogenically cooled ions was successfully demonstrated for library-based identifications and quantification of isomeric peptides [17–19], drug molecules [20] and carbohydrates [21,22]. This analytical method integrates UV photofragmentation (UVPD) spectroscopy with broadband high-resolution MS [17]. It measures the abundances of all UV-induced photo fragments of analyte ions at once as a function of UV wavelength. The resulting two-dimensional data array, 2D UV-MS fingerprint, reflects the structure of the ions. Cryogenic cooling enables vibrational resolution in electronic spectra of mid-size biomolecular ions [23], which greatly sharpens the fingerprints. Unlike LC and ion mobility, optical spectroscopy reflects molecular transitions between quantum states, which are fundamental to ions. This makes 2D UV-MS spectra highly reproducible standards that can be shared across different laboratories. The fingerprints are, first, measured for each expected or possible isomer of a molecule; they are tagged by the mass of the compound and stored as a library. Next, the 2D UV-MS spectra of an analyte mixture with an unknown number and identity of these isomers is measured. To determine the relative concentrations of the mixed

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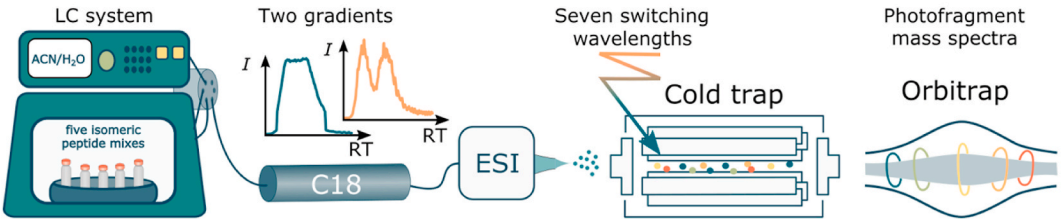

Fig. 1. Schematic view of the experimental setup combining (via electrospray ion source) an LC system with the UV-MS instrument including a cold ion trap, OPO laser and an Orbitrap mass analyzer for high resolution detection of photofragments.

isomers, the 2D UV-MS data array of the mixture is linearly decomposed in the basis set of the library data arrays. The coefficients of the best fit provide the relative concentrations of the mixed isomers.

Although highly specific and accurate, so far, the method has remained too slow for practical applications and has been limited to offline quantification of biomolecular isomers. Recording a full fingerprint at, typically, as many as several hundreds of subsequent wavelengths requires up to an hour. The feasibility to shorten this acquisition time was simulated earlier in the quantitative analysis of the mixtures of isomeric biomolecules [19]. In that modelling, the decomposition of the 2D data arrays was performed only at a few, but the most isomer-specific wavelengths, which had been pre-selected from the whole spectral range of the measurements. Such wavelengths are associated with characteristic spectral features of the isomers (peaks, UV absorption onsets). For each set of the wavelengths, which are not unique, the decompositions of data arrays measured for mixtures of the isomers were performed and RMSD (root-mean-square deviation) was estimated. The best sets (small number of wavelengths, but low RMSD) were kept as characteristic ones. The modelling predicted that the use of such few wavelengths should not decrease the accuracy of the quantification significantly but will shorten the acquisition time to a scale compatible with online LC separation. Herein, we implement this approach and squeeze the acquisition time of 2D UV-MS fingerprinting as much as by two orders of magnitude. In order to evaluate the performance of this accelerated measurements, we compare the accuracy of quantification for mixtures of two amylin (30–37) isomeric peptides, NVGSDTY-NH_2 and NVGSisoDTY-NH_2, quantified by the 2D UV-MS method using a broad spectral range and one of the sets of specific wavelengths that were determined earlier [19]. Finally, as a proof of principle, we demonstrate the online quantitation of these peptides by 2D UV-MS fingerprinting coupled to LC separation.

2. Material and methods

The workflow of our direct infusion measurements and the experimental hardware have been described elsewhere [17,24]. Briefly, we use electrospray ionization to produce ions from an analyte solution of the mixed isomeric peptides. The precursor ions with a desired *m/z* ratio are selected by a quadrupole mass filter, accumulated and thermalized in an octupole ion trap. Subsequently, the ions are transferred to a cryogenic octupole ion trap kept at T = 6 K, where they are collisionally cooled by He buffer gas to vibrational temperature of 10–12 K [23]. Subsequently, the cold ions undergo photofragmentation by a 1–2 mJ pulse of a UV optical parametric oscillator (OPO), whose wavelength can be rapidly changed using a preprogramed computer control. The appearing charged photofragments and the remaining precursor ions are ejected from the trap and guided into the Orbitrap mass analyser, which monitors the entire fragment mass spectrum. The wavelength is then changed and the whole cycle is repeated.

