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Fast Quantification Without Conventional Chromatography, the Growing Power of Mass Spectrometry

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Abstract

Mass spectrometry (MS) in hyphenated techniques is widely accepted as the gold standard quantitative tool in life sciences. However, MS possesses intrinsic analytical capabilities that allow it to be a stand-alone quantitative technique, particularly with current technological advancements. MS has a great potential for simplifying quantitative analysis without the need for tedious chromatographic separation. Its selectivity relies on multistage MS analysis (MS^n), including tandem mass spectrometry (MS/MS), as well as the ever-growing advancements of high-resolution MS instruments.

This perspective describes various analytical platforms that utilize MS as a stand-alone quantitative technique namely, flow injection analysis (FIA), matrix assisted laser desorption ionization (MALDI) including MALDI-MS imaging, and ion mobility, particularly high-field asymmetric waveform ion mobility spectrometry (FAIMS). When MS alone is not capable of providing reliable quantitative data, instead of conventional liquid chromatography (LC)-MS, the use of a guard column (i.e., fast chromatography) may be sufficient for quantification. Although the omission of a chromatographic separation simplifies the analytical process, extra procedures may be needed during sample preparation and clean-up to address the issue of matrix effects.

The discussion of this manuscript focusses on key parameters underlying the uniqueness of each technique for its application in quantitative analysis without the need for a chromatographic separation. In addition, the potential for each analytical strategy and its challenges are discussed as well as improvements needed to render them as mainstream quantitative analytical tools. Overcoming the hurdles for fully validating a quantitative method will allow MS alone to eventually become an indispensable quantitative tool for clinical and toxicological studies.

1) Introduction:

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been established as the gold standard approach for quantitative assays. It is widely used in pharmaceutical, biomedical, and toxicological studies.[1-3] The vast majority of quantitative studies utilize atmospheric pressure ionization, particularly electrospray ionization (ESI) due to its wide applicability for the analysis of molecules with varying structures, molecular weights and polarities.

Despite the superiority of LC-ESI-MS/MS in terms of sensitivity and specificity, it has some limitations, namely long run times and the use of additives that can adversely affect ionization. For example, the use of triethanolamine (TEA) was critical for the quantification of a drug delivery agent in cells as it eliminated peak tailing on a cyano column;[4] however, ion suppression was observed in the ESI source, compromising the sensitivity of the analytical strategy.[4] In addition, stationary phases, such as those utilized in hydrophilic interaction liquid chromatography (HILIC) are challenging to use as minor changes in the analytical conditions may render the quantitative data unreliable. While the use of chromatographic separation will, most likely, remain essential for multianalyte analysis, the intrinsic analytical capabilities of mass spectrometry can be sufficient for the quantification of few targets, without the use of chromatographic separation. As such, quantitative analysis is simplified, and the speed of data acquisition is significantly enhanced.

The key advancements that make MS a stand-alone quantitative technique are 1) the ever growing development in MS/MS in terms of ion dissociation and accumulation for better signals; 2) the continued development of fast high resolution mass analyzers that are suited for rapid analysis; 3) the wide variety of desorption ionization strategies, notably matrix assisted laser desorption ionization

(MALDI) that are advantageous in tolerating the presence of salts and contaminants; and 4) the exponential growth in the development and applications of ion mobility, particularly those with high selectivity, such as high-field asymmetric waveform ion mobility spectrometry (FAIMS).[5-7] Figure 1 summarizes the various analytical strategies of MS as quantitative tool without the use of chromatographic separation.

