Title: Coupling liquid chromatography to Orbitrap mass spectrometry

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Abstract

The Orbitrap mass analyzer has become a mainstream mass spectrometry technique. In addition to providing a brief introduction to the Orbitrap technology and its continuing development, this article reviews the most recent publications quoting the use of the Orbitrap detection for a variety of chromatographic separation techniques. Its coupling to reversed-phase liquid chromatography (LC) represents undoubtedly the most ubiquitous approach to both small molecule and proteomic analyses. Multidimensional LC separations have an important role to play in the proteomics applications while an ultra-high-pressure LC is more frequently encountered in the area of metabolomics and metabolite analysis. Recently, special chromatographic techniques such as hydrophilic interaction chromatography and its variations have also been also cited with the Orbitrap detection.

Key Words

Orbitrap

LC-MS

High resolution mass spectrometry

1. Introduction

The volume and quality of knowledge acquired in chromatographic experiments depends directly on the advances in analytical instrumentation. The application of mass spectrometers (MS) as detectors for liquid chromatography separations is no exception. Recent years have witnessed a significant shift from using single- and triple-quadrupole mass spectrometers towards employing mass spectrometers that provide accurate mass of analytes, such as of time-of-flight (TOF), Fourier-transform ion cyclotron resonance (FT ICR), and Orbitrap detectors.

The Orbitrap mass analyzer was first described in 2000 [1] and has now reached the status of a mainstream mass spectrometry technique. Combination of the Orbitrap analyzer with an external accumulation device such as a linear ion trap enables multiple levels of fragmentation (MS^n) for the elucidation of analyte structure and allows coupling with continuous ionization sources such as atmospheric pressure chemical ionization source, electrospray (ESI) or nanospray. The Orbitrap analytical performance can support a wide range of applications from routine compound identification to the analysis of trace-level components in complex mixtures, be it in proteomics, drug metabolism, doping control or detection of food and feed contaminants [2-5]. In this review we specifically address chromatographic applications where the Orbitrap is used as the detector.

2.1. Principle of Orbitrap Detection

Since its commercial introduction in 2005 [6], Orbitrap mass spectrometry has grown into a blossoming tree which includes quite a number of instruments of different layout and complexity (Figure 1). The common feature of all these instruments is the use of the Orbitrap mass analyzer preceded by an external injection device based on trapping ions in RF-only gas-filled curved quadrupole (the C-trap, Figure 2). The C-trap allows storage of a significant ion population and then its injection into the Orbitrap analyzer in a short pulse so that each mass-to-charge (*m/z)* population forms a sub-microsecond pulse. These short ion packets are focused on the entrance aperture of an outer curved electrode of the Orbitrap analyzer which surrounds the curved central electrode sustained at a high voltage. As ions enter the space between electrodes tangentially at

an offset from the Orbitrap equator, a strong electrical field inside the trap pushes them towards the equator thus initiating axial oscillations, while rotation around the central electrode keeps ions from falling onto the central electrode. This “excitation by injection” is described in greater detail in [7, 8]. Strong dependence of rotation on ion energies, angles, and initial positions forces each ion packet to spread quickly over the angular coordinate forming a thin rotating ring. The whole ring then oscillates along the central electrode harmonically with a period proportional to $(m/z)^{1/2}$ and produces an image current on split outer Orbitrap electrodes.

A broadband detection of this signal is followed by a fast Fourier transform (FT) to convert the recorded time-domain signal into a frequency, and then into *m/z* spectrum. This method of detection brings Orbitrap analyzers into the family of FT mass spectrometers which was, until recently, represented by FT ICR alone. Linearity of detection and very high fidelity of frequency determination are inherent to FT mass spectrometry and thus allow very high resolving power, mass accuracy and dynamic range to be achieved. Due to weak dependence of sensitivity on detection time, Orbitrap analyzers have an important advantage for chromatography: the dynamic range goes down much slower with increase of repetition rate in comparison to other accurate-mass analyzers (*e.g.*, TOF). In this context is it worth mentioning that the Orbitrap analyzer shows an insignificant trade-off in sensitivity *vs* resolving power.

