

# Thermo Scientific LTQ Orbitrap

## An Overview of the Scientific Literature

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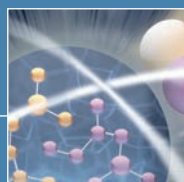
*LTQ Orbitrap*



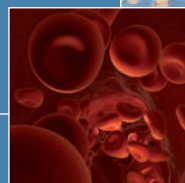
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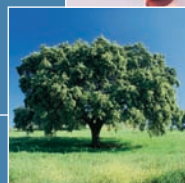
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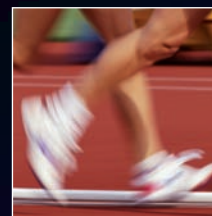


*Doping Control*



*Environmental*





# Thermo Scientific LTQ Orbitrap

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# INTRODUCTION/KEY ATTRIBUTES OF ORBITRAP MS PERFORMANCE

## 1. Introduction

The Orbitrap™ mass analyzer was first described in 2000 and has now reached the status of a mainstream mass spectrometry technique.<sup>1</sup> The key performance characteristics of the Orbitrap mass spectrometer are, above all, easily and routinely achievable ultra-high resolution (> 100,000) and reliable mass accuracy, together with the fact that all these characteristics are available to the user any time without compromising sensitivity. Combination of the Orbitrap detector with an external mass analyzer such as a linear ion trap enables multiple levels of fragmentation (MS<sup>n</sup>) for the elucidation of analyte structure and allows coupling with continuous ionization sources such as atmospheric pressure chemical ionization source (APCI), electrospray (ESI), nanoelectrospray (NSI), or with matrix-assisted laser desorption/ionization (MALDI).

The analytical performance of the Orbitrap mass spectrometer supports 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 contaminants in food and feed. Main application areas have been reviewed extensively, and several papers provided insight into the design and operation principles of the analyzer.<sup>2-21</sup> A publication describing the technological developments implemented in the latest commercially available Orbitrap model, the Thermo Scientific LTQ Orbitrap Velos, has appeared recently.<sup>22</sup>

This report attempts to provide a comprehensive overview of the published literature mentioned in the context of various application areas. Due to overwhelming success of the Orbitrap product line, it should be noted that inevitably and regretfully some important publications might have been omitted.

## 2. Key Attributes of Orbitrap MS Performance

The most coveted attributes of the Orbitrap mass analyzer are its very high mass resolution and, consequently, its reliable mass accuracy. It is the analysis of very complex mixtures where the benefits of these features can be truly appreciated. For analysis of metabolites and small molecules, in general the ability to measure the mass of a compound with adequately high accuracy can directly determine its elemental composition simply by eliminating most other possibilities. Another key aspect for the utility of mass accuracy is represented by a tool based on a so called mass defect which acts as a powerful filter when processing metabolism data.<sup>23</sup> In addition, the use of high-resolution and accurate mass offers the possibility to combine both qualitative and quantitative workflows without compromising the quality of either strategy.<sup>24</sup>

In the context of proteomics, the precursor mass is used as a constraint for data base searches. Mass accuracy is thus a crucial parameter. Uncertain or incorrect determination can either lead to identification results that are not specific enough (if the mass accuracy window is set too wide), or, even worse, to missed identifications (false negatives, if the window is set too narrow).<sup>25</sup> In biomarker discovery studies, the resulting accuracy translates into improved alignment and quantification across spectra.<sup>26</sup>

Acquiring tandem (MS/MS) mass spectra of peptides with a high mass accuracy is an interesting alternative to classical data acquisition schemes where fragment ions are detected at lower mass accuracy and unit resolution in a linear ion trap or a triple quadrupole. When acquiring fragmentation spectra in the Orbitrap detector, the lower number of spectra and a higher detection limit (compared to the detection of the fragmentation spectra in a linear ion trap) are offset by the additional specificity of identifications. In other words, maintaining the same false positive ratio one still obtains similar number of protein identifications using either fast but unit resolution ion trap detection or slower but high resolution Orbitrap detection. A much greater degree of confidence in the identification of peptides with unexpected modifications can be obtained by choosing to acquire (fewer) high mass accuracy tandem spectra compared to acquiring (considerably more) lower mass accuracy tandem spectra.<sup>27</sup>

The Orbitrap detector can reliably deliver mass accuracy better than 1 ppm.<sup>16, 28-31</sup> The mass accuracy can be further improved when using an internal calibrant. Employing known background ions from ambient air has proven to be a very useful, intelligent way of routinely achieving reliable sub-ppm mass accuracy.<sup>28</sup> Another approach relies on certain background ions present in nearly all scans. These provide a large number of measurements allowing for very precise mass estimations and robust landmarks for aligning and calibrating multiple files.<sup>26</sup> Recently, a non-linear recalibration of the 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. Often, such a result limits the peptide to a single composition and represents, therefore, the highest useful accuracy.<sup>25</sup>

# SEPARATION TECHNIQUES USED WITH THE ORBITRAP MASS ANALYZER

## 3.1. Reversed-Phase Liquid Chromatography

With complex mixtures so prevalent in most application areas, some sort of sample simplification is required before its introduction into the mass analyzer. Liquid chromatography (LC) is one of the methods that became popular because of its ability to seamlessly couple with electrospray and nanoelectrospray ionization techniques thus ensuring a high degree of automation and throughput.

The employment of reversed-phase (RP) LC coupled to Orbitrap instruments is truly ubiquitous. In proteomics, the majority of routine protein identifications are performed with this technique often following a digestion of gel separated intact proteins or fractionation of complex peptide mixtures after sample digestion using isoelectric focusing or another form of chromatographic separation, such as Strong Cation Exchanger (SCX). It is of note that a comparison of two sample preparation methods, a one-dimensional sodium dodecyl sulfate gel electrophoresis of intact proteins *versus* isoelectric focusing of enzymatic digest of the sample, showed that both methods delivered a comparable result with no apparent bias for any functional protein class.<sup>32</sup> While C18 columns are used for peptide analyses, C8 or C4 media are usually preferred for larger peptides and proteins.<sup>33</sup> In the area of small molecule analysis, reversed-phase LC is similarly widespread. A detailed study comparing various reversed-phase separation media has been carried out to develop efficient and robust methods for LC/MS-based metabolomic profiling studies.<sup>34</sup>

## 3.2. Ultra-High Pressure Liquid Chromatography

The use of ultra-high pressure LC (U-HPLC) in combination with Orbitrap detection, both for peptide and small molecule analyses, is of a particular interest. Fast acquisition rates are required so as to provide sufficient data points across narrow chromatographic peaks.<sup>35</sup> Publications integrating the Orbitrap mass analyzer to U-HPLC cover areas of plasma phospholipids, drug metabolism, pesticides and veterinary drug residues, and hormones.<sup>24, 36-39</sup>

A transient acquisition time of 0.4 s provides a mass resolution of 30,000 at  $m/z$  400 (and 25 data points across a peak of width at baseline of 10 s).<sup>38</sup> Acquisition of MS/MS spectra can be conveniently performed either in the parallel-scanning linear ion trap or in the Orbitrap mass analyzer 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 7,500, resulting in 20 data points collected across a peak). The positive outcome of the above 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.

Applied to the analysis of serum, interfacing with U-HPLC delivered linear response to the concentration of metabolites over 3.5 orders of magnitude with limits of detection less than 1  $\mu\text{mol/L}$ .<sup>35</sup>

## 3.3. Multidimensional Liquid Chromatography

Combinations of several chromatographic media form the basis of multidimensional LC separation strategies used for complex peptide mixture analyses.<sup>40</sup> The separations can be performed either in-line or off-line of the mass spectrometer and frequently involve the use of SCX in combination with RP chromatography.<sup>41</sup> This strategy can also employ U-HPLC to improve peptide identification in proteomic samples.<sup>42</sup>

Combining a reverse phase separation with a special chromatographic enrichment step(s), such as hydrophilic interaction chromatography (HILIC) is being mentioned in the context of phosphopeptide analysis and metabolomics (see Sections 4.4 and 7 herein).

## 3.4. Gas Phase Separation

The ultimate goal would be to eliminate separation steps altogether. Gas phase fractionation holds the promise for being a greatly simplified, fully automated approach. Its effectiveness was demonstrated by comprehensive analysis of the proteotypic peptides in an unfractionated cell lysate.<sup>43</sup> Such a method can be further refined. For instance, the C-trap, connecting the linear ion trap and Orbitrap mass analyzer, can be filled repeatedly with different  $m/z$  ranges, holding, ultimately, up to a million charges. Each range is then separately analyzed in the Orbitrap detector, creating a survey spectrum composed of hundreds of single spectra. The composite spectrum affords over 6,000-fold dynamic range and sub-femtomole sensitivity, all using a straightforward chip-based nanoelectrospray. Moreover, relative quantitation of the same peptide with different modifications was successfully demonstrated. This approach allows highly confident identification and modification mapping and is an alternative to MALDI peptide mapping and LC-MS/MS.<sup>44</sup>

If sample fractionation can not be avoided, the identified proteins should be evaluated for any discernible bias.<sup>45</sup>

# THE ORBITRAP MASS ANALYZER USED IN VARIOUS APPLICATION AREAS

## 4. The Orbitrap Mass Analyzer used in Various Application Areas

Scientific papers from the field of proteomics make up the majority of the Orbitrap publications (Figure 1). Another large group of published articles deals with the analysis of metabolites, including metabolite identification, structural characterization, and quantitation in the context of metabolism studies, environmental analysis and doping control. Research focused on metabolomics and lipid analysis is also quite frequently quoted, and there are a couple of publications from the areas of nucleic acid analysis and fundamental physical chemistry.