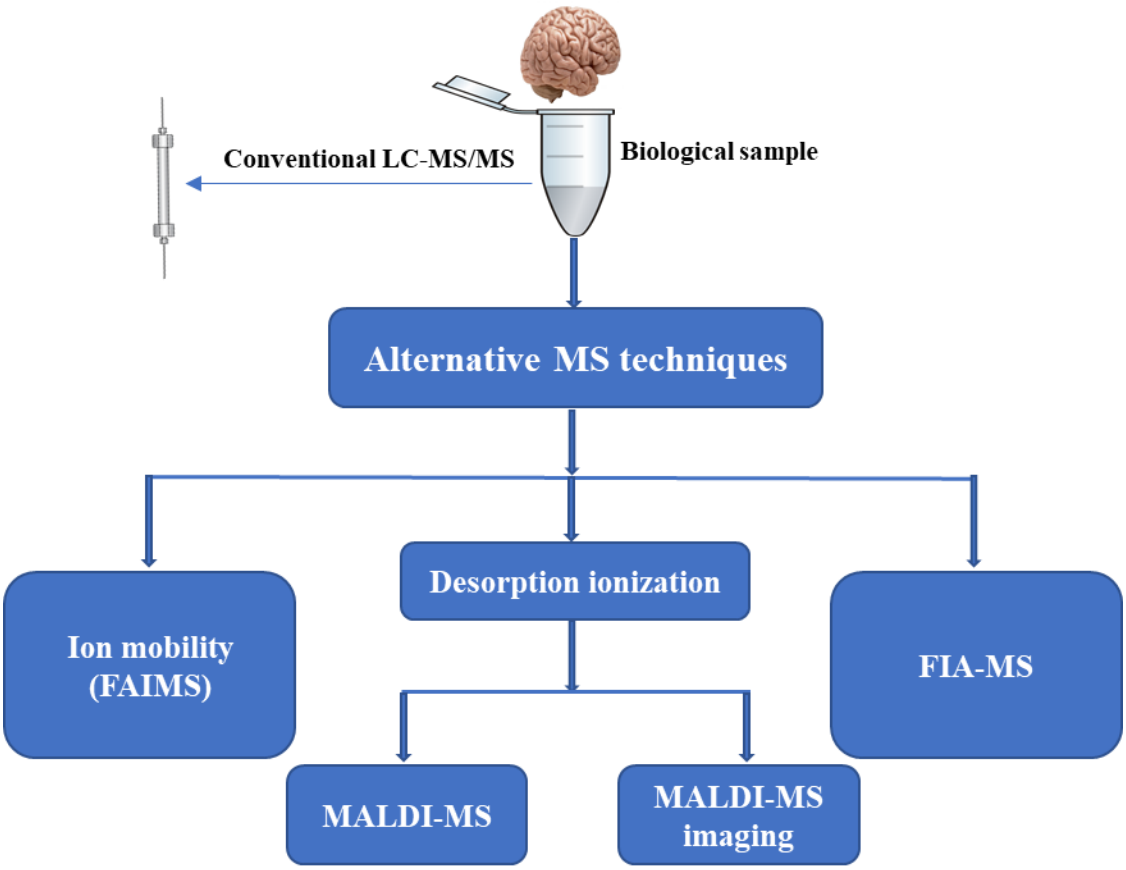


Figure 1. The main analytical capabilities of MS as a quantitative tool without chromatographic separation. Though not shown in the figure, high resolution instruments will enhance selectivity in quantitative assays.

Alongside all MS-based advancements, sample preparation and clean-up are also critical elements for successful quantification without chromatographic separation. For example, flow injection analysis (FIA)-MS/MS of 27 analytes in tobacco was achieved after the isolation of the target analytes from the

complex matrix by homogenization of the powdered sample followed by liquid-liquid extraction in which both the organic and aqueous phases were used for subsequent analysis.[8] Note that despite progress made in utilizing MS alone as a quantitative tool, the vast majority of reported methods fall within fit-for-purpose approach and there is need for the development of fully validated methods as per regulatory guidelines. Method validation[9-11] adds another level of complexity to a quantitative method, but it is a critical element to move quantitative assays towards regular applications in clinical and environmental laboratories.

This perspective paper will focus on the various strategies and approaches that allow MS alone to serve as quantitative tool. It will discuss recent progress, current hurdles and future perspectives in the field with emphasis on development at the MS-front rather than sample preparation, which will be highlighted throughout the manuscript, as required.

2) Flow injection analysis-Mass spectrometry (FIA-MS)

FIA-MS analysis relies on the intrinsic separation capabilities of MS as both separation and detection occur within the MS instrument. Identification and quantification are based on the ability of the MS to resolve analytes according to their mass-to-charge (*m/z*) ratios as well as analysing the product ions. The success of FIA-MS is due to advancements in MS instrumentation where, in many occasions, improved ion optics allow for fast scanning rates, high resolving power, and enhanced sensitivity (better ionization and ion accumulation). In addition, modern instruments have autosamplers and injectors that can precisely and repeatedly inject microliter volumes, ensuring reliability in quantification. Compared with LC-MS, FIA-MS offers several advantages: it is a high-throughput technique due to a fast analysis time; it is a low-cost analytical approach; and it simplifies the analytical procedure. Solvent optimization is simplified as it allows the use of any solvent compatible with MS, for which the analyte is soluble. In fact, it is recommended that solvents that can donate/capture a proton, such as methanol and water, should be used or added unlike in LC-MS/MS where solvent selection is a balance between ionization efficiency

and chromatographic separation.[12] For example, a simple, fast FIA-MS/MS method for the quantification of the drug metformin in dog serum was successfully developed and validated using a solvent mixture of water/acetonitrile (30:70, v/v) with 0.1% formic acid.[13] The method was as sensitive as published LC-MS/MS methods,[14, 15] but it required only 2 minutes run time resulting in savings in instrument time and utilized solvents to obtain the same quantitative data. Published LC-MS/MS methods for the quantification of metformin required longer run times including the use of gradient elution and HILIC technology.[14-16]

Both FIA-MS, and FIA-MS/MS have been utilized in quantifying a myriad of analytes in complex matrices and successfully applied in metabolomics, clinical diagnostics, toxicology, and environmental analysis.[17] As expected, MS/MS enhances selectivity and sensitivity. It also minimizes analyte-to-analyte interferences, such as those arising from isomeric and isobaric compounds. However, the ability of MS/MS to discriminate such interferences is lost when multiple analytes yield similar precursor and product ions.[18] This limits the utilization of FIA-MS/MS to analytes whose precursor → product ion transitions are unique, unless combined with some level of chromatographic separation as discussed below.