The process of capturing ions in the C-trap and injection into the analyzer takes just several milliseconds so it could be easily interfaced and synchronized to any external device such as a linear ion trap mass spectrometer or even directly to an ion source. The process of detection requires a much longer period of time than injection as resolving power is directly proportional to the number of detected oscillations while sensitivity is proportional to square root of this number. For a commercial Orbitrap analyzer, nominal resolving power of 100,000 FWHM (full width at half maximum peak height at *m/z* 400) requires 1 to 1.5 seconds detection time. The C-trap enables several intriguing modes of operation:

- The C-trap supports multiple fills. An injection of a fixed number of ions of a known reference compound can be followed by the injection of analyte ions. Both sets of ions are then detected simultaneously in the Orbitrap. This procedure allows for a robust internal calibration of each mass spectrum, with r.m.s. errors below 1 ppm [9].
- Multiple injections of ions fragmented or selected under different conditions can be stored together and acquired in a single Orbitrap mass spectrum (to be implemented in the future).
- Ions can be fragmented by injecting them into the C-trap at higher energies to yield fragmentation patterns similar to those in triple-stage quadrupole mass spectrometers [10].
- The C-trap represents a 'T-piece' which allows one to interface it to additional devices, such as collision cells, ion/molecule reaction cells, or ETD [11].

2.2. Performance Characteristics

2.2.1. Mass Accuracy

One of the most coveted attributes of a mass analyzer is undoubtedly its mass accuracy. Its usefulness can be gleaned from the fact that correct elemental composition is the first crucial step to structural elucidation of unknown molecules. The mass of chemical elements is based on a convention defining carbon ^{12}C = 12.000 u. All other elements are either slightly above or below their integer value (*e.g.*, ^{1}H = 1.007825 u, ^{16}O = 15.994910 u) [12]. The ability to measure a compound's mass with adequately high accuracy can directly determine its elemental composition simply by eliminating most other possibilities. In many cases, however, additional information other than just the accurate mass measurement will be needed to obtain correct elemental composition, among others restrictions for the number of elements considered, Lewis and Senior chemical rules, or isotopic patterns [13-15]. Other aspects of the usefulness of mass accuracy for small molecule analysis undoubtedly include the application of mass defect, as discussed elsewhere [16].

Metabolomics and lipidomics are disciplines largely driven by the performance of the analytical instrumentation used. Maintaining high mass accuracy in metabolomics applications throughout

148 the duration of the chromatographic experiments makes it possible to extract ion chromatograms
149 with a sufficiently high degree of accuracy. This ability opens the possibility to perform both
150 metabolic profiling and targeted analysis of known compounds in a single experiment. Using
151 selective *m/z* ratios is more powerful for distinguishing quantitative differences of known
152 compounds, while unsupervised profiling is able to show qualitative differences, for known and
153 unknown components. The use of high-resolution and accurate mass thus offers the possibility to
154 combine qualitative and quantitative workflow without compromising quality for either strategy [17].
155 Such applications typically require 5 ppm or better mass measurement accuracy [18].
156
157 In the context of proteomics the *precursor masses* are used as constrains for database searches.
158 Thus, the mass accuracy is also here an extremely important parameter, whose incorrect
159 determination can lead either to identification statistics that are worse than they need to be (if
160 mass accuracy window for database search is set too wide) or to missed identification (false
161 negatives) if the window is set too narrow [19]. In biomarker discovery studies, the resulting
162 accuracy translates into improved alignment and quantification across spectra [20].
163
164 Acquiring *tandem mass spectra* with a high mass accuracy is an interesting alternative to
165 classical data acquisition schemes where fragment ions are detected at much lower mass
166 accuracy and resolution in the linear ion trap or triple quadrupole. When acquiring fragmentation
167 spectra in the Orbitrap detector, the lower number of spectra and a higher detection limit
168 (compared to the detection of the fragmentation spectra in a linear ion trap) are offset by the
169 additional specificity of identifications. In other words, maintaining the same false positive ratio
170 one still obtains similar number of protein identifications using either fast but low resolution ion
171 trap detection or slower but high resolution Orbitrap detection. A much greater degree of
172 confidence in the identification of peptides with unexpected modifications can be obtained by
173 choosing to acquire (fewer) high mass accuracy tandem spectra compared to acquiring
174 (considerably more) lower mass accuracy tandem spectra [21].
175