### 4.1. Proteomics Studies

The mass spectrometric analysis of an entire eukaryotic proteome has been, until recently, considered beyond the capabilities of the current technology. The effort capturing the complete yeast proteome, however, demonstrated the feasibility of such an undertaking.<sup>46</sup> The detected proteins spanned 4 orders of magnitude in abundance and represented 89% overlap with fused tandem affinity tag (TAP) or green fluorescent protein (GFP) tag genome-wide experiments. Moreover, 510 proteins were identified exclusively by this MS approach, and the identifications were not biased against low-abundance proteins.

Most research groups take advantage of a unique **parallel acquisition mode** available on the LTQ Orbitrap series of instrumentation: highly resolved and accurate Orbitrap measurements for the parent ions are complemented with tandem mass spectra detected in a linear ion trap with high speed and sensitivity. LTQ Orbitrap hybrid instruments offer the additional option of transferring the product ions (created in the linear trap) to the Orbitrap mass analyzer for analysis with high resolution and high mass accuracy.

A slightly lower duty cycle is offset by having high mass accuracy and resolution for product ion detection which facilitates the search for modified peptides.<sup>27</sup> Studied proteomes being so complex, methods of separating intact proteins and/or peptides, combined with enzymatic digestion, are usually included in the general proteomics workflow. Each step has to be controlled for efficiency, bias and reproducibility. Studies focusing on comparison of various sample preparation techniques offer valuable insight. Using three different sample preparation approaches (in solution enzymatic digest followed by RP LC; one-dimensional SDS-PAGE followed by RP LC; and two-dimensional PAGE) a comprehensive catalogue of endometrial fluid proteins has been compiled. The protein identifications obtained by these three different methods were only partially overlapping, with one-dimensional SDS-PAGE followed by RP LC affording the most identifications.<sup>47</sup>

Enzymatic digestion of proteins is a critical step in the sample preparation workflow. An LTQ Orbitrap mass spectrometer was utilized for a detailed assessment of less usual digestion methods.<sup>48</sup> Recently, an approach that combines a complete solubilization, buffer exchange, and highly efficient digestion with the ease of use of a simple filter device has been reported. Peptides eluted after digestion on the filter were pure, allowing single-run analyses of organelles and an unprecedented depth of proteome coverage.<sup>49</sup> In addition to the above mentioned examples of yeast, embryonic stem cell, and endometrial fluid proteomes, other proteome-wide studies have been reported in the literature.<sup>32, 46, 47</sup> The urine proteome was analyzed via one-dimensional electrophoretic separation followed by in-gel digestion and RP LC separation coupled to an LTQ Orbitrap mass spectrometer, and afforded identification of over 1,500 proteins with essentially no false-positive identifications.<sup>50</sup>

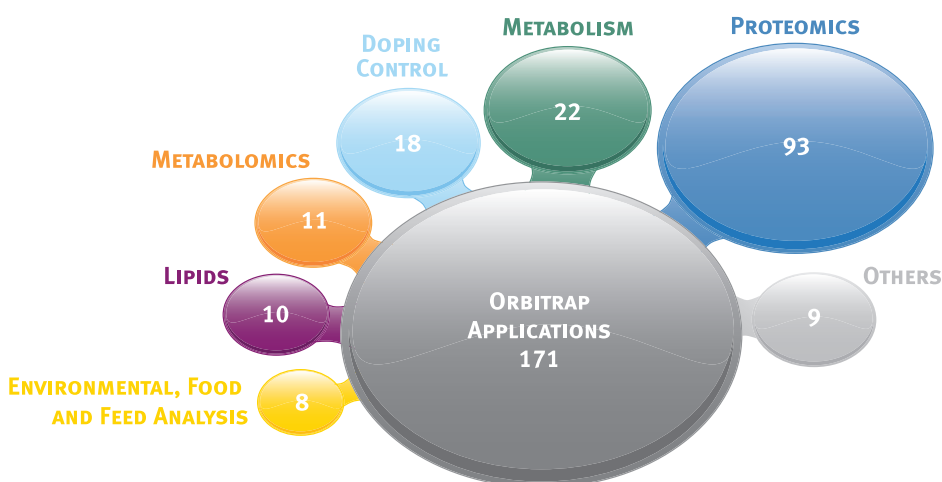


Figure 1. Representation of specific application areas using the Orbitrap mass analyzer in published peer-reviewed journals with the number of publications stated.

# THE ORBITRAP MASS ANALYZER USED IN VARIOUS APPLICATION AREAS

The tear film proteome was similarly analyzed, with the confidence in peptide identifications further boosted by including information from MS<sup>3</sup> fragmentation.<sup>51</sup> The saliva proteome was characterized as a result of a collaboration among several laboratories using very different approaches and instrumentation.<sup>52, 53</sup> The depleted human serum proteome showed practicality of isocratic solid phase extraction-liquid chromatography coupled to an Orbitrap mass spectrometer.<sup>54</sup>

The studies focusing on the proteomics of organelles have to deal with an additional problem of organelle co-purification. Clever use of mild protease treatment for mitochondria purification enabled mapping of cytoplasm-exposed mitochondrial proteins.<sup>55</sup> Kidney glomeruli isolated by laser capture microdissection were characterized to a depth of more than 2,400 proteins.<sup>56</sup> Pancreatic islets of Langerhans were another type of ‘miniorgan’ analyzed using the LTQ Orbitrap mass spectrometer. With 6,873 proteins detected by at least 2 peptides at FDR < 1% this study represents the **largest proteome characterized** to date.<sup>56</sup>

Applications related to human disease studies seem to be where all of the key performance features of LTQ Orbitrap technology come together. As an example, *ab initio* calculations, accurate mass measurements, isotope modeling and *de novo* peptide sequencing were applied to the qualitative and quantitative characterization of the amyloid beta peptide population in biological matrices. Targeting the metabolism of amyloid peptides is currently the leading experimental approach to the treatment of Alzheimer’s disease. The high resolution measurement results in this study provided insight into the formation of amyloid protein, increased the understanding of drug mechanism and contributed to drug efficacy studies.<sup>57</sup>

## 4.2. Peptidomics

Analysis of endogenous peptides complements the classical proteomics approach. The Orbitrap mass analyzer was used for a combined study of the peptidome and proteome of cerebrospinal fluid where highly accurate mass measurements in both MS and MS/MS modes allowed unambiguous identification of neuropeptides.<sup>58</sup> Similarly, this type of instrument was used for profiling endogenous peptides in human synovial fluid.<sup>59</sup> The mass spectrometric analysis of bioactive peptides arising from the proteolytic processing of precursor proteins in neurons and endocrine cells identified 400 peptides. This information served to predict processing sites of regulated secretory pathway proteins. Due to the reliably high mass accuracy of the Orbitrap analyzer, a false discovery rate was minimized to 0% even though entire IPI human database was used with no defined enzyme specificity.<sup>60</sup>

Besides human neuropeptides, there is an interest in neuropeptides from other species leading to a better understanding of the biochemical interactions in general.<sup>61</sup> The Orbitrap instrument was also used to derive sequences of non-ribosomal peptides from *Trichoderma atroviride*. Based on the reliable accuracy of the mass measurement in MS/MS mode it was possible to exclude the presence of hydroxyproline residues within the sequence. Detailed characterization of the peptaibome (*i.e.*, linear antibiotic non-ribosomal peptides containing  $\alpha$ -aminoisobutyric acid) of *Trichoderma atroviride* ATCC 74058 confirmed at least 20 peptaibols of the trichorzianine group and 15 novel peptaibiotics, whose sequences did not match any sequences published in compound databases or in the literature.<sup>62</sup>

## 4.3. Top-Down Proteomics

Biologicals (such as recombinant therapeutic proteins or vaccines) are a growing trend within the pharmaceutical industry. This fuels interest in mass/top-down analysis of proteins. Orbitrap technology offers an attractive alternative to peptide mapping for characterization and monitoring of post-translational modifications on intact proteins as it requires minimal sample preparation, thus offering high speed analysis with relatively minor (method induced) sample modifications. The first applications using this type of mass analyzer referred to proteins of moderate size, up to 25kDa. This size limit has long since been surpassed, and analysis of monoclonal antibodies is mentioned in several recent publications. A technique for rapid characterization of variable regions of monoclonal antibodies was described, relying on in-source fragmentation.<sup>64</sup> This approach has a unique advantage as it fragments all charge states of a protein at the same time and, thus, greatly improves the sensitivity of the fragment ions over a true MS/MS experiment.

