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HRMS in Clinical Research: from Targeted Quantification to Metabolomics

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Introduction to HRMS

Until recently there have been two main schools of thought regarding MS technologies. One school of thought considered the triple-quadrupole as the gold standard for quantification by SRM acquisitions (ion transitions). The second school of thought held that ion trap or high-resolution MS (HRMS), while often considered for qualitative analysis, performed rather poor for quantification. But during the last few years, reports describing the capability of HRMS instruments to perform sensitive and reliable quantification of a large variety of analytes in HR-full scan mode, have appeared. While “hurdles” have been mentioned that may slow down the shift to HRMS, such as cost, the inutility to record large HR full scan data, etc., gradually all of these hurdles are being overcome. New, affordable HRMS instruments in quantitative clinical research analyses demonstrate the sensitivity and selectivity of these instruments for this work. Accelerating the migration is the fact that triple-quadrupole is poor when used for a global view of an biological extract. Triple-quadrupoles have slow full scan acquisition speed, while HRMS instruments have high resolution at fast scanning speeds. Therefore HRMS can be selective and compatible with UHPLC, whereas recording thousands of ions in a chromatogram allowing a global view. **Figure 1** compares an HRMS response (in blue) and a low resolution triple-quadrupole MS response (in grey). It is easy to see that triple-quadrupole MS yields a very broad mass distribution, requiring further fragmentation for adequate selectivity and sensitivity.

There are three key aspects to consider in HRMS: resolution, mass accuracy (MA), and the mass-extraction-window (MEW). Resolution is calculated by the equation in Figure 1. For example, the triple-quadrupole response in Figure 1 is a very broad dispersion with low resolution, typically 500. By comparison the HRMS response has much higher resolution, ≥ 70,000 (at m/z 200). MA is the delta between the theoretical and the measured mass (m/z). MA is important because even if you have high mass resolution, accuracy relative to the theoretical m/z is required for selective detection. The last important aspect to consider is the MEW. MEW is the very narrow mass range used to extract the ion(s) of interest and to construct the extracted ion chromatograms (XIC). The MEW is centered on the theoretical mass and any mass shift of the analyte should be inside the MEW range.

Basically the primary difference between HRMS and triple-quadrupole MS is that when performing triple-quadrupole MS analyses, the molecule to be determined is selected for monitoring before the analysis, in contrast to the HRMS where all ions are recorded first (full scan) and then the molecule to be determined is extracted for monitoring after the acquisition (XIC). Therefore with HRMS it is possible to perform retrospective data mining, even years later.

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Quantitative Analyses

Using HRMS to perform both quantitative and qualitative analysis, allows a laboratory to have one technology and more efficient workflows. For instance, performing research discovery as well as targeted and productive analysis with the same technology simplifies the work significantly.

To evaluate the quantitation performance of HRMS five criteria were chosen: detection selectivity in HR full scan, accuracy and precision of mass determination, LOD and dynamic range/linearity of calibration curves, the level of accuracy of unknown samples in comparison to the quantification performed with triple-quadrupole MS analyses, and ease of use and bottleneck in the workflow. Identical extracted plasma samples (QCs, calibrants and unknowns) were injected on a triple-quadrupole MS and a Thermo Scientific™ Q Exactive™ HRMS, and the results compared. Samples used for comparison purposes included drugs, endogenous metabolites, xenobiotics and peptides like hepcidin. Classical sample preparations (e.g. protein precipitation, SPE) were used, as well as usual HPLC conditions (e.g. C18, 2.1x 50mm, 1.9 to 3.5 uM columns).

According to the literature, higher detection selectivity of full scan acquisition with HRMS in comparison to SRM acquisition performed on triple-quadrupole, is reached when the resolution is ≥ 50,000 and the MEW is ≤ 10 ppm. The case showing the higher HRMS detection selectivity is illustrated in **Figure 2** with the SRM detection, normetanephrine and an isobaric interference, a metabolite of amphetamine, coelute. Because the SRM transitions are the same for both compounds, the chromatography had to be extended to resolve the two compounds. With HRMS, the delta of mass is big enough to easily resolve the compound of interest and its interference.