The 2D UV-MS spectra of the two peptides (Asp- and isoAsp-containing isomers) have been measured offline in the whole wavelength range (255–290 nm) with the step of 0.05 nm. The respective 2D data arrays were normalized to the precursor ion intensity and to the energy of UV pulse, processed and stored as a library. Seven wavelengths (Fig. S1) that correspond to the spectral regions with no UV absorption and to the intense resolved isomer-specific peaks have been selected in these spectra to distinguish the isomers. Each 2D UV-MS data array of the library was truncated to contain the fragment MS measured only at these selected wavelengths (Fig. S2). To assess the performance of the "accelerated" method, we measured the truncated 2D UV-MS fingerprints only at these wavelengths for three mixtures of the two peptides and for their pure solutions. For calculating the identity and the relative concentrations of the mixed peptides, these reduced data arrays were decomposed in the basis set of the two truncated library data arrays. It is worth mentioning that the rapid computer-controlled switching between the widely spread UV wavelengths would be challenging with a grating-controlled dye laser, but became possible with an angle-tuned UV OPO.

For the online implementation of 2D UV-MS method, a chromatographic system, which includes a P680 gradient pump and an ASI-100 autosampler (Dionex, Sunnyvale, CA) with a Zorbax 300SB-C18 column (length 150 mm, i.d. 2.1 mm, Agilent Technologies, Santa Clara, CA), was coupled to the electrospray ion source, as shown schematically

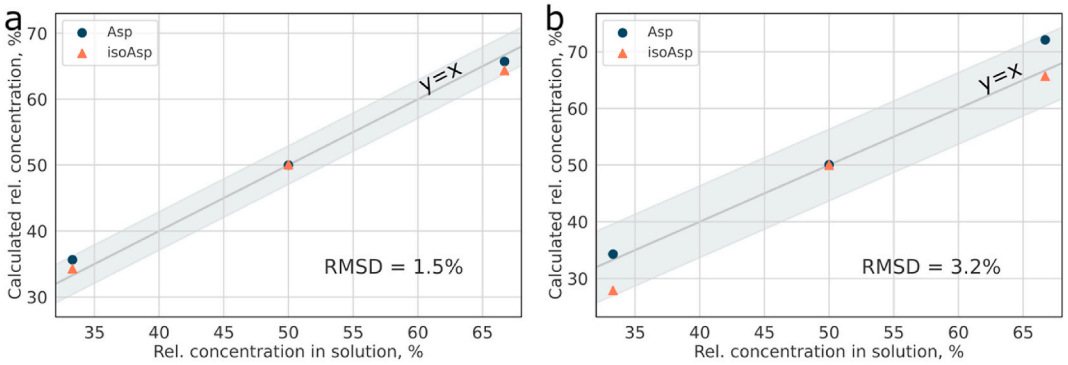

Fig. 2. Calculated relative concentrations of two isoforms of amylin(30–37) peptide as a function of their actual concentrations in three solution mixtures (1:1, 1:2 and 2:1) for 2D UV-MS spectra measured (a) in the whole wavelength range and (b) at the seven selected wavelengths The y = x solid line indicatively shows the ideal correspondence of the calculated and the prepared concentrations, and the grey area around this line corresponds to the confidence interval of doubled RMSD.