The Orbitrap detector can reliably deliver internal mass accuracy below 1 ppm [6, 9, 22-24]. This accomplishment can be aided by exploiting the use of laboratory ambient contaminants [9] as internal calibrants within each single LC-MS run. Certain background ions present in nearly all scans provide a large number of measurements allowing for very precise mass estimations and robust landmarks for aligning and calibrating multiple files [20]. Recently, an approach relying on non-linear recalibration of the Orbitrap mass scale has been reported, bringing the deviations in mass measurement for each peptide typically within 100 parts per billion (ppb) without an additional requirement for having an internal standard (lock mass) added to the sample. The deviation of mass values determined from charge pairs is used to convert mass precision to mass accuracy, and then applied to database searches. Often, such a result limits the peptide to a single composition and, therefore, the highest useful accuracy [19]. It is also believed that such values of mass accuracy are close to the hardware limits of the Orbitrap in its current implementation. Further progress in this direction would require a considerable improvement in resolving power.

2.2.2. Resolving power

For a given sample analyzed, the resolving power is a key parameter affecting the correct assignment of masses for analytes. The accurate mass can only be relied upon when measured at sufficiently high resolution; overlapping nearly isobaric species will skew the mass reading (Figure 3). This can have important consequences for screening approaches. If, for instance, the extraction window is set too wide, compensating for possible matrix interferences and/or inadequate resolving power, then 1) mass accuracy will be compromised, and 2) hidden interferences will contribute to the integrated peak area detected for the compound of interest. In effect, there is a serious risk of having *a false positive*. If, on the other hand, the user sets an extraction window too narrow, the compound showing a higher mass deviation than expected due to the presence of an unresolved interference could go completely undetected. There is a risk of *a false negative* as illustrated in Figure 4.

204 The analysis of residues and contaminants in food and animal feed safety is one of the areas
205 where the importance of adequate mass resolution has been demonstrated. For consistent and
206 reliable mass assignment (<2 ppm) of analytes at low levels in complex mixtures, a high resolving
207 power (> 50,000) was found to be required. At lower resolving power settings, the error in the
208 mass assignment increased due to co-elution of analytes with interferences of similar mass. This
209 negatively affected selectivity and quantitative performance because narrow mass-extraction
210 windows could not be used [25].
211
212 The benefits of employing high-resolution extracted ion chromatography can be also
213 demonstrated on metabolomics and lipidomics analysis of human serum samples. The
214 combination of incorporating high resolution and accurate mass is essential for quantifying
215 targeted analytes (absolute or relative) as well as identifying unknown components in a single
216 analysis. Incorporating this type of mass spectrometer for metabolomics offers the possibility of
217 obtaining both sets of information, with no compromises, in a single analysis [17].
218
219 The resolving power of the Orbitrap is directly proportional to the acquisition time (i.e. the longer
220 the acquisition time the higher the resolving power). An acquisition time of 0.4 s provides a mass
221 resolution of 30,000, translating to about 25 data points across a peak of width at baseline of 10 s
222 [26]. Ongoing developments of the Orbitrap mass analyzer are addressing the trade-off between
223 resolving power and scan speed.
224
225 2.3. Most Recent Developments in Orbitrap Technology
226 The latest additions to the 'tree' in Fig.1 are represented by the LTQ Orbitrap Velos™ and
227 Exactive™. The LTQ Orbitrap Velos (Figure 5) utilizes Orbitrap as an accurate mass detector for
228 a linear trap mass analyzer (referred to as a hybrid mass spectrometer). Though this instrument
229 clearly descends from the previously described LTQ Orbitrap hybrid mass spectrometer [6, 9, 10],
230 a number of important modifications enhance its performance:

- A stacked-ring ion guide on atmospheric-to-vacuum interface increases transmitted ion currents by a several-fold. This in turn permits reduction of ion fill times which leads to increased speed of analysis of low-abundance components in complex mixtures, especially when used with Orbitrap detection.
- A standard linear ion trap has been replaced by an integrated dual-pressure linear trap with an appropriately modified RF circuitry. This helium-filled device contains a high-pressure region for speedy mass selection and dissociation followed by a low-pressure region for very fast scanning. The corollary to this innovation is a two-fold increase in the linear trap scan rate [27].
- Improved integration between the C-trap and the HCD (higher-energy collision induced dissociation) cell provides better vacuum which translates into an increased efficiency and speed of fragmentation [28].