The chromatogram of Dasatinib in a plasma extract at the

Figure 1

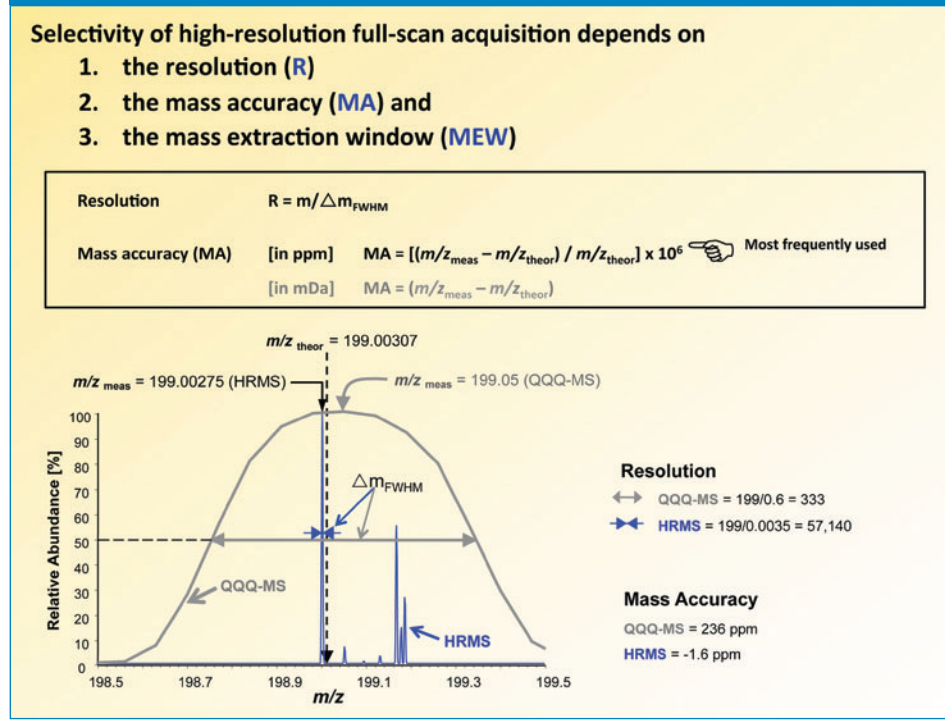
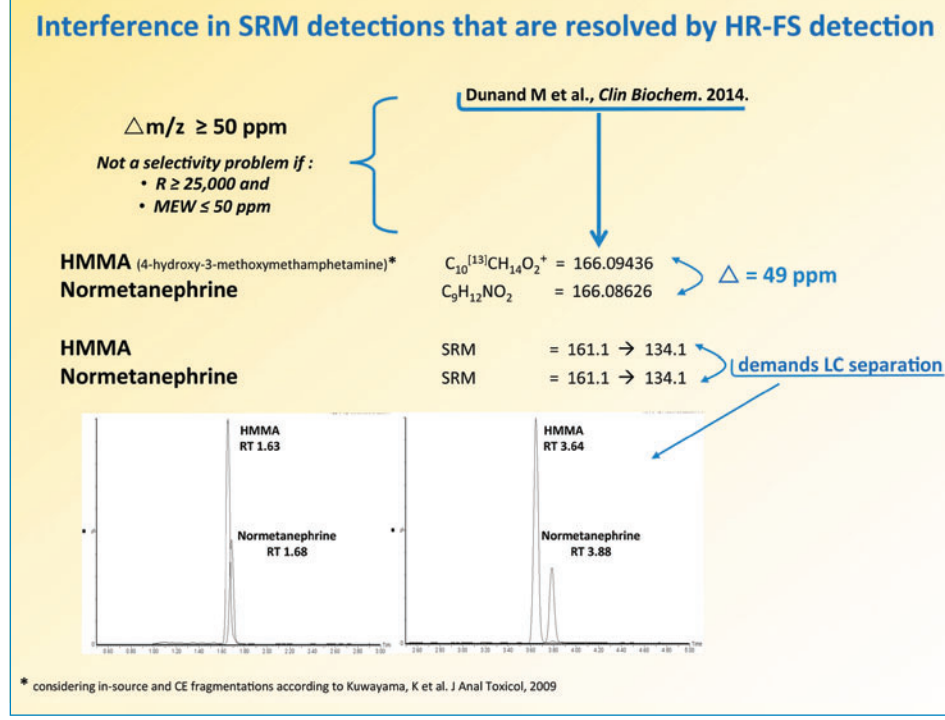


Figure 2



limit of quantification (one picogram on column) is shown in **Figure 3**. Two acquisition types were performed simultaneously: full scan and data independent acquisition monitoring the product ions of interest. Calibration curves are very comparable between the 2 types of acquisition.