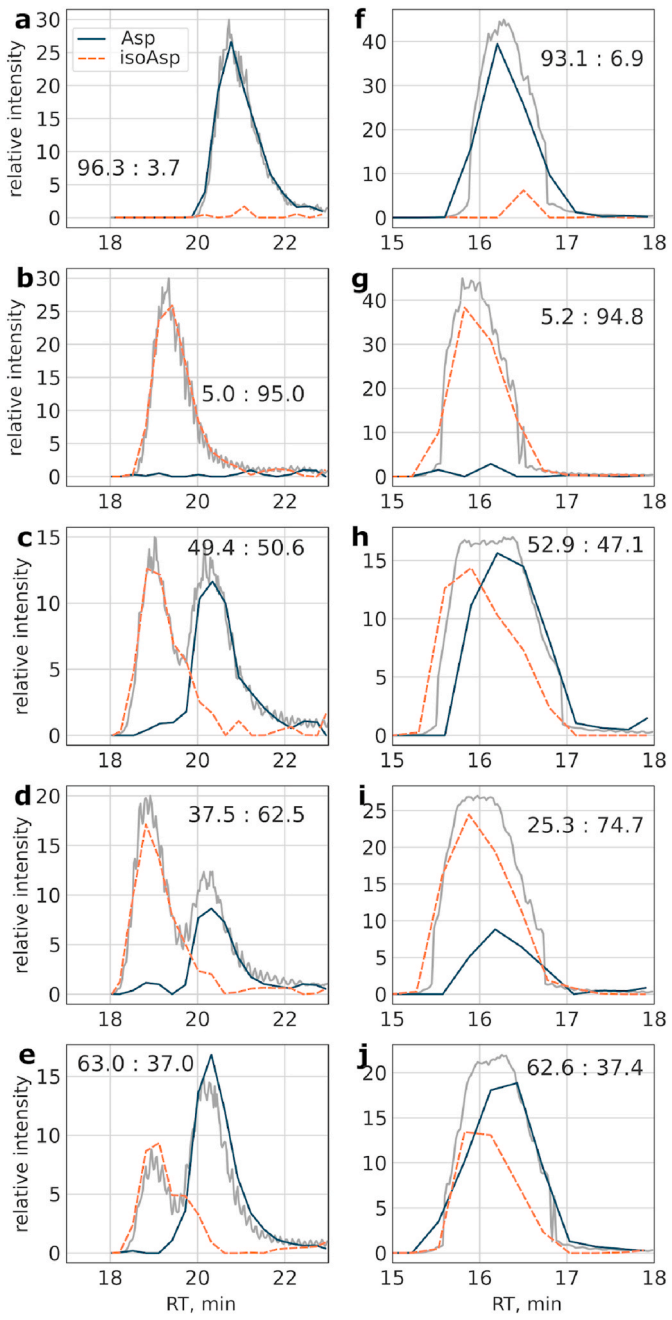

Fig. 3. Chromatograms measured by UV-MS method (a–e) with a resolving gradient, (f–j) with a non-resolving (steep) gradient, (a, f) only for the Asp-containing peptide, (b, g) only for the isoAsp-containing peptide, (c, h), (d, i), and (e, j) – for mixtures of the Asp- and isoAsp–containing peptides with relative concentrations of 1:1, 1:2, and 2:1, respectively. The grey lines show the chromatograms of the precursor ions; the blue solid and red dashed lines show the chromatograms calculated by the decomposition of the 2D UV-MS data array for the peptides with Asp and isoAsp residues, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

in Fig. 1. The HPLC-grade water mixed with acetonitrile (ACN) and 0.2% of acetic acid was used as the solvent. Two different LC gradients were used to illustrate the capabilities of the rapid UV-MS measurements for both resolved and non-resolved chromatographic peaks. In the former case, the gradient included a 5-min equilibration period at 2% concentration of ACN, which was subsequently increased to 5% ACN in 2 min, to 10% ACN in 20 min and to 90% in 5 min. Finally, the column was flushed for 10 min, followed by a re-equilibration. The non-resolving gradient consisted of the same equilibration part followed by a steep increase of ACN concentration to 15% in 5 min, with the same washing and re-equilibration procedures. The OPO wavelength was tuned subsequently through the set of the seven selected values, continuously cycling this tune back and forth during the whole LC gradient time. The details of UV-MS data array decomposition are explained below.