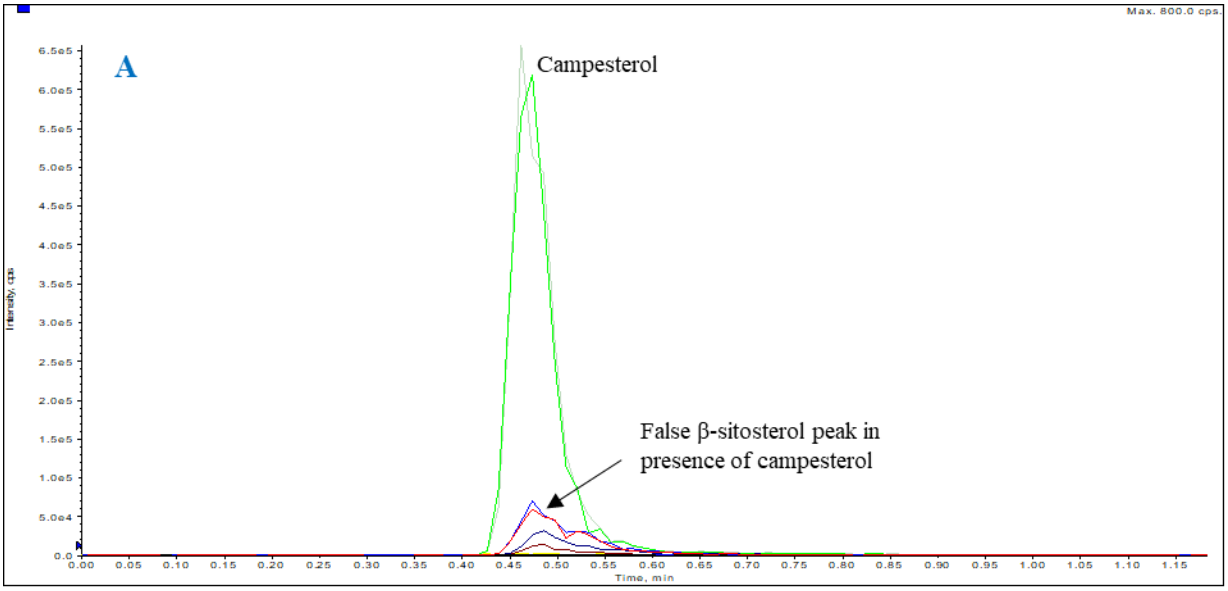
Analyte-to-analyte interferences are common in MS analysis, particularly when dealing with isomers, isobars, and structurally similar compounds. Isomers and isobars may interfere with analyte quantification due to the their intrinsic similarities in ionization and fragmentation, producing identical *m/z* values to that of the analyte, whereas structurally similar compounds may also undergo in-source fragmentation that leads to the formation of a precursor ion of another compound which may be the actual analyte of interest.. For example, during the LC-MS/MS analysis of chlorophenoxy acid herbicides, similar MRM transitions originating from different herbicides were observed.[19] A chromatographic separation was needed to eliminate any false identification and quantification. For complex matrices, in-source fragmentation is a major source of inaccuracy and it poses a major issue in FIA method

utilization. In fact, metabolite(s) can undergo in-source fragmentation to yield another metabolite, such as the generation of nucleotide-diphosphates from nucleotide triphosphates, triose-phosphates from hexose-phosphates. Similarly, O- and N-glucuronides may convert to parent compound[20, 21] during ionization. Such in source fragmentation will result in erroneous identification and quantification of the target analyte(s).

Impurities present in reference standards may also result in analyte-to-analyte interference and compromise the selectivity of the analytical method. Interference from minor impurities in the internal standards was found to cause an interference >20% of the LLOQ, requiring re-optimization of the method where alternate internal standard or changing the LLOQ was evaluated.[22] Regulatory guidelines stipulate that interferences should not exceed 20% and 5% of the LLOQ for the target analyte and IS, respectively.[10, 11] In fact, reference standards even when reported at high purity may contain trace-level impurity of other reference standards. Therefore, selectivity should be evaluated by using the highest concentration for each reference standard prepared individually and monitoring all the transitions to be used during measurement. This way, false signals will be easily identified to ensure the accuracy of the method.

Chromatographic separation demonstrated the ability in eliminating interferences during quantitative analysis.[21, 23] For example, during an ultra-high-performance liquid chromatography (UHPLC)-MS/MS analysis of triterpenols and sterols in sediments, isomers sharing similar MRM transitions were chromatographically resolved[24] and would have otherwise remained unresolved in FIA-MS. As such, to maintain some separation when FIA-MS fails in achieving the desired outcomes, a shorter column can be used. Typically, this is a guard column and it can be argued that the use of a guard column constitutes a modified version of FIA-MS;[25] the terminology is more commonly referred to as “fast chromatography”.[26, 27] Using a guard column provides a compromise between combining the simplicity and high throughput of FIA-MS with the capability to distinguish interferences with chromatography.

Recently the use of a FIA-MS/MS method for the simultaneous determination of tocopherols and plant sterols was implemented to simplify an LC-MS/MS approach.[28, 29] However, interferences were observed among the plant sterols during FIA-MS/MS. For example, β-sitosterol ionizes as $[M + H - H_2O]^+$ at *m/z* 397 but a similar ion is also formed from the in-source ionization of campesterol $[M + H - 4H]^+$. Due to the structural similarities, the MRM transitions of both ions are exactly the same and the presence of campesterol can lead to false identification of sitosterol (Figure 2A). Utilizing the chromatographic separation through a guard column, however, allows for the utilization of "retention time" to identify analytes, as shown in Figure 2B. The separation was considered suitable for the intended application, while still achieving a fast analysis time of less than 1 minute. This illustrates that when two transitions lead to false positives, an additional dimension of selectivity is required. It is, therefore, expected that fast chromatography-MS will be highly explored in the future as it combines the advantages of both FIA and chromatographic separation. We anticipate it to become an additional mainstream method for quantitative analysis.