Mass accuracy (MA) are also important in preventing false detections. Using narrow mass-extraction-windows (MEW), e.g. 10 ppm, the drug of interest is selectively extracted from the full scan acquisition and depicted where as thousands of ions are recorded from this plasma extract, as seen in **Figure 4**. Using a too wide, or too narrow MEW can result in false positive or negative detection, respectively. However, the MA variations of modern HRMS instruments are robust and help avoid false positive and false negative detections when using MEW of ± 10 to 25 ppm.

The **Figure 5** illustrated the LOD and dynamic range differences between triple-quadrupole MS performing SRM acquisition (top panel) and HRMS performing full scan (bottom panel) for a Sunitinib plasma extract. In Figure 5 the chromatograms on the left represent the limit of detection on both technologies. On the right, the calibration curves are displayed as peak areas against drug levels for a better comparison and are depicted at the low, medium and high levels. As can be seen, the dynamic range of the triple-quadrupole MS is smaller compared to the HRMS full scan response. So although sensitivity between the two instruments may differ by only a factor of two-to-three in many cases, the dynamic linear range can differ significantly, in favor of the HRMS instrument (Q Exactive Focus MS in this case).

Figure 3

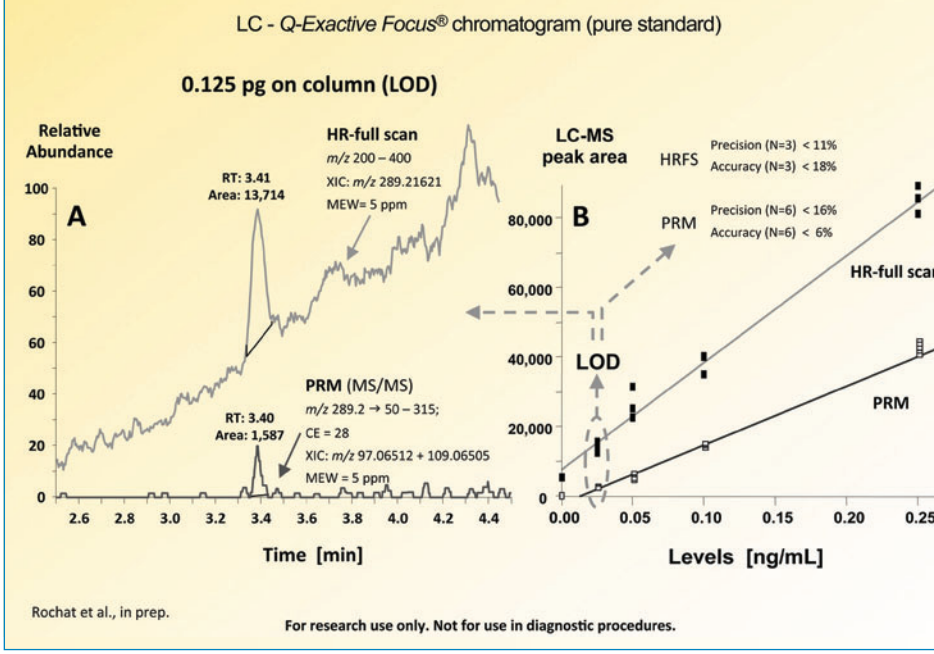
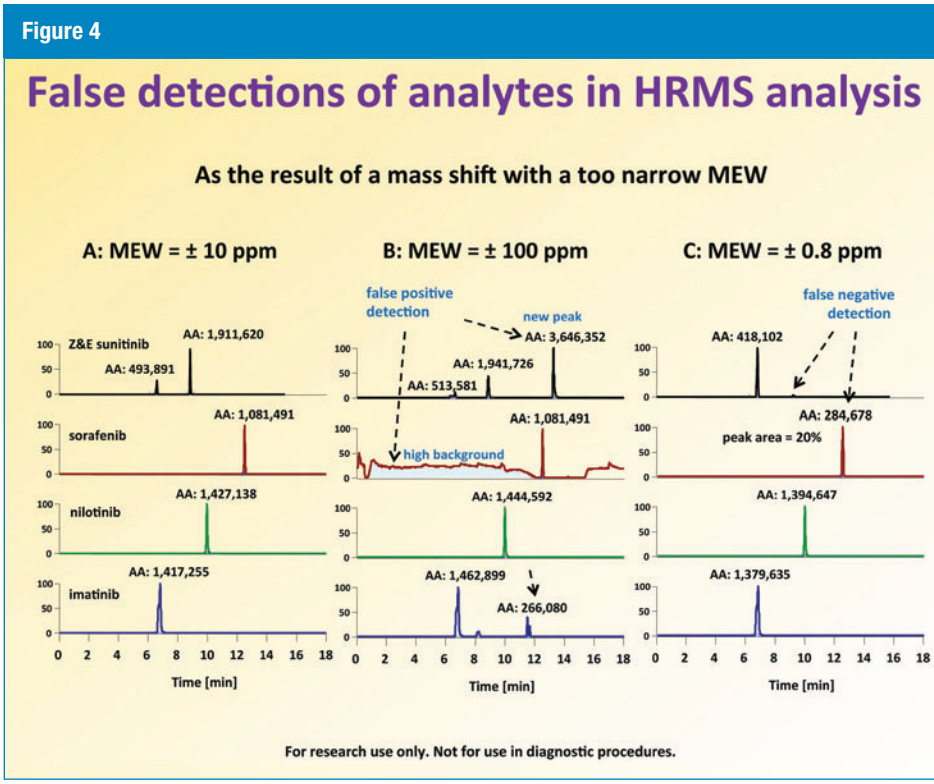