In addition to the aforementioned hybrid Orbitrap-based instruments, a non-hybrid mass spectrometer (Exactive^{TM}) has been developed consisting of a stand-alone Orbitrap analyzer with an atmospheric-pressure ionization source (API; Figure 6) [29]. The Exactive allows combining high resolution, mass accuracy and dynamic range inherent to the Orbitrap mass analyzer with such features as fast polarity switching (full cycle of one positive and one negative scan within one second), bench top design, and scan speeds of up to 10 spectra/s.

In the Exactive system, samples are introduced into the API source and the ions formed are transferred from the source through four stages of differential pumping using RF-only multipoles into the C-trap. There ions are accumulated and their energy dampened using a bath gas (nitrogen). Ions are then injected through three further stages of differential pumping using a curved lens system into the Orbitrap analyzer, where mass spectra are acquired via image current detection (as described previously in section 2.1).

In order to control the ion population within the trap, a pre-scan is used in the Exactive instrument to estimate ion current and then to calculate the ion injection time for the subsequent analytical scan. For high scan rates over wide mass range, the previous analytical scan is used as a pre-scan in order to optimize the scan cycle time without compromising automatic gain control. Ion gating is performed using a fast split lens setup that ensures the precise definition of the ion injection time. In addition, the Exactive instrument introduces a feature of broad-band fragmentation without mass selection ("All Ions MS/MS") which can be implemented using an optional HCD collision cell after the C-trap. This allows an independent confirmation of identify for compounds detected in the precursor scan, and is exemplified by ample references below.

One way for achieving higher resolving power over fixed acquisition time has been outlined by increasing the frequency of ion oscillations in the Orbitrap analyzer. This was achieved by decreasing the gap between the inner and outer electrodes, thus providing higher field strength for a given voltage. Resolving power in excess of 600,000 at m/z 195 and isotopic resolution of proteins above 40 kDa was achieved [30]. Note, this work was done on an experimental prototype which is not a commercially available instrument.

3. LC Separations Coupled to the Orbitrap Detector

High-resolution mass spectrometry coupled to LC is a very powerful combination. The Orbitrap publications most frequently cite the use of reversed-phase chromatography. The use of ultra-high pressure LC (UHPLC) in combination with the Orbitrap detection, both for peptide and small molecule analyses is of a particular interest. Additionally, combinations of several chromatographic media, such as strong cation exchanger and reversed phase, form the basis of multidimensional LC separation strategies used for complex peptide mixture analyses. Similarly, combining a reverse phase separation with a chromatographic phosphopeptide enrichment step can also be viewed as a multidimensional LC technique. Hydrophilic interaction chromatography (HILIC) is being mentioned in the context of phosphopeptide analysis, metabolomics and bioanalysis. The following sections focus in more detail on individual LC techniques coupled to the Orbitrap detection.

3.1. Reversed-phase LC

Out of the many LC techniques coupled to MS, the reversed-phase LC is undoubtedly encountered most frequently, in both proteomics and small molecule applications.

In proteomics applications, which typically rely on a limited amount of starting biological material, the use of capillary columns with 75-100 μm internal diameter are very common, if not vitally critical. An added benefit of using these columns are that the low flow rates (in the order of several hundreds of nanolitres per minute), are optimal for high sensitivity of electrospray MS [31]. Linear gradients starting from 0-5% acetonitrile in aqueous formic acid (usually 0.1% v/v) to final 40-60% acetonitrile concentration are normally employed for the elution of peptides. Even though there are ready-made columns available, many research groups prepare their own capillary columns using a wide variety of stationary phases. In general, C18 phase is used for peptide separations, with particle size 3-5 μm, pore size 300 A.