Practical tips and issues regarding analyses of 150 kDa monoclonal immunoglobulin gamma antibodies using an on-line LC/MS approach were discussed by Bondarenko *et al.*<sup>33</sup> Charge state species with about 50 charges per intact protein molecule were observed with Orbitrap detection. After deconvolution the authors were able to characterize disulfide isoforms of an antibody, glutamine and pyro-glutamate variants of a heavy chain, and the glycosylation profile of intact and reduced IgG. Several glycoforms with different numbers of terminal galactose residues could be distinguished with base-line resolution in the mass spectrum. For the light chain (approximate MW 23 kDa) an isotopic resolution was obtained enabling exact monoisotopic mass determination of the species. Remarkably, these analyses were carried out in a true high-throughput fashion with RP LC/MS coupled to the mass spectrometer.

Data interpretation is a complex issue, as it has to accommodate a multitude of modifications, mutations, insertions, and deletions characteristic of individual protein forms.<sup>65</sup>

#### 4.4. Post-Translational Modifications

Phosphorylation, being a key regulator of many events in eukaryotic cells, has been the focus of many proteomic studies. The acquisition of large-scale phosphorylation data sets can pinpoint conserved regulatory inputs and reveal kinase-substrate relationships.

The relatively low abundance of phosphopeptides calls for enrichment step(s) to improve chances for their detection and characterization. Large numbers of phosphorylation sites can then be detected in a single experiment using the LTQ Orbitrap where the accuracy of the measurement and advanced software features (Ascore) afford the desired confidence.<sup>66</sup>

Ion metal affinity chromatography (IMAC) is an established technique for enrichment of both phosphoproteins and phosphopeptides. Coupling these two IMAC-based enrichments together with SCX/RP separation and the LTQ Orbitrap mass spectrometer yielded quantitative phosphoproteomic analysis of an epidermal growth factor pathway in mammalian cells, resulting in the identification of close to 5,000 phosphopeptides.<sup>67</sup> Similar characterization was accomplished for the *Drosophila* phosphoproteome detecting close to 13,000 phosphorylation events.<sup>68</sup> A large-scale phosphorylation analysis of  $\alpha$ -factor-arrested yeast using a multidimensional separation strategy involving preparative SDS-PAGE and IMAC yielded the confident identification of 2,288 non-redundant phosphorylation sites from 985 proteins. This study also utilized the Ascore algorithm to determine the certainty of site localization for the entire data set.<sup>69</sup>

An approach for phosphopeptide enrichment combining two separations on RP with a metal dioxide enrichment step in between has been described.<sup>70</sup> A large-scale phosphorylation analysis of mouse liver done with the Orbitrap mass spectrometer utilized a combination of SCX, IMAC and immunoprecipitation for phosphotyrosine-containing peptides. This study identified 5,635 phosphorylation sites within 2,328 proteins, with an unexpectedly high frequency of C-terminal phosphorylation.<sup>41</sup> A valuable insight into how phosphorylation regulates the cell cycle was gained using stable isotope labeling with a two-step strategy for phosphopeptide enrichment with subsequent Orbitrap detection. A quantitative atlas of mitotic phosphorylations in human cell line had thus been constructed, based on the identification of > 14,000 different phosphorylation events along with relative quantitative data for the majority of these sites. More than half of those had not previously been described in the literature.<sup>71</sup>

An interesting study utilized anion-exchange chromatography and reversible binding to titanium oxide in order to enrich for chicken eggshell matrix phosphoproteins. Identifications were made by using LC-MS<sup>n</sup> following neutral loss scanning. More than 150 different phosphorylation sites were identified, with some proteins detected as eggshell matrix components for the first time.<sup>72</sup>

Two phosphopeptide enrichment methods (using IMAC and titanium dioxide) were compared for the analysis of peptides obtained by enzymatic digestion of fission yeast proteins separated by preparative SDS-PAGE. In total, 2,887 distinct phosphorylation sites were identified from 1,194 proteins with an estimated false-discovery rate of < 0.5% at the peptide level. The findings from the two methods showed that they are complementary.<sup>73</sup>

Recently, a variant of hydrophilic interaction chromatography termed electrostatic repulsion-hydrophilic interaction chromatography (ERLIC) has been reported for enrichment and identification of phosphopeptides.<sup>74</sup> In addition to simply separating phosphopeptides from non-phosphorylated species, ERLIC could also separate phosphopeptides from each other with good resolution. A relatively high content of phosphotyrosine-containing peptides had been reported among approximately 5,500 phosphopeptides identified and confirmed in this study.

Phosphorylation sites can also be studied as a function of stimulus, time, and subcellular location, providing insight into phosphopeptide temporal dynamics. More than 6,600 phosphorylation sites have been detected on over 2,200 proteins from epidermal growth factor stimulated HeLa cells using LTQ Orbitrap technology. The study revealed that a majority of proteins contained multiple sites showing different kinetics of phosphorylation.<sup>75</sup>

The relative paucity of phosphotyrosine (Tyr (p)) has greatly limited its identification in large-scale phosphoproteomic experiments. The Higher Energy Collisional Dissociation (HCD) which is available on the LTQ Orbitrap models equipped with an extra collision cell, is a useful tool for pinpointing Tyr (p) containing peptides making use of the diagnostic immonium ion ( $m/z$  216.0426).<sup>76</sup> Using an anti-Tyr (p) peptide immunoprecipitation enabled identification of 414 unique tyrosine phosphorylation sites from murine brain.<sup>77</sup> Mapping *in vivo* phosphorylation sites in *Arabidopsis* identified 2,172 high-confidence, unique phosphorylation sites from 1,346 proteins. The distribution of phosphoserine, phosphothreonine and phosphotyrosine sites were 85.0, 10.7, and 4.3%, respectively, which highlights the unexpected extent of tyrosine phosphorylation in plants.<sup>78</sup>

# THE ORBITRAP MASS ANALYZER USED IN VARIOUS APPLICATION AREAS

Recently reported quantitative phosphorylation study provided a global picture of the cellular functions of a member of the protein-tyrosine phosphatase (PTP) family of enzymes, PTP1B, in mouse fibroblasts. Stable isotope labeling by amino acids in culture, SILAC, was employed to analyze alterations in Tyr (p) signaling of PTP1B deficient mouse fibroblasts. In total, 124 proteins containing 301 phosphotyrosine sites under basal, epidermal growth factor-, or platelet-derived growth factor-stimulated conditions were identified. The strategies developed for the analysis of cellular PTP1B function and substrate specificity can be applied to other members of this important enzyme family and have the potential to define the functions of other PTPs in the context of cellular signal transmission networks.<sup>79</sup>

Protein phosphorylation is a general and fundamental regulatory process, not restricted only to eukaryotes. Ser/Thr/Tyr phosphorylation is also present on many essential bacterial proteins. A global, gel-free, and site-specific analysis of the *B. subtilis* phosphoproteome from digested cell lysates identified 78 unique phosphorylation sites on 78 proteins: 54 on serine, 16 on threonine, and eight on tyrosine.<sup>80</sup> Similarly, 81 phosphorylation sites were detected on 79 *E. coli* proteins, with distribution of Ser/Thr/Tyr phosphorylation sites 68%/23%/9%. Despite their phylogenetic distance, phosphoproteomes of *E. coli* and *B. subtilis* show striking similarity in size, classes of phosphorylated proteins, and distribution of Ser/Thr/Tyr phosphorylation sites.<sup>81</sup>

In addition to phosphorylation, acetylation and methylation, together with ubiquitination and identification of formylated lysine residues form part of the known spectrum of linker histone variability.<sup>82</sup> Distinguishing between these modifications is not a straightforward task but the reliable accurate mass measurement is proving extremely useful for such challenging applications. During investigation of a recombinant mutant of the murine dioxin receptor by matrix-assisted laser desorption/ionization-time of flight tandem mass spectrometry (MALDI-TOF), a peptide with a nominal mass increment of +28 u was observed. However, dimethyl-lysine (+28.031300 u), formyl-lysine (+27.994915 u), and mutation of lysine to arginine (+28.00615 u) could explain such a nominal mass increment and cannot be distinguished using the mass accuracy routinely achieved with MALDI-TOF instruments. The authors described precise assignment of a novel *N,N*-dimethyl-lysine modification in the receptor by using sub-ppm mass accuracy for the parent ion with the LTQ Orbitrap hybrid instrument.<sup>83</sup>

Glycosylations represent another large subset of fragile post-translational modifications. The ability of multiple levels of fragmentation ( $MS^n$ ) on the LTQ Orbitrap systems was instrumental to the structural elucidation of a pentasaccharide impurity in the preparation of the drug Fondaparinux.<sup>84</sup>

Another study tried to distinguish sialic anomers of sodium salt of 1-O-octadecyl-3-O-(*N*-acetyl)neuraminyl- $\alpha$ -glycerol by mass spectra. The observations led to a hypothesis that a conformation having the large aglycone in the equatorial orientation affects the collision-induced dissociation fragmentations.<sup>85</sup>