Figure 4



The accuracy of determined levels in real samples is crucial and this is why, we have compared the results obtained with SRM or high resolution full scan analysis. **Figure 6** shows the Passing-Bablok analysis of almost 700 plasma extract sam-

Figure 5

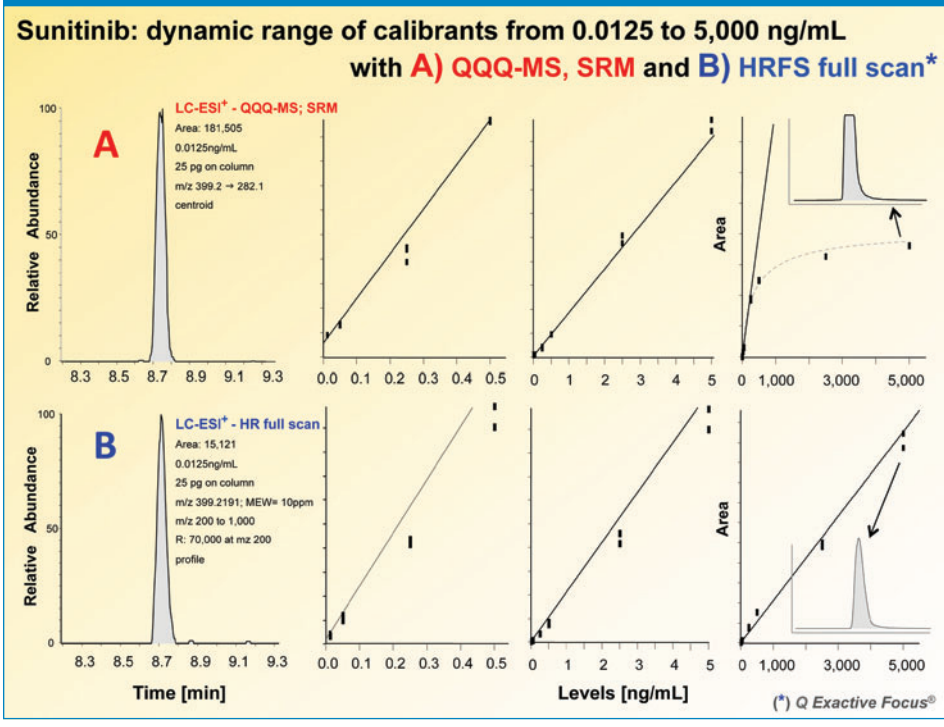
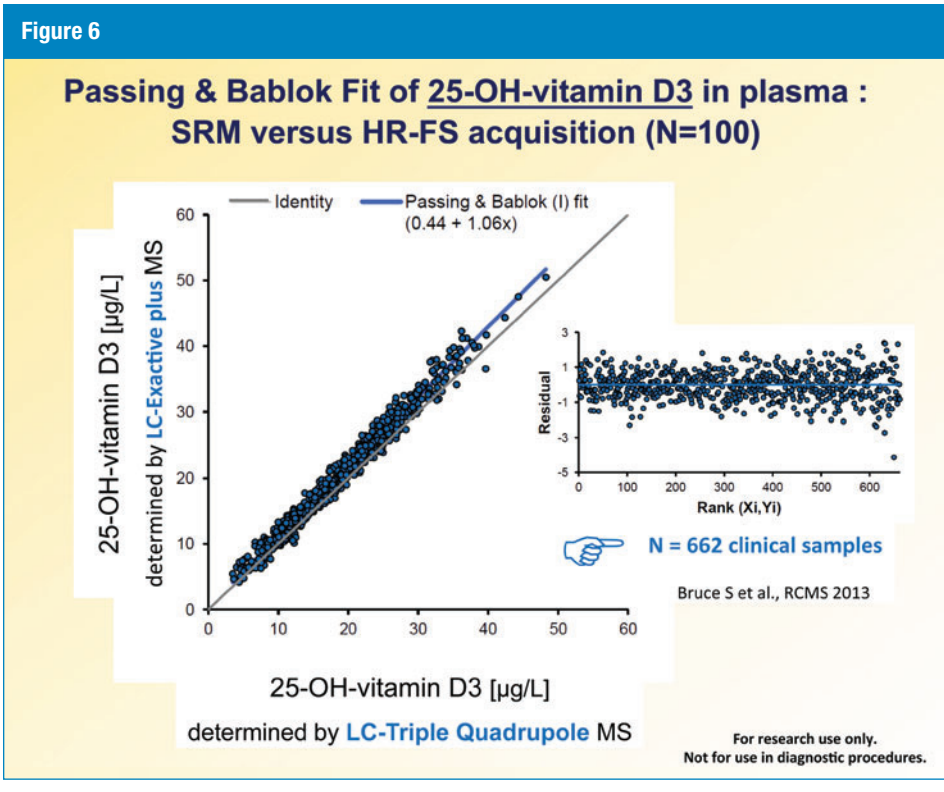