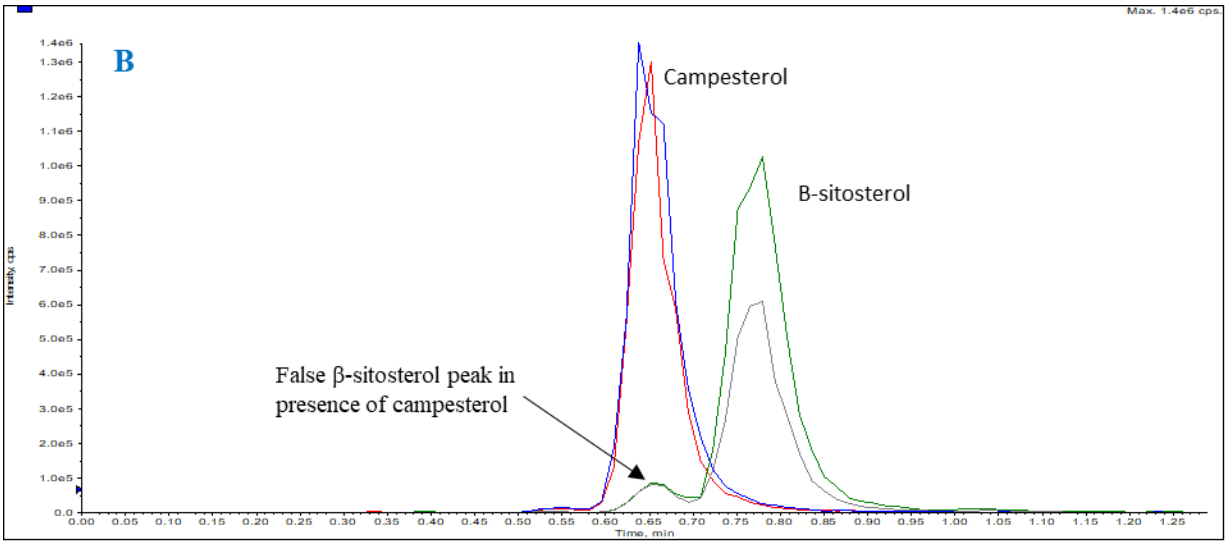


Figure 2. A) Interference from campesterol shows a false β-sitosterol peak and would be indistinguishable from the real peak due to co-elution in case of FIA-MS/MS. **B)** Fast chromatography-MS/MS showed successful separation and the false peak is distinguishable from β-sitosterol peak.

Another dimension that can effectively allow for the discrimination of false positives is the utilization of multistage MS analysis in a quantitative mode. FIA-MS/MS/MS (FIA-MS^3) was effectively utilized in identifying and quantifying two isomeric compounds (methamphetamine and phentermine) in a blood sample.[30] Using MS^3, selectivity is enhanced, allowing for compound unique second generation product ions. Similarly, the incorporation of high-resolution mass spectrometry (HRMS) will further introduce an additional level of selectivity through accurate mass measurements and FIA-HRMS will be highly effective in discriminating interferences from isobaric compounds. HRMS and HRMS/MS were successfully employed to resolve and identify isomeric and isobaric compounds in biological samples in conjunction with LC.[31, 32] Thus, HRMS enhances selectivity leading to accurate identification and quantification. FIA-HRMS was successfully applied in identifying and detecting target and suspect psychoactive compounds in recreational drugs on the basis of accurate mass measurements.[33] In

addition, FIA-HRMS was employed in the quantitative analysis of multi-analytes in tobacco and in enzyme kinetic studies to monitor the successive phosphorylation of thymidine.[8, 34]

A major setback in the utilization of FIA-MS is matrix effects, and whether it's through ion enhancement or suppression, it compromises the analytical data.[35] Similar to LC-MS, using suitable internal standards, can correct for matrix effects to ensure that the analytical method is accurate and reliable.[36] Stable isotope labeled internal standards (SIL-IS) are highly preferred because they behave similar to the target compounds, whereas the behavior of structural analogues might not exactly mimic that of the target analytes in MS. In addition to the use of SIL-IS , efficient sample preparation can minimize matrix effects since most of interfering compounds are removed. To further enhance the applicability of FIA-MS, it is highly recommended that intra- and interlaboratory comparisons be performed. Such evaluations will allow for the assessment of method reproducibility and support the adaptation of FIA-MS for quantitative analysis. Siskos et al.[37] reported a high interlaboratory reproducibility when FIA-MS/MS normalized results on metabolomic analysis in human serum and plasma across six different laboratories were compared.

The vast majority of FIA-MS methods utilize ESI as ionization source. However, the use of other ionization sources for quantification was also reported, including the use of the second most widely used soft ionization method, namely MALDI-MS.

3) MALDI-MS

The use of MALDI-MS as a quantitative tool is usually overlooked, especially for small molecules where the matrix interferes to a large extent in the analysis by forming intense cluster ions in the low *m/z* range (commonly termed "chemical noise"). However, MALDI-MS has significant advantages as it offers a simplified sample preparation, the formation of predominantly singly charged ions, substantial tolerance for additives and salts, and a much faster speed of analysis (i.e., higher throughput).[38-40] In

fact, validated quantitative MALDI-MS methods for single analytes exist in the literature.[41-43] A targeted MALDI-time of flight (ToF) validated method was used to quantify a drug delivery agent within a cell culture.[26] The method was robust, simple and fast and it was superior to the gold standard LC-MS/MS in terms of sensitivity, linear range, solvent consumption and speed of analysis.[4] In comparison with LC-MS/MS, the time of analysis for 100 samples was only 8 minutes (versus over 11 hours) and the amount of solvent consumed was over 20 times less.[26] However, seven matrices were tested prior to selecting a suitable one, and this is a bottleneck during method development. The challenge in identifying a suitable matrix highlights the difficulties that could be encountered when using MALDI-MS for multianalyte analysis. The sample spotting technique is also critical factor in MALDI analysis.[44, 45] These are experimental in nature and should be tested prior to adopting a sample/matrix spotting strategy. Common spotting strategies include: a) mixing the analyte and matrix solution then spotting (dried droplet method); b) spotting the matrix first, dry then spot the analyte/matrix solution (two layer method); c) spotting the analyte first, dry, then spot the matrix (bottom-layer method); d) placing the sample between two matrix spots (sandwich method); and e) homogeneously spray-coating an entire target plates with matrix.[44-46]

In addition, significant sample preparation, in many cases, are needed including the use of antibody purification, for example, to measure anthrax lethal toxin.[41] In addition to small molecules, immunoaffinity MALDI-MS (iMALDI-MS) can be applied for the accurate quantification of peptides. It is a promising approach that widens the use of MALDI-MS to lower molecular weight compounds and peptides. Target peptides can be selectively captured from biological matrices for subsequent quantification. This approach allows for specific detection with an enhanced sensitivity (attomole levels) and is ideal for clinical diagnosis.[47, 48]