Over the last few years, a novel fragmentation technique called Electron Transfer Dissociation (ETD) has been introduced and become well established for the analysis of post-translational modifications on peptides and proteins, especially after its implementation on the LTQ Orbitrap systems.<sup>18-20</sup> Protein arginine (Arg) methylation serves an important functional role in eukaryotic cells, and typically occurs in domains consisting of multiple Arg in close proximity. Localization of methylarginine (MA) within Arg-rich domains poses a challenge for mass spectrometry based methods; the peptides are highly charged under electrospray ionization (ESI), which limits the number of sequence-informative products produced by collision induced dissociation (CID). Also, loss of the labile methylation moieties during CID precludes effective fragmentation of the peptide backbone. To determine extent of Arg methylation on peptides in relatively complex mixtures, a method was developed that employed nano-LC coupled to alternating CID/ETD for peptide sequencing and MA localization/characterization, and an Orbitrap detector for accurate precursor measurement and relative quantification of MA-peptide stoichiometries.<sup>86</sup> Another study described a direct mapping of  $\alpha$ -GlcNAc glycosylations using ETD on an LTQ Orbitrap mass spectrometer.<sup>87</sup>

Advanced knowledge of peptide fragmentation behavior and the ability to choose easily among the multiple fragmentation techniques available on the Orbitrap-based systems led to the development of an intelligent data acquisition 'decision tree'. Essentially, data dependent decision for MS/MS can be made in real time for the optimum dissociation technique (CID, ETD, HCD) based on precursor molecular weight and charge state. The examples of highly modified peptide and intact protein analysis confirmed that the system is delivering enormous analytical power while maintaining the simplicity of operation users have come to appreciate.<sup>20</sup>



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# QUANTITATIVE ANALYSIS OF PROTEOMES

## 5. Quantitative Analysis of Proteomes

Quantitative interrogation has now become an integral part of most proteomic studies. High mass accuracy and resolution, large ion capacity, and the extensive dynamic range provided by LTQ Orbitrap instruments deliver considerable improvement in the number of quality peptide ratio measurements compared to similar analyses done on an ion trap alone.<sup>88</sup> Of the many choices of methods involving isotope labels for relative protein and peptide quantitation, SILAC seems to be a very good option for providing a proteome-wide, unbiased approach. In conjunction with one-dimensional gel electrophoresis and isoelectric focusing of peptides, SILAC delivered identification and quantitative data for over 5,000 proteins in mouse embryonic cells.<sup>32</sup> Recently, the SILAC methodology was applied to the quantitation and characterization of intact proteins. The quantized mass offset between fragments provided information about the number of labeled residues simplifying protein identification and characterization.<sup>89</sup>

Quantitative proteome analyses also contributed to the understanding of parasite biology. The protein expression changes in trophozoite stages of the malarial parasite *Plasmodium falciparum* following chloroquine and artemisin treatment were investigated using stable isotope labeling combined with anion exchange chromatography, followed by SCX/RP separation and the Orbitrap detection. Of the 1,253 trophozoite proteins detected, 30% were novel. Quantitative results were obtained for more than 800 proteins.<sup>90</sup>

Relative quantitation between differentially treated samples using stable isotopes is well established. Absolute quantitation, on the other hand, is a relatively new concept in proteomics. Usually, it requires addition of a labeled standard late in the experimental workflow, introducing variability due to sample preparation. The SILAC method has recently been applied to label recombinant proteins which are then used as internal standards by adding them directly into cell lysates (*i.e.*, introduction early in the sample processing workflow reduces the amount of variability).<sup>91</sup> Authors devised a strategy to quantify peptides at or below their signal-to-noise level on hybrid ion trap instruments which extended the regular detection limit by at least an order of magnitude, and accurately quantified down to 150 attomole of protein in a cell lysate without any fractionation prior to LC/MS.

Label-free quantitation has gained credibility thanks to the recent improvements in mass spectrometry and chromatographic techniques. This approach, based on accurate mass and elution time as unique identifiers, benefits considerably from the stable mass accuracy measurement provided by the Orbitrap mass analyzer. Quantitative comparisons now become feasible and economically attractive on the scales of whole proteomes.<sup>92, 93</sup> A complete workflow was described for the differential proteomics analysis of human plasma or single islets of Langerhans. These represent 2,000 - 4,000 cells each, and demonstrate the ultra-sensitive analysis of nanogram protein quantities.<sup>56</sup>

Having in mind such a wide choice of methods available for protein/peptide quantitation, any attempts to compare their individual merits should be greatly appreciated. One such study compared a spectral counting approach against a stable isotope labeling approach in the context of a multidimensional LC setup. The quantitative approach based on stable isotope labeling demonstrated high reproducibility among biological replicates. The spectral counting method, on the other hand, showed poor correlation probably due to the lack of reproducible sampling for proteins with low spectral counts.<sup>94</sup>

Another analytical approach to protein quantitation is the use of isobaric labeling tags (TMT<sup>®</sup> or iTRAQ<sup>™</sup>). These methods rely on the measurement of specific reporter ions in the low  $m/z$  region of MS/MS spectra of target peptides which is currently achievable via either a pulsed-Q dissociation (PQD) or a higher energy collision-dissociation (HCD) techniques on the LTQ Orbitrap systems.<sup>95, 96, 97</sup> It is important to note that a very good resolution is required, particularly at low mass range, to eliminate interferences from the true reporter ions. For example, a fragment of arginine at  $m/z$  115.087 will affect the quantitation channel using reporter ion at  $m/z$  115.108 unless resolving power of approximately 15,000 at  $m/z$  115 is used. Recent reports indicate that it is possible to combine multiplexed TMT or iTRAQ mass tagging approaches also with ETD fragmentation, specific benefit being the ability to preserve the fragile modifications within the peptide sequence through the fragmentation process.<sup>87, 98</sup> Reporter ion ratios measured by ETD had similar accuracy and precision as those obtained by CID technique. In a quantitative analysis of modified peptides from crystalline, ETD spectra consistently provided confident sequence information, localization of the glycosylation site and quantitative information, all in the same spectrum.<sup>87</sup>

In the context of complex mixtures, quantification accuracy was found highly correlated with signal-to-noise ratio (S/N), and algorithms had been developed that significantly reduced measurement variance at low S/N. After evaluating the interplay between mass measurement accuracy and S/N, a balance has to be found between both parameters resulting in the greatest identification and quantification rates.<sup>99</sup>

# QUANTITATIVE ANALYSIS OF PROTEOMES/ PROTEOMICS DATA PROCESSING

An example for the absolute quantitation of an endogenous peptide hormone based on high resolving power and accurate mass was given by Thomas and co-workers analyzing insulin and its synthetic analogues.<sup>100</sup> They employed antibody-coated magnetic beads which resulted in very clean extracts from urine samples enabling chromatography and ionization at the nano-scale level. The high resolution full scan data provided confirmation of identity and quantitative information. Elucidation of CID spectra enabled all synthetic and animal insulins to be differentiated from their human counterpart, which was particularly important for Lispro variant possessing the same molecular mass as human insulin. The method presented in this paper has been fully validated, with LOD 0.5 fmol/mL, precision < 20%, recovery of ~30% and linearity 2-40 fmol/mL.

Targeted absolute quantitation of peptides is a fast developing field with triple quadrupole instruments being traditionally used for this type of work. Nevertheless, high resolution Orbitrap-based analyzers can deliver comparable assay precision, accuracy, linearity and sensitivity. Unlike selective reaction monitoring (SRM), the technique tandem mass spectrometers must use to obtain selectivity and sensitivity, Orbitrap analyzers can take advantage of high resolution and accurate mass measurements in full scan mode to gain sensitivity and selectivity. Some advantages of using the Orbitrap approach are: ease of setting up a method (no need for building and optimizing transitions) and capturing all information instead of just the targeted list obtained on a tandem instrument.<sup>101</sup> Other groups use Orbitrap MS/MS data as a starting point to configure SRM assays, thus greatly expediting the development of the targeted SRM assays.<sup>102, 103</sup>

## 6. Proteomics Data Processing

Data processing offers an enormous space for exploration. The commercial software packages available provide sound but fairly basic tools for data interpretation. Many research groups are thus developing specific algorithms aimed at various steps within the proteomics workflow. The accurate determination of the monoisotopic mass and charge state of precursor ions can significantly improve peptide identifications.<sup>104</sup> An outcome of extensive data processing with similar feature detection algorithms was the observation that more than 11% of the MS/MS spectra in large shotgun experiments are composed of product ions from different molecular species.<sup>105</sup> In most of those cases, the isolation width of the instrument captured two precursor ions of similar mass, and the resulting MS/MS represents a composite spectrum. Only one peptide at maximum can be identified from such a composite spectrum. The authors achieved a higher identification rate by creating two separate peak lists differing in the precursor mass based on the two simultaneously detected parent ions within the isolation width.