3. Results and discussion

Fig. 2 shows the plots of the calculated vs prepared solution concentrations of the isomeric peptides for the fingerprints measured continuously in the whole spectral range and at the seven pre-selected wavelengths. To illustrate the quality of the fits, Figs. S3 and S4 show the integral UV- and mass spectra of the full-range decompositions and compare them with the measured data. The calculated root-mean-square deviation (RMSD) for the decompositions in the full spectral range is only twice smaller than with the seven-wavelengths (1.5% and 3.2%, respectively). This result experimentally demonstrates that cold ion UV spectroscopy performed at a few specific wavelengths only (instead of in the entire spectral range) may allow for an accurate quantification of isomers. The great benefit of the particular technical implementation of this approach herein is the shortening of the data acquisition time by two orders of magnitude. The measurement included the accumulation of fragment ions from five laser pulses (0.5 s at 10 Hz repetition rate of the OPO) for every single microscan of the Orbitrap analyser, the averaging of three microscans per each MS scan, and the acquisition of two scans at each wavelength. The latter is required for avoiding an occasional MS detection of photofragments at two subsequent wavelengths of the set in the single cycles. This technical problem can be fixed by fully synchronizing the working cycles of the OPO and the MS instruments, which, however, requires an access to the commercial software of the latter. The current workflow of the measurements results in the total acquisition time of 3 s at each wavelength, or 21 s for all seven wavelengths. Such acquisition rate appears to be fast enough to demonstrate the 2D UV-MS identification of the peptide online with HPLC separation. Approximately three full cycles of the measurements could be performed within a chromatographic peak of 1 min duration. Solving the described above problem of synchronisation should shorten the full scan to ~10 s, which is well below the typical timescale of peak elution in many LC measurements; combination with a fast (6 Hz) ultrahigh field Orbitrap analyser and a 20 Hz OPO may further shorten the acquisition timescale of UV-MS.

Fig. 3 shows the chromatograms measured with the UV-MS detection for five mixtures of Asp- and/or isoAsp-containing isomeric peptides at their relative concentrations of 1:0, 0:1, 1:1, 1:2 and 2:1. These experiments were performed under both resolving and non-resolving LC conditions. The measured fragment MS were normalized to the intensity of the precursor ions and to the OPO power. Linear decomposition of the resulting 2D data arrays in the basis set of the library data arrays (Fig. S2) allows for calculation of the relative concentrations at every segment within an elution peak. The decomposition was performed for each segment of the elution peak that corresponds to each cycle of seven wavelengths (see Fig. S5 as an example). With these calculations, the elution peaks of the isomeric peptides could be restored from the total chromatogram. Finally, the integration of the corresponding elution peaks allows for the determination of the relative concentrations of the two isoforms. The accuracy in the quantification of the isomers by decomposition of the time-integrated UV-MS fingerprints is expectedly higher for the non-resolving (steep), than for the resolving LC gradient (Fig. S6), because only a fewer time segments were available for the former.

In conclusion, we experimentally demonstrated a fast, sub-minute, 2D UV-MS sensing of mixtures of two isomeric peptides (amylin (30–37) with Asp or isoAsp residue) using just seven pre-selected wavelengths, instead of, typically, many hundreds of them in a wide

spectral range. Although accelerated by hundred times, the accuracy in quantification of relative concentrations of the mixed isomers degrades only by a factor of two. The acceleration enables coupling of the 2D UV-MS fingerprinting to online LC separation. As a proof of principle, we demonstrate an isomeric profiling of the LC elution peaks by 2D UV-MS sensing, which still allows for 4–6% accuracy. Further work is required to fully assess the benefits and limitations of the accelerated fingerprinting before advising a workflow for its implementation as an analytical tool. In particular, an algorithm for a rational pre-selection of isomer-specific wavelengths is to be developed, tests with non-fully LC-resolved multicomponent mixtures are to be performed for evaluating the synergy of the two techniques, etc. Nevertheless, the demonstrated herein principle of identification of large non-volatile isomeric molecule by sensing them at a few UV wavelengths, instead of the time-consuming continuous wavelength scans, should be generally valuable and efficient whenever a vibrational spectral resolution is achievable (e.g., by cryogenic cooling). For instance, it can be extended to the identifications of isomeric biomolecules by the currently popular methods of IR ion spectroscopy, where the acquisition is still on a 10 min timescale at best [25].

Credit author statement

Anna A. Lobas:: equal contributions to performing experiment, data treatment and writing the manuscript. Elizaveta M. Solovyeva: equal contributions to performing experiment, datssa treatment and writing the manuscript. Erik Saparbaev: preparation of experiments and technical support. Mikhail V. Gorshkov: discussion of the data and correcting the manuscript. Oleg V. Boyarkin: concept of the experiment, data discussion, writing the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2021.122412.

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