To expand the use of MALDI-MS towards multianalyte analysis, new matrices should be developed that allow for the analysis of small molecules with minimal chemical background interference. Various

strategies have been adopted including systematic searches for matrix chemicals with higher matrix suppression effects, such as the lipid matrix 4-phenyl-α-cyanocinnamic acid amide (Ph-CCA-NH2).[49] Other approaches include the use of nanoparticle matrices with high molecular weights, or immobilizing conventional matrices on nanoparticle or polymeric surfaces.[50, 51] Both metal- and carbon-based nanoparticles have been evaluated. For example, gold nanoparticles were shown to enhance the analysis of small organic molecules with diverse polarities, such as amino acids, carbohydrates, nucleosides, and nucleic bases.[52] Gold nanoparticles were also surface-modified with α-cyano-4-hydroxycinnamic acid (CHCA), a conventional matrix, for the effective analysis of protein digests.[53] Another class of carbon-based nanoparticles are nanodiamonds (NDs), and are mainly used for sample preparation and clean-up in MALDI-MS analysis. Particularly, they facilitate the isolation of biomaterials, such as carbohydrates and proteins, prior to MALDI-MS analysis.[53, 54] In such applications, NDs are used in conjunction with conventional matrices. The use of NDs alone, as potential matrices for carbohydrates was demonstrated in 2010[55] and boron doped NDs was subsequently tested for metabolites[56], showing promising MALDI-MS qualitative results. The first report of the utility of functionalized NDs for the analysis of small molecules demonstrated the promise of such novel matrices for the analysis of pharmaceuticals as background noise was reduced, while analyte signal was enhanced[57]. Notably, this work revealed that: a) tested pharmaceuticals were favorably ionized in the negative ion mode, which is desirable when using biological samples as interferences from endogenous compounds are minimized in comparison with the more widely used positive ion mode; and b) coating the NDs with an amino acid (lysine) surprisingly surpassed the performance of NDs covered with some conventional matrices, probably due to the presence of the amine functional group.[57]

The above developments are qualitative in nature but quantitative data using nanoparticles has emerged recently[58, 59], albeit in non-validated methods. This highlights the need for additional research to move the use of nanomaterials as MALDI matrices towards the validated quantitative sphere.

Despite its potential, MALDI-MS's current major quantitative impact is in MALDI-MS-based pharmacological assays and it is rapidly evolving in pharmaceutical R&D and in chemical biology. MALDI-MS has shown sufficient sensitivity in quantitative methods for single-analyte assays[26] whereas relative quantification of MALDI-MS enables the generation of pharmacological concentration-response curves and the calculation of pIC50s (negative log of the IC50) even in intact cells.[60] The fast analysis time of high-speed MALDI-TOF mass spectrometers are ideally suited for high-throughput screening in a 1536-well format[38, 61] as well as for mechanistic cell-based assays for the profiling of drug-like compounds.[62] As stated earlier, MALDI-ToF-MS-based assays require careful optimization of the choice of the matrix, the buffer components and the sample preparation method[62-64] alongside the use of proper internal standards. Finally, such assays allow for the verification of signal identity by accurate mass determination and MS/MS analysis.[62]

The most promising impact of MALDI-MS as an analytical research tool, however, falls within mass spectrometry imaging (MSI). MALDI-MSI is currently a main MS imaging technology that has wide applications in biomedical and life sciences.[65] However, MALDI-MSI is facing major challenges in terms of data reproducibility, especially for quantification. As discussed below, the lack of a homogeneous "blank" matrix as well as high and often variable ion suppression effects in tissues[66] demand utilizing new innovative approaches to make MALDI-MSI a routine quantitative tool.

3.1) MALDI -mass spectrometry imaging

Quantitative MALDI-MSI (qMSI) is primarily making an impact in the pharmaceutical R&D industry; however, it is also used for clinical biomarker studies.[65, 67, 68] The ideal qMSI experiment would combine high spatial resolution, high speed, high sensitivity and high molecular specificity ("4S-criteria for performance" [65]) (Figure 3A, B) without chromatographic separation.

In recent years, significant progress in MALDI-MS instrumentation has been achieved with respect to the speed of analysis[69, 70] and enhanced molecular specificity, namely improved resolving power, mass accuracy and/or orthogonal intra-instrument separation, such as those achieved by trapped ion mobility (tims)-ToF.[70, 71] In addition, instrumentation advancements resulted in higher spatial resolution[71-73] and higher sensitivity.[71, 73-75] . While most commercial instruments support spatial resolutions of 20 µm, some recently published MALDI-MSI experiments offer step-sizes or pixel-sizes of <2 µm.[73, 76, 77] However, the ability to detect analytes decreases by the square of the step-size. As such, sample preparation methods for very small crystals (e.g., sublimation), required for sub-micron MSI, often do not allow for high sensitivity. Specificity has also been enhanced by optimizing analytical conditions to target a specific subclass of compounds. For example, the utilization of metal assisted laser desorption ionization enhanced the selective ionization of target compounds depending on the metal being utilized. The use of silver and gold assisted laser desorption showed favorable ionization for lipids, particularly cholesterol, fatty acids, and triglycerides.[78, 79] Such optimization allows for the successful utilization of MALDI imaging for specific targets to answer specific biological questions.