Interesting concepts are emerging for processing peptide MS and MS/MS data. Combining various (simultaneously acquired) data sets offered by the LTQ Orbitrap system, such as, MS with MS/MS, or MS/MS with MS<sup>3</sup>, or even MS/MS at unit resolution with MS/MS at high resolution, is a frequently appearing topic. An improvement of identification sensitivity for low-abundant proteins was reported by combining MS and MS/MS information. The benefit of this approach is that 'one-hit wonders' from MS/MS database searching can be further substantiated by MS information.<sup>106</sup> Extracting the high-accuracy mass data from the Orbitrap mass analyzer and combining it with the high-sensitivity data analyzed in the LTQ™ portion of the analyzer might also bring certain benefits. Such hybrid data sets reportedly identified more peptides than when using two separate searches for the Orbitrap and LTQ data sets. In a wider context, it serves as a clear demonstration of the utility of parallel data acquisition that only this unique hybrid instrument can provide.<sup>107</sup>

A simple post-acquisition treatment of data can have a considerable impact on the peptide identification rate. Removing certain features from ETD spectra (e.g., charge-reduced precursors, unique neutral losses) led to a 28.6% increase in unique identifications while maintaining ~1% false discovery rate.<sup>108</sup>

Several groups have undertaken development of software tools relevant for quantitation. Some of them accept both high- and unit-resolution mass spectrometry data and can cater to multiple labeling strategies as well as label-free analyses.<sup>109</sup> An integrated suite of algorithms was developed to combine identifications from liquid chromatography tandem mass spectrometry with peptide accurate mass and retention time locations from high-resolution LC/MS. The analysis workflow (msInspect/ATM) extends and combines existing open-source platforms for LC-MS/MS and LC/MS data analysis, and is freely available to the community.<sup>110</sup>

Orbitrap instruments uniquely bring together a powerful set of tools for *de novo* sequencing: namely, mass accuracy, MS/MS, MS<sup>n</sup> and multiple fragmentation techniques. For example, using HCD fragmentation for *de novo* sequencing ensures high mass accuracy/resolution for fragments and presence of immonium and other low MW ions. Efficient deconvolution of such informative spectra enables their easier manual or automated processing. Peptide *de novo* sequencing by precision mass spectrometry employing direct sequence lookups, rather than comparisons of spectra to a database, proved to be quite effective and allowed the use of databases in novel ways, such as searching for products of alternative splicing or products of fusion proteins.<sup>112</sup> It seems that combining *de novo* sequencing with conventional and sequence similarity searches brings certain advantages. Such an approach enabled an in-depth analysis of the blood-feeding insect vector of Chagas disease, which falls within the category of today's great majority of under-sequenced genomes.<sup>113</sup>

An issue closely related to *de novo* sequencing is peptide identifications with borderline statistical confidence. They constitute a significant bottleneck in the reliable and reproducible characterization of proteomes. An entire workflow has been developed taking advantage of the independent interpretation of corresponding MS/MS spectra by *de novo* sequencing software followed by mass spectrometry-driven BLAST sequence-similarity database searches. In a model study with *Ceanorhabditis elegans* proteins it enabled rapid assignment of more than 70% of the hits which had borderline statistical confidence.<sup>114</sup>

The renewed interest in top-down proteomics is accompanied by the development of relevant software tools. Data interpretation is somewhat complicated by the fact that it has to accommodate a multitude of modifications, mutations, insertions, and deletions characteristic of individual protein forms.<sup>65</sup> ProSight software is currently the most developed suite of tools for the interpretation of high resolution MS and MS/MS spectra. It relies on shotgun annotated peptide database (*i.e.*, a peptide database incorporating all known protein-modifying events) to work with peptide fragmentation data with < 5 ppm mass deviation. It thus takes full advantage of the increased capabilities of the current generation of hybrid FT mass spectrometers.<sup>115</sup> Recently, a precursor ion independent top-down algorithm for use in automated assignment of protein identifications from tandem mass spectra of whole proteins has been reported.<sup>116</sup>



# USE OF ORBITRAP INSTRUMENTS IN METABOLOMICS

## 7. Use of Orbitrap Instruments in Metabolomics



Metabolomics has a lot in common with proteomics, namely the analysis of complex mixtures in a high-throughput fashion and often with the same ultimate goals. The widest range and most secure identification of metabolites will be made on instruments operated at high resolution, making use of exact mass measurement of both precursor and fragment ions.<sup>4,117</sup> This has been demonstrated on the studies of plasma metabolome, namely oxysteroids, which represent an interesting subset of compounds implicated in neurodegenerative disorders such as Alzheimer's disease and multiple sclerosis.<sup>118</sup>

Employing the Orbitrap mass analyzer within the metabolomic workflow allows for considerable simplification. The attainable resolving power allows, in some instances, for chromatographic separation to be eliminated altogether. Instead, a direct introduction of biological samples is performed. Such methods have been validated by studies of the matrix effect, linearity, and intra-assay precision of quantitation. The same analysis can include MS<sup>n</sup> fragmentation data as well, providing the basis for the verification of structures proposed based on accurate mass measurement of the precursor ion.<sup>119</sup>

For LC/MS-based metabolomic profiling studies, the focus is on the development of efficient and robust LC/MS methods for the identification of a large number of metabolites in biological samples using both positive and negative electrospray modes. A detailed study 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 Å) and large surface area (e.g., > 400 m<sup>2</sup>/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.<sup>34</sup>

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.<sup>120</sup> 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 phosphatidylcholines elute quickly from the column making the separation very robust.<sup>121</sup> 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.<sup>122</sup>

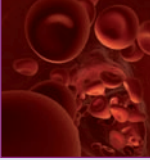
Similarly, in the study of selenized species the reversed-phase column showed elution of a considerable fraction of selenium in the void. Retaining these polar compounds on HILIC columns enabled their detection and characterization. The comprehensiveness of the Se-species identification using HILIC coupled to an LTQ Orbitrap mass spectrometer was verified by using inductively-coupled plasma MS confirming that nine compounds observed with Orbitrap detection 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. Another incontestable advantage over TOF MS was the sub-ppm mass accuracy being preserved in mass spectra up to MS<sup>4</sup> while the large fragmentation window allowed for following the lineage over the whole isotopic pattern range.<sup>30</sup>

In metabolomics applications, great stress is put on time- and cost-effectiveness. The LTQ Orbitrap platform demonstrated an aptitude for both when applied to the studies of the inhibition of the ergosterol pathway analysis in fungi. Data indicating the point of pathway interruption were obtained in seconds.<sup>123</sup> Other groups focused their efforts on optimizing the separation conditions for metabolite extracts. The results of initial studies led to the design of an automated dual-capillary LC system which afforded reproducible detection of approximately 900 features from *Cyanospora sp.* metabolite extracts.<sup>34</sup>

Another trend in improving the effectiveness is the use of U-HPLC which operates with sub-2 μm chromatographic particles at pressures up to 15,000 psi. Fast acquisition rates are required so as to provide sufficient data points across narrow chromatographic peaks. Applications using U-HPLC coupled to the Orbitrap mass spectrometer have been mentioned in more detail under section 3 above.<sup>35,38</sup>

# LIPID ANALYSIS

## 8. Lipid Analysis



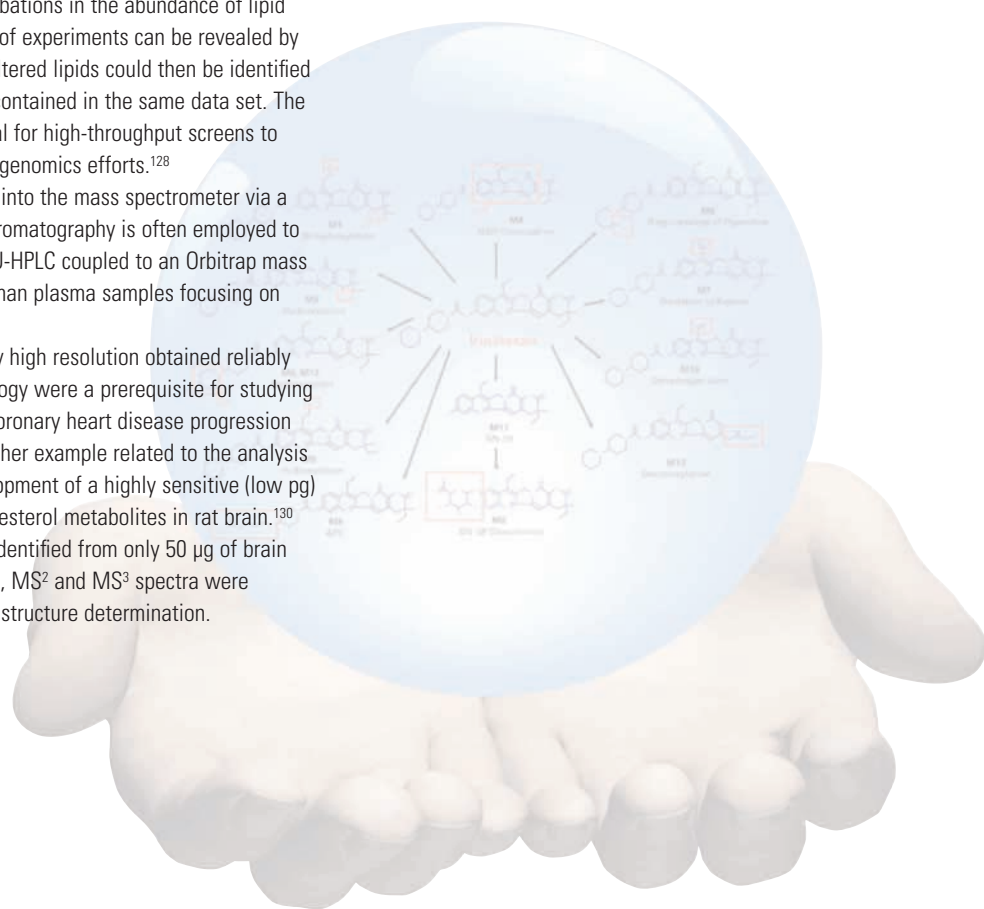
Although the transcriptome, proteome, and interactome of several eukaryotic model organisms have been described in detail, lipidomes remain relatively uncharacterized. High resolution survey spectra from an Orbitrap mass analyzer enabled direct profiling of total lipid extracts. Lipids of major classes could be recognized by accurately determined precursor masses with no recourse to MS/MS. Such an automated 'shotgun lipidomics' analysis was applied to lipidome-wide absolute quantification of individual molecular lipid species in *Saccharomyces cerevisiae*. Absolute quantification of 250 molecular lipid species covering 21 major lipid classes was achieved, providing 95% coverage of the yeast lipidome with 125-fold improvement in sensitivity compared with previous approaches.<sup>124</sup>