303 The ubiquitous employment of reversed-phase LC and LTQ Orbitrap for proteomics makes it
304 difficult to capture all the references. In fact, the majority of routine protein identifications are
305 performed with this technique often following a digestion of gel separated intact proteins or
306 fractionation of complex peptide mixtures after isoelectric focusing. Nevertheless, the effort
307 capturing the complete yeast proteome is definitely worth mentioning [32]. The detected proteins
308 spanned 4 orders of magnitude in abundance and no discrimination against membrane or low
309 level regulatory proteins was observed. To date, the largest number of confident protein
310 identifications within a single proteome has been a study of embryonic stem cells [33]. The
311 comparison of two sample preparation methods, a one-dimensional sodium dodecylsulfate gel
312 electrophoresis of intact proteins vs. isoelectric focusing of enzymatic digest of the sample,
313 showed that both methods delivered a comparable result with no apparent bias for any functional
314 protein class.
315
316 Large-scale phoshopeptide mapping is an exciting area of proteomics. Using nano-LC with C18
317 material packed into self-pulled columns coupled to the LTQ Orbitrap provided an insight into
318 tyrosine phosphorylation in plants, an area that has been largely underestimated to date [34].
319 Most phosphopeptide identification and quantitation strategies, however, rely on some form of
320 phosphopeptide enrichment prior to the reversed-phase separation (discussed in section 3.3).
321
322 While C18 material is typically being employed for peptide analyses, C4 or C8 are preferred for
323 larger peptides and proteins. A recently published study provides a practical guidance for
324 analyses of 150 kDa monoclonal immunoglobulin gamma antibodies using an on-line LC-MS
325 approach. The accuracy of mass measurements of intact antibody achieved with the Orbitrap
326 detection was within +/- 2 Da (15 ppm). Moreover, the glycoforms of intact antibodies separated
327 by 162 Da were baseline resolved. The on-line reversed-phase LC/MS analysis also delivered an
328 isotopic resolution for the light chain (approximate MW 23 kDa) [35].
329
330

For LC/MS-based metabolomic profiling studies, the focus is on the development of efficient and robust LC-MS methods for the identification of large number of metabolites in biological samples using both positive and negative electrospray modes. A detailed study performed with the LTQ Orbitrap compared various LC stationary phases in conjunction with multiple mobile-phase systems. It benchmarked the selection of the best mobile and stationary phase based on the separation efficiency of a 45-component metabolite mixture. A material with small pore size (e.g., <100 A) and large surface area (e.g., >400 m^2/g) provided the greatest retention of small, polar analytes. In this study, the optimum mobile phase contained 10 mM ammonium acetate in water (pH 5.3, adjusted with acetic acid; A) and 10 mM ammonium acetate in 90% acetonitrile/10% water which allowed for detection in both positive- and negative-ESI mode. The exponential gradient offered better separation efficiency [36].

Environmental applications are yet another area making use of reversed-phase LC coupled to the Orbitrap MS. In one such example, the Orbitrap was utilized for exact parent mass measurement for 1H-benzotriazoles and benzothiazoles in drinking water and surface water. The method was extended for monitoring at least one product ion simultaneously detected in the linear ion trap. Albeit the product MS spectrum is being acquired at low resolution, the information serves for the confirmation of compound identity. The method allowed the quantification of these residuals down to the detection limits of 0.01 μg/L [37].

3.2. Ultra-high-pressure LC

Ultra-high-pressure LC (UHPLC) operates with sub-2 μm chromatographic particles and a fluidics system capable of operating at pressures up to 15,000 psi, providing an increased chromatographic efficiency compared to conventional HPLC using larger particles. It allows using a wider range of linear velocities while maintaining good chromatographic resolution, and thus providing more rapid analysis times [38].

Consequently, fast acquisition rates are required so as to provide sufficient data points across narrow chromatographic peaks. An acquisition rate of 0.4 s on the LTQ Orbitrap provides a mass resolution of 30,000 (and 25 data points across a peak of width at baseline of 10 s observed in [39]). Acquisition of MS/MS spectra can be conveniently performed either in the parallel-scanning linear ion trap or in the Orbitrap at resolution of 7,500 with a scan time of 0.1 s (i.e., a full scan at resolution 30,000 followed by an MS/MS scan at resolution 7500, resulting in 20 data points collected across a peak). The positive outcome of this theoretical estimate was verified for metabolic profiling of serum samples. The results confirmed that peak widths of 5-10 s allow the collection of an adequate number of data points across the chromatographic peak while maintaining good sensitivity and mass accuracy [39].