Figure 6



ples and compared the results obtained with a triple-quadrupole and an Thermo Scientific™ Exactive™ Plus HRMS in full scan mode resulting in a perfect match for the determination of the vitamin D in plasma. Many other comparisons have been made in our lab or published in peer-reviewed articles and show similar results.

Finally, the ease of use, user-friendliness and robustness of HRMS instruments are also important considerations. Modern HRMS instruments are very user friendly considering that the analyst doesn't have to tune the collision energy for the ion transitions. Furthermore, analysis is straightforward; once an injection is made, a full scan is collected and treated post-run (XIC). If a problem is observed, the full scan acquisition gives a global view of the sample extracts allowing easier troubleshooting.

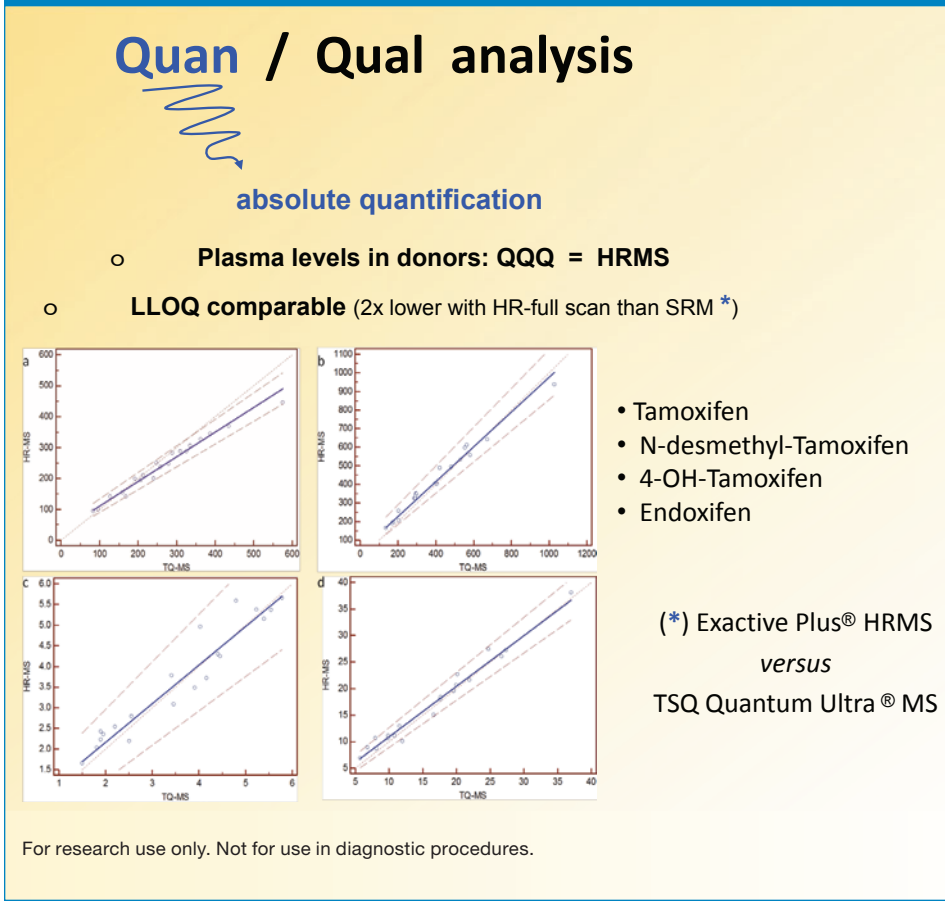
Qualitative/Quantitative Analysis

The true power of HRMS is highlighted when performing qualitative/quantitative (quan/qual) analysis, and is illustrated in the quan/qual analysis of tamoxifen in human donor plasma samples. Tamoxifen is an anti-cancer drug. In 2005, >50,000 women were treated with tamoxifen in the US. There is a long history of tamoxifen drug metabolism studies because the drug is metabolized in toxic as well as very bioactive metabolites. Our quan/qual study has shown that the limit of quantitation of tamoxifen and 3 metabolites were essentially the same (<3x) between the tested triple-quadrupole and HRMS instruments, as illustrated in **Figure 7**.

Tamoxifen studies using a triple-quadrupole, have found as many as 22 metabolites in donor plasma samples. However, using HRMS, 40 metabolites have been observed from the full scan, many of them never described before. Post-runs, we extracted potential tamoxifen metabolite ions.

This shows the in depth investigation capabilities of HRMS

Figure 7



and full scan acquisition in the study of the fate of a compound in biomatrices. Indeed, we found up to 7th tamoxifen metabolite generation (7 biotransformation steps) in the donor plasma.

Relative Quantification: Metabolomics

Genes, proteins and metabolites which lead to genome, proteome and metabolome, are the 3 bricks for life. Analysis of the metabolome, is called metabolomics, but why it is so important?

The biomedical perception is to say: could these hundreds of metabolites that we can measure now via HRMS, give us a more global information about a person's biological status?

The future in clinical practice and clinical analysis is very probably targeted metabolomics, which is the determination of 100's of metabolites that have been previously identified.

