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Mass spectrometric recommendations for Quan/Qual analysis using liquid-chromatography coupled to quadrupole time-of-flight mass spectrometry

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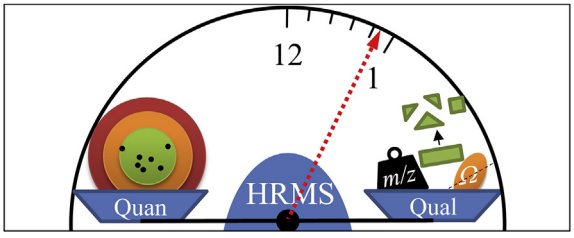
HIGHLIGHTS

- A widely applicable Quan/Qual method using high-resolution MS is proposed.
- Resolution, scan mode, scan rate and smoothing affect Quan/Qual performance.
- Recommendations are provided for future Quan/Qual method development.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: High-throughput simultaneous quantitative and qualitative (Quan/Qual) analysis is attractive to combine targeted with non-targeted analysis, e.g. in pharmacometabolomics and drug metabolism studies. This study aimed to investigate the possibilities and limitations of high-throughput Quan/Qual analysis by ultra-high performance liquid chromatography (UHPLC) coupled with high-resolution mass spectrometry (HRMS), to develop a widely applicable Quan/Qual UHPLC-HRMS method and to provide recommendations for Quan/Qual method development.
Methods: A widely applicable 4.25-min UHPLC method for small-molecules was used to investigate and optimize mass spectrometric parameters of a Synapt G2S for Quan/Qual analysis. The method was applied on a rat metabolomics study investigating the effect of the fasting state and administration of a dosing vehicle on the rat plasma metabolic profile.
Results: Highly important parameters for high-throughput Quan/Qual analysis were the scan mode and scan rate. A negative correlation was found between the amount of qualitative information that a method can provide and its quantitative performance (accuracy, precision, sensitivity, linear dynamic range). The optimal balance was obtained using the MS^E scan mode with a short scan time of 30 ms. This 4.25-min Quan/Qual analysis method enabled quantification with accuracy and precision values $\leq$ 20% at the lowest quality control (QC) level and $\leq$15% at higher QC levels for 16 out of 19 tested analytes. It provided both parent *m/z* values and fragmentation spectra for compound identification with limited loss of chromatographic resolution and it revealed biologically relevant metabolites in its application to the metabolomics study.
Conclusion: Quan/Qual method development requires balancing between the amount of qualitative data, the quality of the quantitative data and the analysis time. Recommendations are provided for MS

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resolution, scan mode, scan rate, smoothing and peak integration in Quan/Qual method development and analysis.

1. Introduction

High-resolution mass spectrometry (HRMS) has become increasingly popular over the last decade for use in quantitative bioanalysis [1]. It offers the possibility to do simultaneous quantitative and qualitative (Quan/Qual) analysis, which is attractive for studies combining targeted and non-targeted analysis, such as those investigating the absorption, distribution, metabolism and excretion (ADME) of a (radiolabeled) drug and pharmacometabolomic studies. Today, Quan/Qual analysis applying HRMS is still mostly applied in non-regulated early stage drug discovery and metabolomics. It is expected, however, that its use will expand to regulated bioanalysis [2], where currently the triple-quadrupole mass spectrometer (QqQ-MS) still runs the show. In order to compete with the contemporary short UHPLC-QqQ-MS analysis methods for large scale studies, a Quan/Qual analysis method needs to be fast and sensitive, and this represents a challenge for the high resolution mass spectrometer. Various Quan/Qual methods have already been published (e.g. Refs. [3,4]), but this work does not only provide a Quan/Qual method that is widely applicable for targeted and non-targeted analysis of small molecules, it focuses on the mass spectrometric challenges encountered when applying Quan/Qual in a high-throughput fashion and provides recommendations how to deal with these challenges. In addition, the applicability of the method is demonstrated in a metabolomics study.

Previously, we evaluated various sub-2 μm particle size ultra-high performance liquid chromatography (UHPLC) columns and mobile phases to develop a fast (4.25 min) and easy-to-use chromatographic method with an excellent chromatographic resolution [5]. In the present study, we aim to evaluate how to optimally use HRMS to achieve reliable quantification and collect qualitative information applying this chromatographic method, minimizing compromises to its chromatographic resolution.