Structural characterization of lipids is another exciting application area for Orbitrap technology. It allowed for rapid, comprehensive, and structure specific profiling of the molecular composition of sphingolipids and glycerophospholipids.<sup>125</sup> The ability to measure in both positive and negative ionization modes delivered unequivocal assignment of the fatty acid distribution in intact lipid A.<sup>126</sup> The advanced data interpretation routines (so called Boolean scans) enable in-depth structural characterization of fragmented precursors.<sup>127</sup> Pronounced perturbations in the abundance of lipid species within the entire series of experiments can be revealed by principal component analysis. Altered lipids could then be identified relying on MS/MS information contained in the same data set. The approach demonstrated potential for high-throughput screens to complement ongoing functional genomics efforts.<sup>128</sup>

Samples can be introduced into the mass spectrometer via a simple infusion, however, RP chromatography is often employed to simplify the complex mixtures. U-HPLC coupled to an Orbitrap mass spectrometer for analysis of human plasma samples focusing on phospholipids.<sup>24</sup>

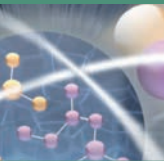
The mass accuracy and very high resolution obtained reliably with the use of Orbitrap technology were a prerequisite for studying lipid oxidation as a marker for coronary heart disease progression and response to therapy.<sup>129</sup> Another example related to the analysis of lipid oxidation was the development of a highly sensitive (low pg) method for identification of cholesterol metabolites in rat brain.<sup>130</sup> Novel oxysterols in brain were identified from only 50 µg of brain injected on column. At this level, MS<sup>2</sup> and MS<sup>3</sup> spectra were sufficiently informative to allow structure determination.

Recently, a study demonstrating specific association between hypertension and lipid profiles has been published. It used a top-down lipidomics approach to monitor the abundance of 95 lipid species originating from 10 lipid classes in the plasma of 70 male individuals, 19 of whom were hypertensive. It was demonstrated that hypertension was specifically associated with reduced levels of free plasma cholesterol and ether lipids, in particular ether phosphatidylcholines and ether phosphatidylethanolamines, while other lipid classes remained practically unaffected. These results may form the basis for novel dietary strategies for the treatment of the metabolic syndrome and hypertension.<sup>131</sup>



# ANALYSIS OF METABOLITES

## 9. Analysis of Metabolites



Metabolite identification studies remain an integral and important part of pre-clinical and clinical drug development programs. Analysis of biological matrices, such as plasma, urine, faeces and bile, pose significant challenges due to large amounts of endogenous components that can mask the drug and its metabolites. The situation is further complicated for human samples. Unlike animal species, where the animal colony is maintained in a controlled environment, human biological matrices contain a number of variables (environmental differences, ethnic diversity, dietary habits, disease states, self-medication, etc.). Even though sample complexity can be partially alleviated by hyphenated separation techniques, high resolution MS has become valued for tackling the problem in a time- and cost-effective manner.

Current approaches to discovery-stage drug metabolism studies (e.g., pharmacokinetics, microsomal stability studies) typically use triple quadrupole instruments for quantitative analysis. This necessitates optimization of parameters such as Q1 and Q3  $m/z$  values, collision energy, and interface voltages. These studies detect only the specified compounds (targeted approach) and information about other components, such as metabolites, is lost. The ability to perform full-scan acquisition for quantitative analysis would eliminate the need for compound optimization while enabling the detection of metabolites, and other non-drug-related endogenous components. The Orbitrap-based mass analyzers provide the tools for combined qualitative and quantitative analysis in a single data acquisition.<sup>36</sup>

The narrow mass tolerance afforded by the Orbitrap-based mass analyzers reduces or eliminates background chemical noise, dramatically increasing sensitivity for confirming or eliminating the presence of metabolites and their isobaric forms. Metabolites of nefazodone were analyzed using this approach.<sup>132</sup> Analysis of lafutidine and its metabolites in human liver microsomal preparation employed the same strategy.<sup>133</sup> The ability to conduct tandem mass spectrometry in parallel with accurate mass measurements was used for the structural characterisation of 14 phenobarbital metabolites in rat urine. Some of the observed metabolites had not been previously reported in the literature.<sup>134</sup> Analogously, several new GSH conjugates of carvediol were reported in another study, which may explain hepatotoxicity during clinical trials and recent clinical issues.<sup>135</sup>

A simple and rapid LC-MS/MS screening method utilizing Orbitrap detection was developed for cyclo-dopa and diketopiperazine alkaloids in crude plant extracts. The alkaloids could be identified based on their MS/MS data, elemental compositions, and retention behavior. Limits of detection at or below 5 ng/mL were achieved in the assays. This screening method considerably reduces the time and cost involved in the identification of the alkaloids, as well as being a simple and convenient approach to the identification of other structural families of natural products.<sup>136</sup>

Interesting is the application of the LTQ Orbitrap mass spectrometer for structural identification of trace level impurities where the authors used hydrogen/deuterium exchange chromatography.<sup>137</sup>

A recent publication addressed the question of quantitation, comparing performance against triple quadrupole SRM detection using a Sciex™ API 4000. Comparable assay precision, accuracy, linearity and sensitivity were observed for both approaches. The concentrations of actual study samples from 15 drug candidates reported by the two methods were statistically equivalent. Authors commented that one of the biggest advantages using full scan high resolution mass spectrometry was that data was obtained not only on the analytes of interest, but also on other components in the sample, and this data can be retrieved at any time, post acquisition. In this case, simultaneous collection of MS/MS data in the ion trap provided structural confirmation of the metabolites. In conclusion, the bioanalytical process could be simplified when using high resolution, since the calculated theoretical mass of an analyte was used directly for quantification of small molecules, and since no compound tuning was necessary.<sup>138</sup>

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.<sup>24</sup> 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 within the acquired mass range, with a degree of specificity equal to most MS/MS assays.<sup>24</sup>

It is worth noting that some assays well established on triple quadrupole platforms have been transferred onto the Orbitrap platform so as to utilize exact mass measurement for metabolite confirmation. The authors pointed out the reduced quality of isotopic patterns on quadrupole-linear ion trap system (even when 'enhanced mass mode' was used) and perceived that as a strong disadvantage of that instrument. The problem was especially exacerbated for minor metabolites with low signal intensity. On the other hand, the LTQ Orbitrap mass spectrometer maintained excellent isotopic pattern fidelity in full FT survey scan mode.<sup>139</sup> Recently, a detailed study of isotope ratio abundances showed that the errors largely remain within a few percent. The observed errors for 10 compounds ( $m/z$  639 to 1,663 Da) did not exceed 3% for detection at RP 7,500, and acceptable errors were shown for resolutions up to 60,000.<sup>140</sup>

# PROCESSING METABOLITE ANALYSIS DATA SETS

The ability of the Orbitrap mass analyzer to clearly observe an isotopic pattern can be used to trigger data-dependent product ion scans. The method has a broader detection capability compared to classical approaches utilising product ion or neutral loss scans, since it is independent of the collision-induced dissociation behavior of the compounds. This feature can be very conveniently used for the analysis of metal-containing compounds as well as compounds with other atoms showing a specific isotopic pattern signature, like sulfur or bromine. Such an approach afforded, for instance, simultaneous detection and structural characterization of novel metabolites from the class of glutathione conjugates.<sup>141</sup>

Metal-containing compounds, e.g. arsenic or selenium metabolites, are gaining prominence due to their nutritional value and implications in potential toxicity. Many metals demonstrate a specific isotopic pattern that can serve as a trigger for data dependent analysis of metal-containing compounds. Detection of the selenium-isotopic pattern in mass spectra down to the intrascan abundance of 0.001 was demonstrated with low or sub-ppm mass accuracy regardless of the concentration.<sup>30</sup> The detection was then complemented with a detailed characterization of these selenium species, all in single chromatographic run.