Another aspect of the UHPLC-Orbitrap analysis is a quantitative linear dynamic range extending over 2.5 – 3.5 orders of magnitude with correlation coefficients greater than 0.993, and limits of detection for metabolites less than 1 μmol/L which lies below the physiological concentrations of many primary metabolites. Sub-2 ppm mass accuracy was readily obtained over a wide concentration range, and extending to concentrations approaching the limit of detection for the metabolites studied. This allowed narrow (highly selective) mass range windows to be employed for quantitation and also provided more definitive metabolite identifications [39].

The ability to obtain mass spectra with a very high degree of mass accuracy at sufficient mass resolution and scan rates opens the possibility for combining targeted analysis as well as unbiased metabolite profiling without any compromise. UHPLC coupled to the LTQ Orbitrap was used for analysis of human plasma samples focusing on phospholipids [17]. Because the theoretical masses can be used for ion extraction, it becomes possible to query the data with a list of theoretical candidate metabolites, without the need for any prior experimental screening, results or evidence. The real advantage, however, is the post-acquisition availability of accurate mass information for any ion in the full scan spectrum, with a degree of specificity equal to most MS/MS assays [40].

Discovery-stage drug metabolism studies (pharmacokinetics, microsomal stability, etc.) typically rely on triple-quadrupole-based approaches for quantitative analysis. This requires the optimization of various parameters (such as Q1 and Q3 *m/z* values, collision energy) but the biggest drawback is that these studies then detect only the specified compounds – information about other components, such as metabolites, is lost. The ability to perform full scan acquisition for quantitative analysis would eliminate not only the need for compound optimization but also enable detection of metabolites and other endogenous components.

The instrumental parameters of a bench-top Orbitrap MS (the Exactive) have been extensively tested in relation to the requirements for a quantitative-qualitative workflow in drug discovery settings. When coupled to UHPLC reversed-phase separation, the Exactive delivered 30-40 points across the peak for peaks that were 3-4 s wide. The mass accuracy and mass resolution were maintained for full dynamic range of the chromatographic peak which was important for accurate quantitation in full scan mode. Selectivity for specific analytes in complex matrices was obtained through mass resolution. Moreover, fragment ion information could be collected without the need to select the precursor ion, and such fragmentation is performed with minimum signal losses [26].

The same strategy – a combined qualitative and quantitative analysis – was applied to the comprehensive residue analysis in food and feed matrices. The sample extracts to be analyzed, in the case of wide-scope screening, are highly complex due to the use of generic sample preparation (often simply extraction/dilution). The lack of selectivity due to such sample preparation step has to be compensated for by the selectivity in the instrumental analysis, moreover, fulfilling the legal residue limits in order of ng/g. A mixture of 151 pesticides, veterinary drugs, mycotoxins, and plant toxins in generic extracts of honey and animal feed (10-250 ng/g) was analysed with UHPLC coupled to the Exactive. The conclusion of the study was that for a

consistent and reliable mass assignment (<2 ppm) of analytes at low levels in complex matrices a high resolving power (>50,000) was required [25].

The statement regarding the necessity of high resolving power for full scan mass selectivity was further reaffirmed in the case of hormone and veterinary drug residue analysis performed on hair extracts fortified with 14 steroid esters using UHPLC coupled to the LTQ Orbitrap. The study showed that false compliant (false negative) results can be obtained when mass resolving power of the MS is insufficient to separate analyte ions form isobaric co-eluting sample matrix ions [41].

Recently, a mass spectrometric approach for detection of gonadotrophin-releasing hormone (GnRH) has been presented with good validation results. The method was designed to determine the non-degraded hormone in regular urine doping control samples. In contrast to the above mentioned UHPLC-Orbitrap examples employing standard column dimensions (2.1 or 4.6 mm internal diameter) this particular setup was using a capillary column (75 μm internal diameter) at flow rate of 750 nl/min. The full scan analysis in the Orbitrap MS enabled the determination of the accurate (monoisotopic) mass and ensured the highest confirmatory potential [42].

3.3. Multidimensional LC

For some types of samples, a single LC separation is not sufficient to minimize the suppression effect of multiple co-eluting components. In proteomics, so called ‘shotgun’ strategies convert a complex protein mixture to an even more complicated peptide mixture. In general, the complexity conversion factor is about 40x (i.e. every digested protein yields approximately 40 peptides). Improvements in resolution can be obtained by using multidimensional separations (LC/LC) [43 and references therein].