Various mass spectrometers of several vendors are being or could be used for Quan/Qual analysis. Examples are the TripleTOF® series from Sciex, the Synapt® and Xevo® series from Waters, the 6500 series QTOF LC-MS and 6200 series TOF LC-MS systems from Agilent Technologies, the LCMS-IT-TOF MS from Shimadzu, the compact, impact™ and maXis™ series ESI-QTOF Instant Expertise™ mass spectrometers from Bruker and the Q-exactive™ from Thermo Scientific, each with their own advantages and limitations [6]. In the present study, we used the Synapt G2S mass spectrometer (Waters). An advantage of this instrument is the variety of scan modes, of which TOF-MS, MS^E and $HDMS^E$ provide an increasing amount of qualitative information. In the TOF-MS mode, the collision energy is at a fixed low level in order to detect the parent ions. In the MS^E mode, low and high collision energy scans are alternated to provide both accurate parent ion masses and data-independent fragmentation spectra of all ions. In the $HDMS^E$ mode, ion mobility separation is applied, providing an additional dimension of separation and an additional identifier of an ion, i.e. the collisional cross section, which is related to the drift time.

To obtain our goals, first the MS resolution mode and scan rate of a Synapt G2S mass spectrometer were optimized to accommodate the high-throughput Quan/Qual analysis. Then the TOF-MS, MS^E and $HDMS^E$ scan modes were evaluated for their quantitative performance in terms of accuracy, precision, linear dynamic range and sensitivity, using a test set of 19 small-molecule drugs, selected for their diversity in molecular masses (151–749 Da), hydrophobicity (log P of 0.91–6.7) and pKa values (ranging from an acidic pKa of 3.77 to a basic pKa of 9.68). The qualitative performance of the MS^E and $HDMS^E$ mode were evaluated based on an *in-vitro* drug metabolism study.

An ideal example application of the developed Quan/Qual analysis method is to simultaneously investigate drug metabolism and changes in endogenous metabolism related to the drug administration. The problem in this use is however that *in vivo* ADME studies often lack (appropriate) control samples and make use of generic blank control samples. In this case, drug metabolites may still be identified based on radioactivity measurements (in case of radiolabeled mass balance studies) or fragmentation patterns. However, differences between endogenous metabolite levels which changed upon drug administration are more complicated to find and may include many false positives that are only related to e.g. fasting state or to a dosing vehicle. In this perspective, Fiebig et al. recently recommended the use of control samples of the same gender and intravenously dosed with the same dosing vehicle, for ADME studies in rat [7]. Here we demonstrate the applicability of the Quan/Qual method for metabolomics studies in a similar set-up, investigating differences in rat plasma metabolome caused by a dosing vehicle, the fasting state or by bench instability (i.e. short-term storage at room temperature). The results show that the developed method is not only fast and widely applicable, but also capable to provide biologically relevant information.

2. Experimental

2.1. Chemicals

Water was obtained from a Milli-Q Purification System from Millipore (MA, USA). Acetonitrile, methanol (both Ultra LC-MS grade) and isopropanol (HPLC-MS grade) were supplied by Actu-All Chemicals (Oss, the Netherlands) and dimethylsulfoxide (DMSO, ≥99.7%) and formic acid (ULC-MS grade) by Biosolve (Valkenswaard, the Netherlands). Ammonium acetate (>99%) and the drug product standards of acetaminophen, tolbutamide, 19-norethindrone, omeprazole, prednisone, buspirone hydrochloride, (+/−)-verapamil hydrochloride, nefazodone hydrochloride and loperamide hydrochloride originated from Sigma-Aldrich (St. Louis, MO, USA). Abiraterone was supplied by Cambridge Major Laboratories, Inc. (WI, USA). Cilag AG (Schaffhausen, Switzerland) provided darunavir ethanolate and midazolam was obtained from Actavis (Dublin, Ireland). Janssen Research and Development (Beerse, Belgium) supplied the drug product standards of galantamine hydrobromide, rilpivirine, risperidone, bedaquiline and simeprevir. An overview of the physicochemical properties of the compounds is provided in Supplementary Table 1. The internal standards acetaminophen-d4, tolbutamide-d9, galantamine-*O*-methyl-d3, risperidone-d4, verapamil-d6 hydrochloride and bedaquiline-d6 (mixture of diastereomers) and the metabolites 1′-hydroxy midazolam and 4-hydroxy midazolam were obtained from Toronto Research Chemicals (Toronto, Canada).