Despite these advancements, key challenges still limit the utility of MSI as a quantitative tool. First, selective ionization and compound aggregation can severely limit the number of observed analyte species[80] even when high-field Fourier transform ion cyclotron resonance mass spectrometers (FT-ICR MS) are used. Second, the dependency of analyte desorption/ionization characteristics for various molecular classes on laser wavelength and physicochemical properties of MALDI matrices are still poorly understood.[49, 81] Finally, ion suppression is non-homogeneous within heterogeneous tissues[66, 82], posing a significant challenge for data reproducibility. For example, high signal variability (≈35%) was observed during the analysis of propranolol in brain and kidney tissues due to varying chemical and morphological environment in the tissue.[66] Such quantification and reproducibility issues can be minimized by applying tissue-specific ionization factors.[83] The approach involves homogeneously spray-coating of the imaged

tissue or a reference tissue (e.g., liver) with IS. In other words, either an off- region of the imaged tissue or a homogeneous reference tissue is sprayed with IS. The resulting MALDI signal of the sprayed IS is then used to normalize MSI data, correcting for tissue-specific or region-specific ion suppression. Such normalization has been adapted and is termed tissue extinction coefficient (TEC).[66, 82, 84, 85]

Most current qMSI workflows employ both the use of stable isotope-labeled internal standards (SIL IS) for pixel-by-pixel normalization of analyte signal and the use of a calibration function derived from MSI of various concentrations of the target analyte that are co-imaged with the sample tissues (Figure 3C).[65, 86, 87] Early studies in pharmaceutical R&D had suggested agreement between MALDI imaging-derived drug quantities and those obtained by conventional LC-MS/MS. This was even true before MALDI-qMSI workflows that used SIL IS as well as spotted dilution series of an analyte for calibration were utilized.[66, 88-90] In a recent comprehensive clinical pharmacology study, 78% of all drug levels determined by qMSI were within a two-fold window of concentrations determined by LC-MS.[67] Despite these promising results, the reported variations may prove problematic within regulatory guidelines in which much lower variations are expected. In fact, it is suggested that when repeating a measurement of experimental samples that the incurred sample re-analysis should be within ±20% of the mean for at least 67% of the repeats.[91]

Future work is expected to further improve accuracy and precision in qMSI by creating strategies for homogeneous coating of matrix with SIL IS as well as by including additional external and internal controls. Condina et al.[92] demonstrated the use of egg white as an external control to monitor detector performance and sample preparation for MALDI MSI of peptide and N-glycan in gynecological tissue. Although mouse liver or gelatin solutions was successful candidates as controls, egg white was chosen because it is readily available, easy to prepare, and capable of supporting multiple MALDI MSI analyses. The Quality of the quantitative data in MALDI-qMSI can be further improved, in some occasions, by the

use of non-linear instead of linear calibration functions,[67] or by innovative computational methods, such as the use of chemical noise background for mass alignment and calibration.[93].

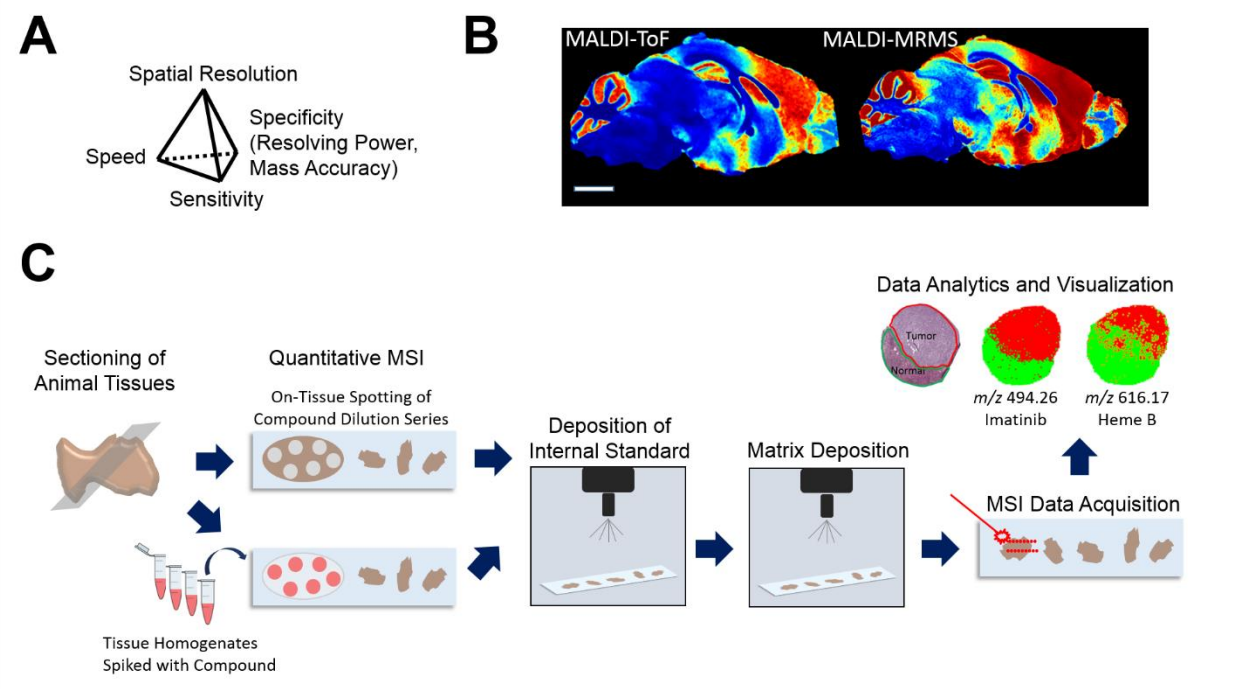


Figure 3. A, "The 4S criteria of MALDI imaging performance". Besides **s**peed, **s**patial resolution and **s**ensitivity, high-quality MALDI Imaging experiments require high molecular **s**pecificity, provided by high resolving power and high mass accuracy; **B**, MALDI-magnetic resonance mass spectrometry (MRMS) offers very high molecular specificity exemplified for the sulfatide SM4s (d18:1;24:1) $[M-H]^-$ (*m/z* 888.6240; scale bar 2 mm), Reproduced with permission from ref.94 Copyright (2019), Bruker; **C**, Typical quantitative MALDI Imaging workflow: For calibration, a dilution series of a target compound is either spotted onto calibrant tissues or spiked into tissue homogenates that are evaluated on the same slide as the respective sample tissues. Internal standards, often deuterated analogs of the target compound, are deposited before or with the MALDI matrix prior to image data acquisition (MALDI images of imatinib and heme distribution in comparison with H&E staining are reproduced with permission from ref. 67.