Multi-phase peptide separation utilizes different properties of peptides such as charge and hydrophobicity to increase resolution. The separations can be performed either online or off-line of the mass spectrometer and frequently involve the use of strong cation exchange (SCX) in

combination with reversed-phase chromatography. On average, a dozen SCX fractions are generated which, after desalting, could be analyzed further using reversed-phase separation with acetonitrile gradient in aqueous formic acid coupled to the LTQ Orbitrap [44]. Also here, UHPLC can be employed to improve peptide identification in proteomic samples [45].

In case of very complex proteomic samples, yet another LC separation dimension could be added on the level of protein separation before their digestion to peptides, such as a strong anion exchange pre-separation [46]. Even though successful attempts have been made to combine three LC separations on-line with the mass spectrometric detection [47], the ion exchange chromatography is usually performed off-line. This is because many protocols employ acetonitrile in SCX elution buffers to improve the resolution of the technique but this then precludes direct loading of SCX fractions on a reversed-phase column. The information content in the data from such multi-dimensional protocols with the LTQ Orbitrap detection is extremely high, which then makes the data processing a very demanding part of the experiment [48].

Phosphopeptide analysis represents a very special subset of multidimensional LC separations coupled to the LTQ Orbitrap. Their relatively low abundance calls for enrichment step(s) to improve chances for their detection and characterization. Ion metal affinity chromatography (IMAC) is an established technique for enrichment of both phosphoproteins and phosphopeptides, and coupling these two IMAC-based enrichments together with SCX/reversed-phase separation and the Orbitrap detection yielded quantitative phosphoproteomic analysis of an epidermal growth factor pathway in mammalian cells identifying close to 5000 phosphopeptides [49]. Similar characterization was accomplished for the *Drosophila* phosphoproteome detecting close to 13,000 phosphorylation events [50].

Several other chromatographic approaches have been developed for phosphopeptide enrichment. For instance, phosphopeptides elute in earlier fractions from the SCX column than most ‘normal’ peptides, thus providing simple enrichment before proceeding with reversed-phase

chromatography separation and the Orbitrap detection [51]. Titanium dioxide is another medium successfully used for phosphopeptide enrichment [52, 53]. Using a combination of titanium dioxide fractionation followed by SCX and reversed-phase LC, more than 10,000 phosphopeptides were detected in 116 LC MS analyses [54]. The phosphorylation sites could be studied as a function of stimulus, time and subcellular location, providing insight into phosphopeptide temporal dynamics after stimulus.

Another approach to phosphopeptide enrichment described two separations on reversed-phase media with a metal dioxide enrichment step in between, again with the LTQ Orbitrap detection [55]. A large-scale phosphorylation analysis of mouse liver encompassed the Orbitrap analysis utilizing a combination of SCX, IMAC and immunoprecipitation for phosphotyrosine-containing peptides [44]. A quantitative atlas of mitotic phosphorylations studied in human cell line resulted in the identification of >14,000 different phosphorylation events and employed SCX, IMAC and titanium dioxide enrichment steps with Orbitrap detection [56].

3.4. Hydrophilic interaction chromatography

Global metabolite extracts can be quite complex and generally include small organic acids and amino acids, nucleotides, carbohydrates, vitamins, and lipids. Polar compounds tend to elute in the void volume from C18 reversed-phase columns. In hydrophilic interaction chromatography (HILIC), an appropriate amount of water (usually 5–15%) in the mobile phase maintains a stagnant enriched water layer on the surface of the polar stationary phase into which the analytes partition. HILIC separates compounds by eluting with a strong organic mobile phase against a hydrophilic stationary phase where elution is driven by increasing the water content in the mobile phase. The highly volatile organic mobile phases such as methanol and ACN used in HILIC provide low column backpressure as well as an increased API ionization efficiency for MS/MS detection [57].

The utility of HILIC in retaining hydrophilic compounds while allowing hydrophobic species to flow through rapidly is a significant advantage for metabolomic profiling experiments. Components such as sphingolipids and phosphatidylcholine lipids elute quickly from the column making the separation very robust [58]. Metabolic profiling of the *rosy* (***ry***) *Drosophila* mutation served as a validation of the HILIC - LTQ Orbitrap platform by detecting the same changes in metabolites as have been reported classically. In addition, completely unexpected changes were detected in several metabolism pathways [59].