2.2. Preparation of standard solutions

Stock solutions of each drug product (2 mg mL^{-1}) and internal standard (0.4 mg mL^{-1}) were prepared in DMSO except for midazolam, which was purchased as a 5 mg mL^{-1} saline solution. For the optimization of mass spectrometric settings, a 4 μg mL^{-1} mixture of all drug products in DMSO was used and diluted to the desired concentration with a final composition of DMSO:acetonitrile:water of 1:6:7 (v/v/v). Working solutions for the calibration standard (CS) and QC samples were prepared from separate primary solutions containing 100 μg mL^{-1} of prednisone and darunavir and 50 μg mL^{-1} of the other 17 drug products in DMSO, which were further diluted in DMSO to the desired concentrations.

2.3. Chromatographic conditions

Chromatography was performed using an Acquity UPLC system (Waters, Milford, MA, USA) equipped with a binary solvent manager, sample manager and column oven. Previously investigated chromatographic conditions for optimal peak capacity were applied in this study [5]. Briefly, a Cortecs UPLC C18 column (50 mm × 2.1 mm, 1.6 μm, Waters, Etten-Leur, the Netherlands) was used, connected to the injection valve with a pre-cut 550 × 0.127 mm Viper tubing with fingertight fitting (Thermo Fisher Scientific Inc., Waltham, MA, USA) and to the ion source with 470 × 0.102 mm peek tubing. Volumes of 1 μL were injected using the partial loop needle overfill mode. The weak and strong needle wash solution consisted of 5% methanol in water and methanol:water:isopropanol:formic acid 40:40:20:0.1 (v/v/v/v), respectively. At 45 °C, gradient elution was applied with a flow rate of 0.9 mL/min, starting at 99% mobile phase A (10 mM ammonium acetate in water) and 1% mobile phase B (acetonitrile:methanol 80:20 (v/v)) for 0.1 min. Subsequently, mobile phase B was increased to reach 90% at 2.25 min, 98% at 2.35 min, where it was kept until 3.25 min and subsequently decreased to reach 1% at 3.35 min until the end of the run (4.25 min).

To reduce carry-over, a shark-tooth shaped gradient was inserted in the washing step between 2.35 and 3.25 min, whereby B changed from 98% at 2.8 min to 20% at 2.9 min and back to 98% at 3.25 min. Additionally, four injection valve switches were programmed, at 2.65, 2.7, 2.75 and 3.3 min.

2.4. Mass spectrometry

A Synapt G2S (Waters, Manchester, UK) mass spectrometer was used with electrospray ionization operating in positive ionization mode. Leucine-enkephalin (*m/z* 556.2771, 0.1 mg mL^{-1}) was used for lock-mass calibration and continuously infused at 20 μL/min. Applied settings for each scan mode used are listed in Table 1. The MS parameters were optimized by both direct infusion and flow injection analysis of the drug mixture. For ion mobility separation, the default settings as advised by the manufacturer were used, with exception of the wave velocity. The wave velocity ramp was set from 1200 m/s to 550 m/s, to give the maximum separation between the smallest and largest molecule in the drug mixture within the 20 to 180 bins of the mobilogram (which is the optimal ion mobility range to maximize separation without experiencing carry-over from slowly drifting ions to the beginning of the next scan). Mass Spectrometric and chromatographic data were obtained with Masslynx software (version 4.1, Waters). Data analysis was performed using Targetlynx, UNIFI (version 1.8, Waters) and Microsoft Excel (MS Office 2010).

2.5. Optimization of mass spectrometric analysis settings

2.5.1. Resolution mode

To compare the resolution modes “sensitivity”, “resolution” and “high resolution”, a 143 ng mL^{-1} solution of the 19 drugs was injected three times per resolution mode applying the Quan/Qual LC-TOFMS method with a scan time of 100 ms. Peak intensities and noise levels (after a Savitzky-Golay smoothing factor of two iterations at a window of one scan), chromatographic peak widths at half maximum of the peak (FWHM, without smoothing) and mass spectrometric peak widths at FWHM were manually determined for three compounds: a low mass (acetaminophen, *m/z* 152.0712), mid mass (verapamil, *m/z* 455.291) and high mass (simeprevir, *m/z* 750.2995) compound. Extraction windows of 0.02 Da around the measured mass instead of the exact masses were applied for all resolution modes to avoid differences in calibration accuracies. The chromatographic peak widths were used to calculate the peak capacity as 1 + FWHM/gradient time, using the gradient time of 135 s. The mass spectrometric peak widths were used to calculate the mass resolution as *m/z*/FWHM.