A new application of targeted metabolomics is something that the World Anti-Doping Agency (WADA) used already and is called the Athlete Biological Passport. This biological passport, which is the record of many metabolites, could be determined for anyone by the mean of targeted metabolomics. Today, most often, targeted metabolomics uses LC-HRMS analyses to quantify (relative quantifications) a few hundreds of metabolites in plasma samples. By this mean, a metabolite phenotype (metabotype) is recorded. The individual metabolite determinations (metabotyping) would give a personal reference value as opposed to a population reference value that is commonly used today. For instance, testosterone/epitestosterone ratio is used in the anti-doping world to check if an athlete has taken external testosterone to improve his performance (the ratio will increase because epitestosterone has a stable level in human). Using athlete individual reference value, the control is more efficient in determining the doping. Similarly, endogenous metabolite ratios could be used for medical purposes. The biomedical knowledge, that is to say the signification of metabolite ratios, still have to be constructed. But the good news is that the analytical platform is now available to build this knowledge: HRMS. Targeted metabolomics analysis will allow a global screening and will reveal an individual's metabotype that may be indicative of something that may need more in-depth medical investigation (e.g. targeted and absolute quantification methods). Similarly, pollutants and xenobiotics will be screened.

Conclusion

Although there is a lot of conclusive evidence for the advantages of HRMS, there may still be some doubt about adopting the technology. But in most cases, HRMS is becoming the new gold standard mainly because of its versatility. It is fully compatible with GLP guidance for validated analyses, and there is no longer a difference in cost between triple-quadrupole and HRMS instruments. The risk of false negative or false positive detection is minimized by proper selection of the mass extraction window (e.g. 10-20 ppm) and appropriate mass calibration. Sensitivity we found on the HRMS tested, is similar with triple-quadrupole MS technology. In addition, it is simpler to develop HRMS analyses and address troubleshooting as it is much easier to look at a problem with a full picture. Add to that the advantage of quan/qual analysis as well as targeted metabolomics (metabotyping) in the field of personalized biology and medicine: for all these reasons, one can see that the adoption of HRMS is the wave of the future.