Similarly, in the study of selenized species the reversed-phase column showed elution of a considerable fraction of selenium in the void. The ability of HILIC column to retain these polar compounds enabled their detection and characterization. The comprehensiveness of the Se-species identification using HILIC coupled to the LTQ Orbitrap was verified by using inductively-coupled plasma MS confirming that nine compounds observed with the LTQ Orbitrap represent 97% of total selenium injected. In comparison, seven out of these nine Se-compounds were missed when HILIC was coupled to TOF MS, which highlights the importance of the higher intra-scan dynamic range of the Orbitrap MS. Further incontestable advantage over TOF MS was the sub-ppm mass accuracy being preserved in mass spectra up to MS^4 while the large fragmentation window allowed for following the linage over the whole isotopic pattern range [23].

Recently, a specific variant of hydrophilic interaction chromatography termed electrostatic repulsion-hydrophilic interaction chromatography (ERLIC) has been reported for specific enrichment and identification of phosphopeptides [60]. In addition to simply separating phosphopeptides from non-phosphorylated species, ERLIC can also separate phosphopeptides from each other with good resolution. A relatively high content of phosphotyrosine-containing peptides has been reported among approximately 5,500 phosphopeptides identified and confirmed in the study.

4. Conclusions

In addition to providing a brief introduction to the Orbitrap technology and its continuing development, this article delivers a broad overview of the most recent publications quoting the use of the Orbitrap detection for a variety of chromatographic separation techniques. The references herein demonstrate the versatility of the Orbitrap analysis and underscore a fundamental principle: mass resolving power determines the true utility of full-scan accurate mass LC-MS for the analysis.

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609

610 Captions to Figures

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612 Figure 1. Orbitrap mass spectrometry pedigree: status in 2009. (ESI: electrospray ionization;
613 nanoESI: nanoelectrospray ionization; APCI: atmospheric-pressure chemical ionization; APPI:
614 atmospheric-pressure photo ionization; LDTD: laser diode thermal desorption; FAIMS: field-
615 asymmetric ion mobility spectrometry; DESI: desorption electrospray ionization; DART: direct
616 analysis in real time; LTQ: linear ion trap; MALDI: matrix-assisted laser/desorption ionization;
617 ETD: electron-transfer dissociation).

618

619 Figure 2. Block diagram of an Orbitrap mass spectrometer

620

621 Figure 3. Resolving power is a key parameter affecting the correct assignment of masses for
622 analytes. The two analytes Imazalil ($[M+H]^+$ = 297.05560, $C_{14}H_14Cl_2N_2O$, RT = 7.26 min) and
623 Flunixin ($[M+H]^+$ = 297.08454, $C_{14}H_{11}F_3N_2O_2$, RT = 7.32) differ by 30 mmu in their exact masses.
624 The mass spectra at three time points (a, b, and c) across the Imazalil elution profile show a
625 mass accuracy better than 2 ppm for all measurements of the high resolving experiment (top), but
626 mass deviations up to 95 ppm were encountered for the measurement at a resolving power set at
627 10,000 (bottom). Such a high mass deviation is due to the presence of an unresolved peak of
628 Flunixin, which is partially coeluting with Imazalil (dashed line). Courtesy of Marcus Kellmann,
629 Thermo Fisher Scientific.

630

631 Figure 4. High resolution prevents a false negative result. Pesticide Sulcotrion™ (*m/z* 329.02475)
632 was measured in a mixture with other 115 pesticides and food toxins in a horse feed matrix. The
633 mass deviation at a resolution of 15,000 is higher than 5 ppm extraction window set by the user
634 due to the presence of an interference (top pane) giving a false negative result (insert, top trace).
635 Sulcotrion can be detected with mass deviation of less than 1 ppm at 50,000 resolution (bottom
636 pane) leading to a confident identification and quantitation (insert, bottom trace). The figure
637 courtesy of Markus Kellmann, Thermo Fisher Scientific.

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639

640 Figure 5. Schematic layout of the LTQ Orbitrap Velos mass spectrometer.

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642 Figure 6. Schematic layout of the Exactive mass spectrometer (including an optional HCD

643 collision cell).

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