Table 1
Main mass spectrometry settings. The following settings were used in the TOF MS, MS^E and $HDMS^E$ mode.

MS Mode	TOF MS	MS^E	$HDMS^E$
Setting:			
Capillary voltage (kV)	0.5	0.5	0.5
Sampling cone (V)	40	40	40
Source offset (V)	50	50	50
Cone gas (N_2, L/h)	50	50	50
Desolvation gas (N_2, L/h)	1200	1200	1200
Source temperature (°C)	150	150	150
Desolvation temperature (°C)	600	600	600
MS function 1:		Low energy	Low energy
Trap CE (eV)	4.0	4.0	4.0
Transfer CE (eV)	2.0	2.0	2.0
MS function 2:	NA	High energy	High energy
Trap CE (eV)		15–35 (ramped)	4.0
Transfer CE (eV)		2.0	15–35 (ramped)
IMS settings:			
Trap DC bias			45.0
IMS wave height (V)			40.0
Variable wave velocity			1200–550 (ramped)

MS, mass spectrometry; TOF, Time-of-Flight, MS^E, mass spectrometry with fragmentation of all ions; $HDMS^E$: High-definition (ion mobility assisted) MS^E; CE: collision energy, IMS: ion mobility separation, DC: direct current.

2.5.2. Scan time and smoothing

To test the effect of the scan time on the Quan/Qual method, a 3.6 ng mL^{-1} and a 36 ng mL^{-1} solution of the 19 drugs was analyzed in five-fold applying the Quan/Qual LC-MS^E method with each of the following scan times: 15, 20, 30, 40, 60, 80 and 100 ms. The peak capacity and signal-to-noise ratios were determined using Targetlynx and the precision was calculated as the coefficient of variation (%CV) of the peak areas. Various smoothing factors were applied while monitoring the peak capacity and signal-to-noise ratio and inspecting the chromatographic peak shape to choose a suitable smoothing factor.

2.6. Evaluation of scan modes for quantitative analysis

The quantitative performance of the Quan/Qual method was tested in the TOF-MS mode (with 100 ms and 30 ms scan time), the MS^E mode (with 30 ms scan time) and HDMS^E mode (with 86 ms scan time). Two 16-points plasma calibration sets (including a double blank and single blank) and five levels of plasma QC samples were prepared to accommodate the various measurable ranges of the 19 different compounds. Hereto, human citrate plasma (of a male volunteer) aliquots of 50 μL were spiked with an appropriate volume combination of a CS or QC working solution and DMSO to obtain a final volume of 90 μL. A 10 μL volume of internal standard mixture in DMSO (containing 1.5 μg mL^{-1} of acetaminophen-d4, tolbutamide-d9 and bedaquiline-d6, 150 ng mL^{-1} of galantamine-*O*-methyl-d3 and 300 ng mL^{-1} of risperidone-d4 and verapamil-d6) was added to all samples except the double blank. The CS samples contained 0, 0.5, 1, 2, 4, 5, 10, 20, 40, 50, 100, 200, 300, 450 and 500 ng mL^{-1} and the QC samples 1, 2, 20, 100 and 450 ng mL^{-1} of each drug in plasma, and twice as much for prednisone and darunavir. The 100 μL volumes were vortex mixed (1 min) with 150 μL of acetonitrile and centrifuged (5 min, 4 °C, 13600 g). Subsequently, 200 μL of the supernatant was transferred to a clean tube, vortex mixed (1 min) with 150 μL of water and analyzed.

After LC-MS analysis of the samples, TargetLynx was used for 2D peak integration of the data in all four data sets and UNIFI was used for 2D and 3D peak integration of the data collected in HDMS^E mode. Lists with peak responses were exported to Excel for further calculations.