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Other desorption-based ionization methods such as desorption electrospray ionization (DESI) and direct analysis in real time (DART) have been used for quantitative analysis.[95, 96] However, they have limited analytical capabilities within the quantification sphere due to the nature of the sample that are placed outside of the MS instrument. Small variations in the angles of sample position, for example, will have profound impact on the quantitative analysis, and such a limitation is particularly concerning if the analysis is intended for clinical applications. As such it is not surprising that validated quantitative methods using either DESI or DART are lacking in the literature. In a rare direct comparison, however, it was shown that DESI-MS was substantially inferior in terms of linear range and sensitivity to four different MS-based quantification modes, such as LC-MS, MALDI-MS and FC-MS.[26]

Regardless of the ionization method being used, ion mobility spectroscopy (IMS) is gaining momentum for advancing MS-based quantitative analysis. Selective IMS devices, such as FAIMS, are the most suited for quantitative analyses, aiding in the elimination of tedious chromatographic separation as discussed below.

4) Ion mobility

Since ion mobility devices separate ions in the gas-phase based on principles different from mass spectrometry, they have been used to simplify quantitative analyses (e.g., reduction of chromatographic run times).[97] This also makes them particularly important companions for FIA-MS/MS, as they help overcome limitations due to isobars, isomers, and endogenous interferences. Ion mobility devices can be broadly grouped into two types, dispersive and selective.[98] Dispersive devices aim to detect all ions present in a single pulsed event (e.g., a drift tube that separates ions in time), whereas selective devices only allow a subset of ions to continually transmit. Since triple quadrupole instruments are typically

used for quantification because of their high sensitivity, the continuous nature and high sensitivity of selective ion mobility devices makes them ideal interfaces for this instrument. Selective devices are based on differential ion mobility spectrometry (DMS)[6, 99] and use a voltage to select the subset of ions to transmit. Of the two most common DMS devices, planar DMS devices offer the potential for the best separation, however FAIMS devices are the most sensitive because the cylindrical electrodes enable the unique advantage of ion focusing at atmospheric pressure.[100, 101] Since sensitivity is critical in quantification, the use of FAIMS is therefore the focus of this section. Furthermore, separation capabilities are adjustable using electrode temperatures, which alter the electric fields (or more correctly the electric field/gas number density, E/N) in FAIMS. By selecting temperatures that allow for just enough separation, sensitivity can be maximized.[102, 103] This point is illustrated in Figure 4 that shows compensation voltage (CV) scans obtained using direct infusion of vicine and convicine extracted from faba bean.[102] Since vicine containing one ^{13}C isotope will interfere with convicine quantification (both use the 306 → 144 transition), FAIMS was used to separate these ions prior to entering the mass spectrometer (note the small m/z 306 peak at the same CV as vicine is due to vicine that contains one ^{13}C isotope). The Figure illustrates the peak separation/sensitivity trade-off; 60 °C was used for the analysis since there is sufficient baseline separation which maximizes sensitivity.

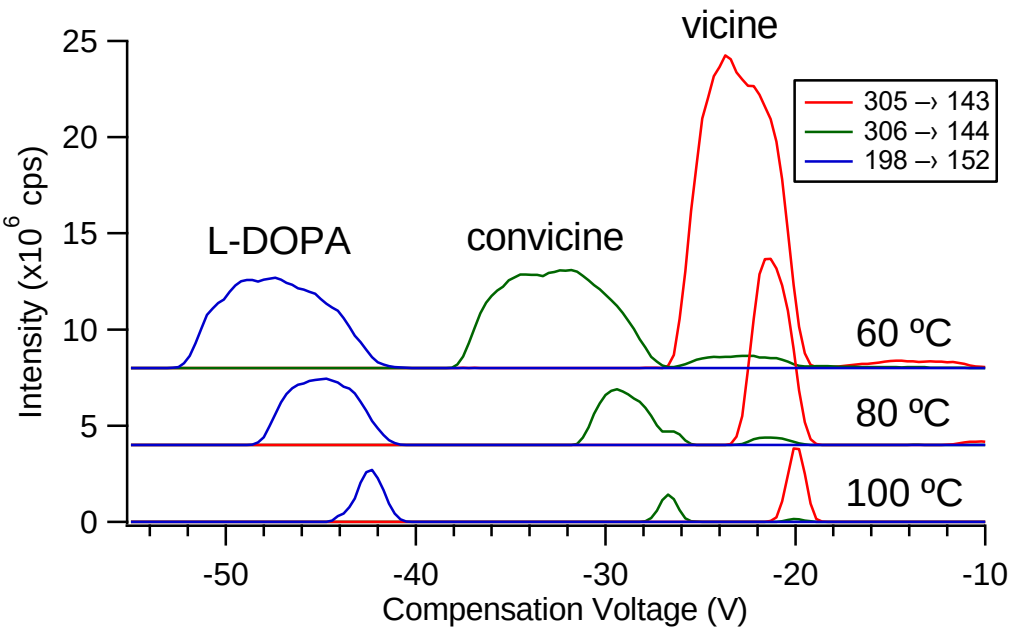

Figure 4. Adjusting peak separation of vicine and convicine by changing inner electrode temperature (and E/N) in FAIMS. A temperature of 60 °C was chosen for the analysis since this gives maximal signal intensity with sufficient peak separation.