The presence of a metal atom in the metabolite of interest offers an exciting possibility to combine the results from the LTQ Orbitrap mass spectrometer with those from inductively coupled plasma mass spectrometry (ICP-MS). ICP-MS delivers sensitivity of ppt or even ppq with linear dynamic range of concentrations spanning 12 orders of magnitude. The LTQ Orbitrap data proved to be highly complementary with its ability to fully structurally characterize molecules based on their MS<sup>n</sup> spectra. A two-pronged approach using ICP-MS and the Orbitrap MS was applied to the speciation of arsenic and, in particular, arsenite phytochelatin complexes. Both of these MS techniques were necessary to identify and quantify the arsenic species in plant root extracts.<sup>142</sup> ICP-MS is not limited just to the compounds containing metals and metalloids; metabolite profiling and identification was conducted using both mass spec platforms for a new bromine-containing antituberculosis compound in drug development.<sup>143</sup>

The unique capability to provide multiple levels of fragmentation (MS<sup>n</sup>) with accurate mass reading has been utilized to elucidate the structure of a sulphated pentasaccharide impurity seen in Fondaparinux preparation. Valuable structural information was generated through sequential MS<sup>10</sup> fragmentation by isolating specific adducts and assessing their fragmentation pathways.<sup>84</sup>

## 10. Processing Metabolite Analysis Data Sets

The rich data sets acquired for metabolite detection and identification represent a bonanza of information but requires advanced data processing techniques for analysis. Multiple post-acquisition data mining tools can incorporate such features as extracted ion chromatograms, mass-defect filters, product-ion chromatograms and neutral loss filter techniques. Such approaches are highly complementary and often lead to the discovery and characterization of uncommon metabolites.<sup>23</sup> Recent inclusion of the multiple mass defect filter in commercial software packages demonstrates that this is a rapidly evolving area.<sup>144</sup>

Analysis of indinavir metabolites in rat liver preparation exemplifies the practicality of multiple post-acquisition data mining techniques. The high resolution extracted ion chromatogram process was shown to be very effective in the detection of common metabolites with predicted molecular weights. The mass defect filter, which searches for metabolites based on the similarity of mass defects of metabolites to those of indinavir and its core substructures, helped find uncommon metabolites not detected by the extracted ion chromatogram processing. The high resolution product ion and neutral loss filters selectively detected metabolites that underwent fragmentation pathways similar to those of indinavir or its known metabolites. As a result, a total of 15 metabolites including two new indinavir metabolites were detected and characterized.<sup>145</sup>

The ability of high resolution MS to discern between odd and even electron species allows for detailed studies of fragmentation behaviour. This information assists modern software packages to build and verify extensive databases of compound fragmentation mechanisms which, in turn, can be used to interpret spectra and fragmentation pathways for 'unknowns'.<sup>146</sup>

High throughput metabolite identification is hindered by the lack of software enabling fast automated annotation of metabolites with their correct elemental formula. A recently published approach seems to address this issue.<sup>147</sup> Computational methods can also significantly improve the mass accuracy. The presence of ubiquitous background ions in LC/MS profiles allows for accurate alignment and internal mass calibration.<sup>26, 28</sup>

# DOPING CONTROL

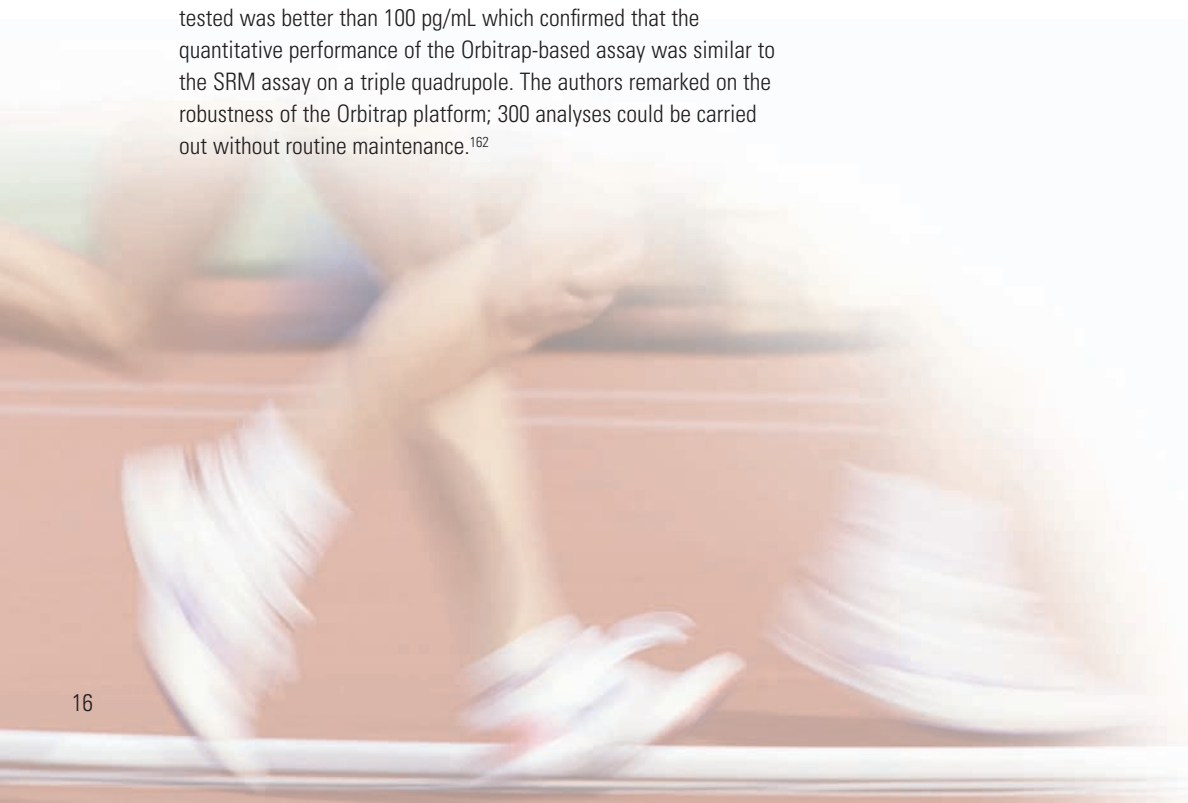
## 11. Doping Control



Metabolite identification and quantitation in the area of doping control is benefiting considerably from accurate mass determination where it helps elucidating dissociation pathways to characteristic product ions. Knowledge of these dissociation pathways supports the identification of novel drugs of abuse, related compounds (designer drugs) as well as their metabolites, which is of utmost importance to drug testing laboratories.

Selective androgen receptor modulators (SARMs) are innovative non-steroidal anabolic agents designed to counteract muscle wasting and osteoporosis. They are prohibited in amateur and professional sports. Dissociation pathways for both positive and negative ESI of these compounds were studied using an LTQ Orbitrap mass spectrometer. Determining diagnostic product ions and common fragmentation patterns then served to establish screening procedures capable of targeting the intact SARMs as well as putative metabolites.<sup>148-153</sup> Other compound classes that have been analyzed in this same manner and for which validated methods have been derived include: human insulin-like growth factor-1 and its analogues; synthetic analogues to human adrenocorticotropin, acetylcholine esterase inhibitors, metabolites of the anti-depressant/antiobesity drug sibutramine, efaproxiral, stanozolol, and proteases.<sup>154-162</sup> In a recent report, a screening method was developed allowing detection of 29 banned compounds, including antiestrogenic activity,  $\beta_2$  agonists, exogenous anabolic steroids, and other anabolic agents. HPLC was coupled to the LTQ Orbitrap mass spectrometer and an in-source collision-induced dissociation was used with atmospheric pressure chemical ionization source. The detection limit for all compounds tested was better than 100 pg/mL which confirmed that the quantitative performance of the Orbitrap-based assay was similar to the SRM assay on a triple quadrupole. The authors remarked on the robustness of the Orbitrap platform; 300 analyses could be carried out without routine maintenance.<sup>162</sup>

The requirement of high resolving power for full scan mass selectivity was highlighted in the case of hormone and veterinary drug residue analysis performed on hair extracts fortified with 14 steroid esters using U-HPLC. 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 from isobaric co-eluting sample matrix ions.<sup>38</sup> 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. This particular setup was using a capillary column (75  $\mu\text{m}$  internal diameter) at a 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.<sup>163</sup>





# ENVIRONMENTAL, FOOD AND FEED ANALYSIS

## 12. Environmental, Food and Feed Analysis



Environmental analysis is yet another area making use of the Orbitrap MS. The emerging demands under European legislation for screening more hydrophilic compounds have translated into development of methods for their monitoring in aqueous environments. The application of accurate mass screening for target analyses of pharmaceuticals, benzotriazoles, illicit drugs, and identification of unknowns in samples of ground water and polar fraction of a landfill soil extracts has been demonstrated.<sup>164</sup>

A method developed for detecting 1H-benzotriazoles and benzothiazoles in drinking and surface water relying on accurate mass measurements of precursor ions was extended to monitoring at least one product ion simultaneously detected in the linear ion trap. Albeit the product MS spectrum was acquired at unit resolution, the information served for the confirmation of compound identity. Such an approach allowed the quantification of these residuals down to the detection limits of 0.01 µg/L.<sup>165</sup> In another study, picogram levels of *N*-nitrosamines in drinking and waste water samples could be quantified affording both high sensitivity and selectivity.<sup>166</sup>

Comprehensive residue analysis in food and feed matrices is a challenging area. 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). Such rudimentary sample preparation steps lack on selectivity which has to be compensated for by that much higher performance of the instrumental analysis. Moreover, the overall sensitivity fulfilling legal requirements of ng/g for residues has to be achieved. The Orbitrap-based analyzers bring in a new paradigm enabling to combine qualitative and quantitative aspects in a single analysis.