An internal standard was chosen for each analyte using the QC samples. Hereto, the CV% of the QC samples was calculated for the peak responses of each analyte, as well as for the peak response ratio with each of the deuterated internal standards. The internal standard giving the smallest variation was selected or no internal standard correction was applied if variation was better without IS.

Log/log transformation was applied and linear calibration curves were constructed in Excel by excluding calibration points with a signal-to-noise ratio below 5 or a relative error of more than 20% from the curve. Additionally, if the precision (the CV% of the calculated concentrations) of a QC level exceeded 20%, all calibration levels up to this point were excluded. A calibration curve was accepted only if minimally 6 non-zero calibration levels were included and no more than 25% of the calibration points (>LLOQ) were excluded. QC samples with a peak area ratio more than 1.5 interquartile ranges below the first quartile or above the third quartile were marked as an outlier [8] and excluded in the calculation of the accuracy and precision of the affected compound. To compare the sensitivity between modes, a detection limit was calculated as 3 times the standard deviation of the lowest QC level.

2.7. Evaluation of scan modes for drug metabolite analysis

The qualitative performance of the MS^E and HDMS^E scan modes was evaluated using incubation samples of rat hepatocytes with buspirone. The hepatocytes were treated as described previously [5] and cell suspensions containing 1.0×10^6 cells mL^{-1} were incubated with buspirone at a concentration of 5 μM. Aliquots were taken at t = 0 min (control) and at t = 120 min and immediately stored at −20 °C until analysis. The incubation samples were precipitated with three volumes of acetonitrile, centrifuged for 5 min at 3000 g and the supernatants were injected on the column.

Data analysis was performed in UNIFI. Separate metabolite identification analysis methods were made for the MS^E and the HDMS^E data, but settings were kept the same as much as possible. The 2D peaks were smoothed (Savitsky Golay, half width 1 and 2 iterations). The MS^E method searched for 3D peaks (*m/z*, RT, intensity) and the HDMS^E method for 4D peaks (*m/z*, RT, intensity, drift time), both with a maximum of 100000 peaks per channel. Buspirone was added as expected component, a wide selection of common phase I and phase II transformations was included and the dealkylation tool [9] was activated. Targeted screening was based on retention time (0.1 min tolerance, burspirone only) and mass (20 ppm tolerance, all potential metabolites based on phase I and phase II transformations). Binary comparison was performed with a mass tolerance of 5 ppm and retention time tolerance of 0.05 min. To clean the obtained peak lists, features with any of the following characteristics were filtered out: only found in the t = 0 h sample, not tentatively identified by the software, eluting outside the retention time window of 0.2–2 min (unchanged buspirone eluted at 1.43), having an intensity < 0.5% of the parent, having < 2 identified high energy fragments, having identification anomalies and/or being < 2.5 × as intense in the 120-min sample as in the 0-h sample.

2.8. Application of the Quan/Qual method to a non-targeted study

The Quan/Qual UHPLC-MS^E method was applied to explore the effect of a dosing vehicle on the metabolite profile of a rat. The study procedures were covered by and in conformance with the animal Health regulation of Jansen Pharmaceutica, Beerse, Belgium. Six groups of three Sprague Dawley rats were included. Three of the groups had fasted for 12 h, the other three groups received a standard diet. Of both the fasted and the fed rats, three received a single oral dose of 10 mL kg^{-1} PEG400, three an empty cyclodextrin (20%) vehicle and three were used as a control. One blood sample was collected per rat, 4 h after vehicle administration, and centrifuged to obtain plasma samples. The plasma samples were stored at −20 °C and transported to the Leiden Academic Center for Drug Research. Duplicate 50 μL aliquots of individual samples were prepared, as well as QC pools, comprising equal volumes of each plasma sample. Sample preparation was performed as described for plasma in section 2.6, whereby the internal standard mixture and a CS working solution was spiked to obtain a plasma concentration of 100 ng mL^{-1} of the drug mixture. The QC samples were analyzed in triplicate at the start, middle and end of the analytical run and the individual samples were randomized in-between.