Often this fine adjustment of selectivity is sufficient to eliminate interferences or reduce chemical background, but a more coarse adjustment of selectivity involves the addition of modifiers (e.g., acetonitrile & methanol) to the gas-phase to produce very different CV spectra due to the ion-dependent interactions occurring with the modifiers in the device.[104-106] Improved understanding of these mechanisms is progressing[107, 108] and will help harness the separation power of using these modifiers in FIA-FAIMS-MS/MS applications in the near future.

Ion transmission time through most FAIMS/DMS devices is typically 20-25 ms, which means that a change in CV requires this time before desired ions are detected at the new CV value. Since peak widths in FIA can be short, the number of analytes that can be detected based on the number of CV switches and the mass spectrometer dwell time is limited. When a large number of analytes are used, the number of CV values should be minimized since ions using the same CV value will not require the CV

change time. Employing wider peaks by adjusting electrode temperatures is desirable to limit the number of CV values, as long as the required separation is maintained

The potential of the first generation FAIMS was illustrated through several applications.[7] For example, eliminating the effect of in-source fragmentation of an N-oxide metabolite (fragment is the same as the original compound) on quantification using fast LC-MS/MS[109], which uses merely a guard column. Note that fast LC is often used instead of FIA in ESI applications in which ion suppression is an issue, since FAIMS is after ESI; as such, it does not help in reducing ion suppression in the ESI source. However, issues with residence time, robustness, sensitivity, and the need for helium restricted routine use of this device.[110] Turbulence in the FAIMS interface region was a primary factor for robustness and sensitivity issues and this region was redesigned using a novel aerodynamic mechanism referred to as the "Coandă Effect".[110] In addition to reducing gas flow turbulence, this redesign prevented ions from preferentially striking the inner electrode in FAIMS. Thus, a smaller gap between electrodes was implemented, which resulted in shorter residence times and higher electric fields (thereby eliminating the need for helium).[110] This redesigned second generation FAIMS (i.e., FAIMS Pro), which therefore offers several advantages over the first generation FAIMS was recently released for proteomic applications (Aug. 2018) and is quickly becoming widely used for proteomic analyses.[111, 112] Once the second generation FAIMS is released for small molecule applications, the improved sensitivity, robustness, and usability should also make it more mainstream in quantitative analyses of analytes with interferences or suffering from high chemical background noise.

5) Conclusions and future perspectives

Mass spectrometry will remain central for quantitative assays in the foreseeable future, primarily within hyphenated techniques. Its various analytical capabilities, however, allow for the elimination of the separation part as discussed above. The examined advancements will continue to grow and cement the role of mass spectrometry within the quantitative sphere as a stand-alone technology. In fact,

combining multiple strategies such as ion mobility with FIA using HRMS analysis will add multiple layers of selectivity and "separation" that indeed will allow MS alone to increasingly invade the multianalyte quantification sphere. Cost and expertise are deterrents; adding ion mobility to an MS instrument, for example, adds cost and a need for additional analytical expertise that is familiar with the power of ion mobility when combined to MS for quantitative workflows.

While ESI will remain a central ionization technique for quantitative assays, MALDI-MS has many advantageous features when used for quantification. The key, however, for quantifying multiple small molecules is finding an optimal matrix that generates minimal interferences at the lower mass range. Efforts should be placed into developing such new matrices that will facilitate the use of MALDI-MS for small molecules in a quantitative fashion. In addition, quantitative MALDI-MS in the imaging sphere has great potential for biomarker and drug discovery as well as understanding the molecular basis of many diseases. qMSI has many layers of challenges, particularly reproducibility. Only multi-center efforts such as those spearheaded by the MALDISTAR initiative can address such a pressing concern (https://www.maldistar.org/). MALDISTAR project aims at improving standardization and reproducibility in MALDI MSI through quality assessment (developing quality control metrics), standardization (development of calibration and cross normalization methods), and development of a reference software whose algorithm will allow analysis and evaluation of data obtained from different instruments.

An additional key challenge for future clinical use is method validation, demanded by regulatory bodies. Method validation safeguards the robustness of an analytical strategy, ensuring accurate quantification. Without method validation, quantitative data is an "estimate" at best, which may be sufficient for a specific research question at the discovery stage. However, fit-for-purpose will not be acceptable if the quantitative data is intended for clinical or pre-clinical applications, such as the introduction of new drugs or diagnostics. Method validation and development go hand-in-hand as validation criteria not

passing during validation will require innovative approaches at the methodology end. Omitting chromatography, though attractive, will create challenges as more of the sample matrix will compete with the analytes during ionization and as such more sample preparation and clean up steps may be needed. In summary, there is a need to place more efforts into method validation for quantitative methods that omit chromatography. Only then, can MS alone become a mainstream quantitative technology within the clinical sphere.

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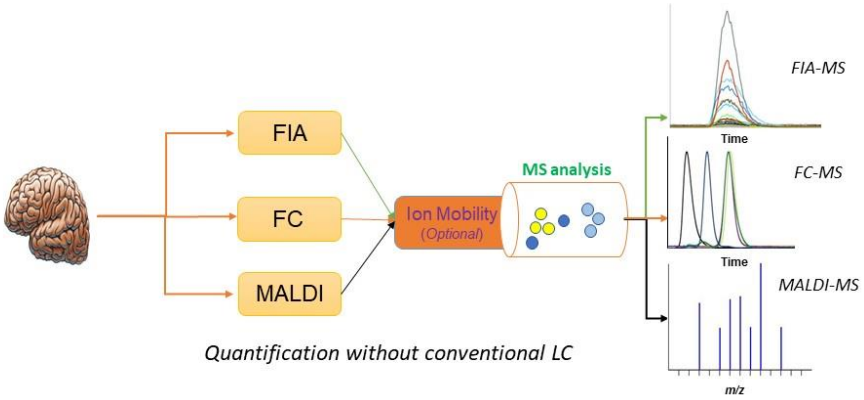

Quantification without conventional LC