The report on stanozolol determination in urine was one of the first illustrating the use of the Orbitrap technology in the field of food contaminant analysis. Initially, the authors used a QqTOF instrument for an accurate mass spectrum of product ions to confirm the identification of stanozolol in the sample, however, the experimental value of some fragments was far from the theoretically expected and most of the fragment masses did not yield realistic elemental compositions within a mass error of 10 mmu. However, at resolution 60,000 a clear explanation was found: the reason for the inaccurate masses in the QqTOF fragmentation spectrum was the presence of doublets having only minor differences in mass which could not have been determined at resolving power (approx. 10,000) achievable by the QqTOF instrument used in the experiment.<sup>167</sup>


Another direct comparison between QqTOF and Orbitrap mass analyzers was performed for hormone and veterinary drug residue analyses. Experiments using resolution of 10,000 failed to detect the steroid esters being assayed in hair extract. The Orbitrap analysis carried out at 60,000 resolution enabled the detection and accurate mass measurement of all 14 steroid esters in this complex matrix at low ng/g concentration levels.<sup>38</sup>

A mixture of 151 pesticides, veterinary drugs, mycotoxins, and plant toxins in generic extracts of honey and animal feed (10-250 ng/g) was analyzed with U-HPLC coupled to the Orbitrap MS. 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.<sup>37</sup>

It is not just small molecules that are of interest from the point of contaminants and residue analysis in food. Recombinant bovine somatotropin (rbST) is a polypeptidic hormone used in dairy cattle to enhance milk production. Its use in Europe is banned. A method has been developed relying on the detection of its *N*-terminal peptide. This peptide obtained by enzymatic digestion of the hormone is specific to the difference between the endogenous and recombinant form of somatotropin. The method allowed the direct detection of 10 ng/mL of rbST in fortified samples as well as in plasma collected from a goat even 2 days after administration.<sup>168</sup>

# DNA AND RNA ANALYSIS/USE OF ORBITRAP TECHNOLOGY FOR FUNDAMENTAL STUDIES

## 13. DNA and RNA Analysis



Analysis of DNA and RNA on a hybrid mass spectrometer used sequential MS<sup>3</sup> experiments to gain extensive information on adducts formed by reaction of active metabolites of heterocyclic aromatic amines with deoxynucleosides. The combination of exact mass and MS<sup>n</sup> provided new information for already known adducts as well as identification and characterization of two new adducts.<sup>169</sup> In another study, high-resolution accurate mass data enabled differentiation between two possible metabolite sequences of siRNA with a monoisotopic molecular mass difference of less than 1 Da.<sup>170</sup> This particular area faces many analytical and sample preparation challenges, and this was the first published report showing reliable identification of siRNA drug metabolites with high confidence and in a more automated manner.

## 14. Use of Orbitrap Technology for Fundamental Studies

Some fundamental aspects of science have also seen a contribution from the Orbitrap mass analyzer, such as elusive processes of gas-phase chemistry. Results from both positive and negative ionization modes were complemented by density function calculations to provide insight into the atom sites involved in protonation/deprotonation reactions.<sup>171</sup>

## 15. Developments on the Front End

### 15.1. Ion Mobility

Dirty matrices present an ever mounting challenge. It is for this reason that some novel ionization and separation techniques are finding their way into the traditional LC/MS setup. Successful LC/MS method development requires isolation of the analyte from the matrix. Even though mass spectrometry imparts an added dimension of selectivity and signal-to-noise improvement through fragmentation and high resolution, isobaric interferences or compounds that fragment poorly pose a distinct challenge. Ion mobility (more specifically, High-Field Asymmetric waveform Ion Mobility Spectrometry, FAIMS) shows a promise in bioanalysis. It separates ions in the millisecond timescale after LC separation and prior to introduction into the vacuum region of a mass spectrometer. It exploits differences in ion mobility at very high electric fields, and can thus facilitate the separation of compounds that are isobaric.<sup>172</sup>

FAIMS has demonstrated improvement in sample throughput for bioanalysis in the discovery pharmaceutical settings. It ensured the removal of endogenous and metabolite interferences thus eliminating the need for liquid chromatography when analyzing simple mixtures.<sup>173</sup> Even though most of the applications of the FAIMS come from its coupling to a triple quadrupole mass spectrometer, the analysis of complex mixtures would undoubtedly benefit from a high resolution Orbitrap-based mass spectrometer. First such examples have been already reported from the field of proteomics.<sup>174</sup> This study showed that it is possible to partially separate phosphopeptides having identical sequences but differing sites of modification by FAIMS with confirmation of the separated species by ETD mass spectrometry.

### 15.2. Novel Desorption Techniques

Albeit most analyses report the use of electrospray or nano-electrospray ionization coupled to an LTQ Orbitrap mass spectrometer for metabolite applications, other types of atmospheric pressure ionization techniques such as desorption electrospray ionization (DESI) offer interesting possibilities. Measurement and characterization of active ingredients directly in pharmaceutical tablets (loratidine in a Claritin tablet) has been reported using DESI coupled to an LTQ Orbitrap mass spectrometer.<sup>175</sup> This study showed that it is possible to partially separate phosphopeptides having identical sequences but differing sites of modification by FAIMS with confirmation of the separated species by ETD mass spectrometry.

Recently, a novel atmospheric ionization technique termed laser diode thermal desorption (LDTD) has been introduced. The LDTD source uses an infrared laser diode to desorb samples, previously dried onto a well of a special 96-well plate. The desorbed gas phase molecules are swept by a carrier gas into a corona discharge region to undergo atmospheric pressure chemical ionization (APCI), and then transferred directly into the mass spectrometer. The analysis and detection of intact desorbed molecules are accomplished within a few seconds enabling a considerable sample throughput (960 samples analyzed in 1.9 hours). The LDTD source coupled with LTQ Orbitrap MS was tested for analysis of polyaromatic molecules. The LDTD source delivered better performance than ESI or APCI in terms of throughput, ease of use, spectral quality and specificity for detection.<sup>176</sup>

# DEVELOPMENTS ON THE FRONT END

## 15.3. Matrix-Assisted Laser Desorption/Ionization

Matrix-assisted laser desorption/ionization (MALDI) has become recently available on the hybrid Orbitrap family.<sup>177</sup> Among others, it offers possibilities in visualizing the distribution of parent drug and/or metabolites in parts of tissues or even whole laboratory animal sections (tissue imaging). Compared to traditional immunocytochemistry, MALDI imaging experiments provide comparable information regarding compound distribution with relatively lower resolution due to instrument limitations, such as laser beam size and certain level of analyte diffusion during sample preparation. However, MALDI imaging is capable of distinguishing isoforms and also it can be performed in a much higher throughput manner to enable a large scale documentation of compound localization.<sup>178</sup> An atmospheric pressure MALDI has also been coupled to the LTQ Orbitrap MS.<sup>179</sup>

Chen *et al.* performed a comprehensive investigation of the neuropeptide expression and their localization in the nervous system of *Homarus americanus*. The authors compared 3 different MALDI mass spectrometers (TOF/TOF, Orbitrap and Fourier transform ion cyclotron resonance analyzers). A modest resolving power of MALDI TOF/TOF instrument was a distinct disadvantage. For instance, it did not allow detection of the presence of both SSEDMDRLGFG ( $m/z$  1213.51538) and FDAFTTGFGHN ( $m/z$  1213.52726) peptide peaks, while both these species could be confidently detected using the Thermo Scientific MALDI LTQ Orbitrap. The study provided useful insights for further understanding of the neuropeptide biological functions.<sup>178</sup>

Analysis of lipids desorbed from nerve tissue was another study which reported an improvement in mass spectral resolution and a decrease in background observed in the spectra collected from the Orbitrap mass analyzer. More accurate mass spectrometric images of the distribution of lipids within nerve tissue could then be obtained. The linear ion trap portion of the instrument was used to collect MS/MS spectra for identity confirmation of the detected lipids. Employment of both mass analyzers thus provided a rapid and reliable means of compound identification based on MS/MS fragmentation and high-resolution/accurate mass.<sup>179</sup>

Both manual and automated MS and MS/MS analyses of isolated protein complexes were performed by Luo *et al.* Using the MALDI LTQ Orbitrap MS the authors readily resolved the composition of the assayed complexes, and noted increase confidence of protein identification compared to MALDI QqTOF or MALDI ion trap instruments. Moreover, sub-femtomole sensitivity was achieved with the matrices used. Similar sensitivity was also obtained for the analyses of phosphopeptides in mixtures employing neutral loss scanning.<sup>181</sup>



# CONCLUSIONS/ACKNOWLEDGEMENT/ REFERENCES

## 16. Conclusions

This concise literature overview is far from being an exhaustive list of publications mentioning the use of Orbitrap mass analyzers. The publications referenced herein discuss the use of Orbitrap technology for mass analysis, for both small molecule and large molecules, for both qualitative and quantitative assays. No other mass spectrometric platform can match the volume of published (and peer reviewed) work or the range of applications that the Orbitrap has achieved. The Orbitrap mass analyzer has truly played a role in advancing scientific knowledge and is helping make our world healthier, cleaner and safer.

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