Targeted data analysis for QC was performed using Targetlynx and non-targeted feature detection using Progenesis QI (Nonlinear Dynamics, Newcastle, United Kingdom). The feature detection list from all samples was exported to Excel and filtered: features with a chromatographic peak width <0.01 min, with a CV% > 30% in the QC samples and a retention time outside 0.2–1.51 min were excluded to minimize the number of false positive peaks. statistical analysis which was performed using MetaboAnalyst 3.0 [10], whereby the samples from PEG-dosed rats and the QC samples were excluded because of too much matrix effect. For the data analysis, zero or missing values were replaced with a small value (half of the minimum positive value in the original data). To make features more comparable, the data was transformed using log normalization and

autoscaling was applied. Significance of features was determined using Analysis of Variance (ANOVA). Only features with a p-value < 0.05 were used to construct a principal component analysis (PCA) plot and to determine the most discriminative features between the groups based on their variable importance in projection (VIP) score.

3. Results and discussion

3.1. Optimization of mass spectrometric analysis settings

Important characteristics of an MS method for high-throughput Quan/Qual workflow are sensitivity, chromatographic peak capacity and mass resolution. Additionally, a practical consideration is the file size.

3.1.1. MS resolution

The sensitivity, mass resolution and file size were first tested in the three available resolution modes on the Synapt G2S: the sensitivity mode, the (normal) resolution mode and the high-resolution mode. When the instrument operates in the sensitivity mode or resolution mode, the ions in the TOF section are reflected once (also referred to as V-mode) before reaching the detector. In the high-resolution mode, ions are reflected three times, following a W-shaped path. The increase in flight time in this mode compensates for the distribution in kinetic energy within the ion packages, resulting in an increase of the mass resolution, however at a cost of sensitivity [11].

In the resolution mode, the 143 ng mL^{-1} academic solution of a low-, mid- and high-mass compound gave a median peak intensity of $1.18*10^6$, a signal-to-noise ratio of $1.0*10^4$ and a mass resolution of 25 k. The mean file size was 1.69 Gb. Supplementary Fig. 1 displays the relative differences of the sensitivity mode and the high resolution mode with respect to these values in the resolution mode.

The high resolution mode was characterized by a severely reduced sensitivity, with median peak intensities and S/N being around 300 and 14 times lower, respectively, than in the normal resolution mode. As a logical result, the high resolution mode gave the smallest file size (the number of detected ions is smaller). Despite this advantage, the low absolute peak intensities will hamper the detectability of low concentration compounds, making this mode less suitable for Quan/Qual analysis, especially when low exposure concentrations are expected.

No big differences were observed between the sensitivity mode and the resolution mode. Even though the peak intensity in the sensitivity mode was around 1.6 times the intensity in the resolution mode, the S/N was 14% lower. This can most probably be attributed to the high concentration test sample used. This allowed the comparison of the 3 resolution modes with the same sample, but resulted in detector saturation for some compounds in the sensitivity mode. Consequently, at lower compound concentrations the S/N difference between the sensitivity mode and the resolution mode may be in favor of the first. The mass resolution was very similar instead of the expected two-fold difference, but this highly depends on the tuning condition of the instrument. The file size was largest in the sensitivity mode, as this file is the richest in data points. It was observed (not shown here) that the difference in file size between the resolution modes becomes much more pronounced upon injection of a matrix sample instead of an academic standard, as can be expected since all matrix ions are also included. Considering the wide applicability we aim for Quan/Qual analysis and the similar performance of the modes in this case, but the potential lower mass resolution of the sensitivity mode on other instruments, the (normal) resolution mode was considered optimal for the Quan/Qual workflow and used in further experiments.

3.1.2. Scan time and smoothing

Another important MS parameter in high-throughput Quan/Qual analysis is the data acquisition rate or the scan time. If the scan time is too long, the chromatographic peak contains too few data points to describe its shape, compromising both the precision (invaluable in quantitative analysis) and the chromatographic resolution (essential for qualitative analysis) [12,13]. On the other hand, a short scan time can introduce more variability and can compromise the sensitivity, because less transients can be accumulated per scan, resulting in a spectrum with a lower signal-to-noise ratio [14].

Fig. 1 shows the practical consequences of these theoretical scan time effects on the Quan/Qual UHPLC method. Panel A displays the median of the peak area precision ($n = 5$) over the 19 analytes per scan time and shows no clear correlation between the two at the evaluated concentration level. In contrast, increasing the scan time had a detrimental effect on the chromatographic peak resolution, as shown in panel B. The chromatographic resolution (expressed as peak capacity) almost linearly decreased with scan time making it increasingly difficult to separate close-eluting isomers with the Quan/Qual method. The sensitivity, on the other hand, expressed as the median signal-to-noise ratio and plotted against the scan time in panel C, increased significantly at the start but seemed to reach a plateau around 60 ms. The optimal balance between the chromatographic resolution and sensitivity was achieved with a scan time from 30 ms to 60 ms, as the loss in resolution and sensitivity compared to their maximum values was minimal for these values, independent of the sample concentration. A scan time of 30 ms was selected for further experiments in TOF-MS and MS^E mode.

If the Synapt G2S operates in a scan mode employing IMS, the minimal scan time is 86 ms. Therefore, the effect of higher scan times (86 ms–300 ms) was tested in the $HDMS^E$ mode. This resulted in a further decrease of the peak capacity to only 83 at a scan time of 300 ms, while the effect on the sensitivity remained marginal (S/N of 500 at a scan time of 86 ms increased to around 750 at scan times of 125 ms up to 300 ms) and still no trend was observed in the precision of the peak areas, suggesting that in $HDMS^E$ mode the minimal scan time of 86 ms is optimal for high-throughput UPLC separation.

In general, the scan times for the Synapt G2S advised by the manufacturer are larger than the applied 30 ms in MS^E mode and especially the 86 ms in $HDMS^E$ mode. Each IMS experiment requires 200 pushes and the TOF-pusher interval at a mass range of m/z 50–1200 is 54 μs, resulting in an IMS duty cycle time of 10.8 ms. With a scan time of 86 ms, and alternating low and high collision energy (MS^E), only four IMS experiments can be combined into one scan. To check the effect of this low number on the variability in the mobilogram, the precision of the drift times of the QC samples used for the quantitative analysis was calculated. Generally, the CV% was around 0.3% (results not shown), so very precise. Only for compounds displaying severe detector saturation as a broad and flattened peak in the mobilogram, the CV% was higher due to the high-concentration samples and in the order of 1.5%.

Not only the scan time, but also the smoothing factor is important for chromatographic resolution, as illustrated in Fig. 2. This figure shows extracted ion chromatograms of rilpivirine, which has an E and Z isomer that can chromatographically be separated. The panels A to C have increasing scan times of 30 ms, 86 ms and 200 ms, and panels D, E and F show the same data after a Savitzky-Golay smoothing factor of two iterations at a window of one scan. This example illustrates that use of a scan time of 200 ms and smoothing (panel F) can severely compromise a carefully optimized chromatographic resolution.

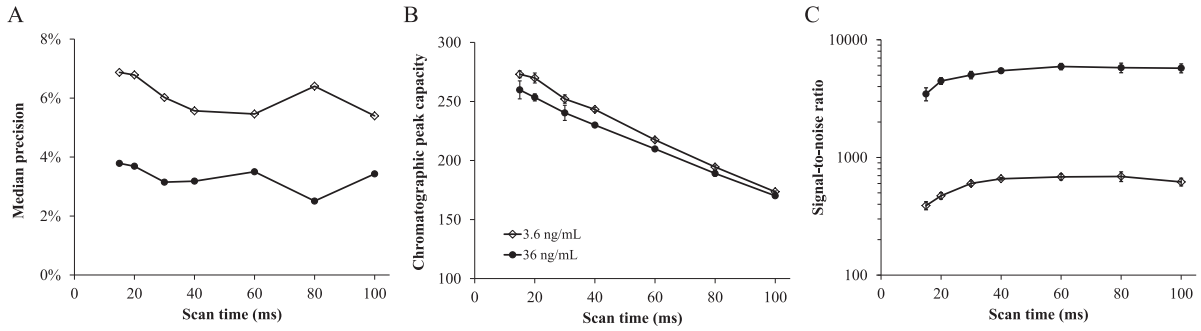

Fig. 1. Effect of the mass spectrometric (MS) scan time on precision, peak capacity and signal-to-noise ratio. Effect of the MS scan time on the mean precision (A), median chromatographic peak capacity (B) and median signal-to-noise ratio (C). Values are calculated over 19 compounds measured in 5-fold per scan time, applying an MS^E scan protocol in (normal) resolution mode and a Savitzky-Golay smoothing factor of two iterations over a single data point window.

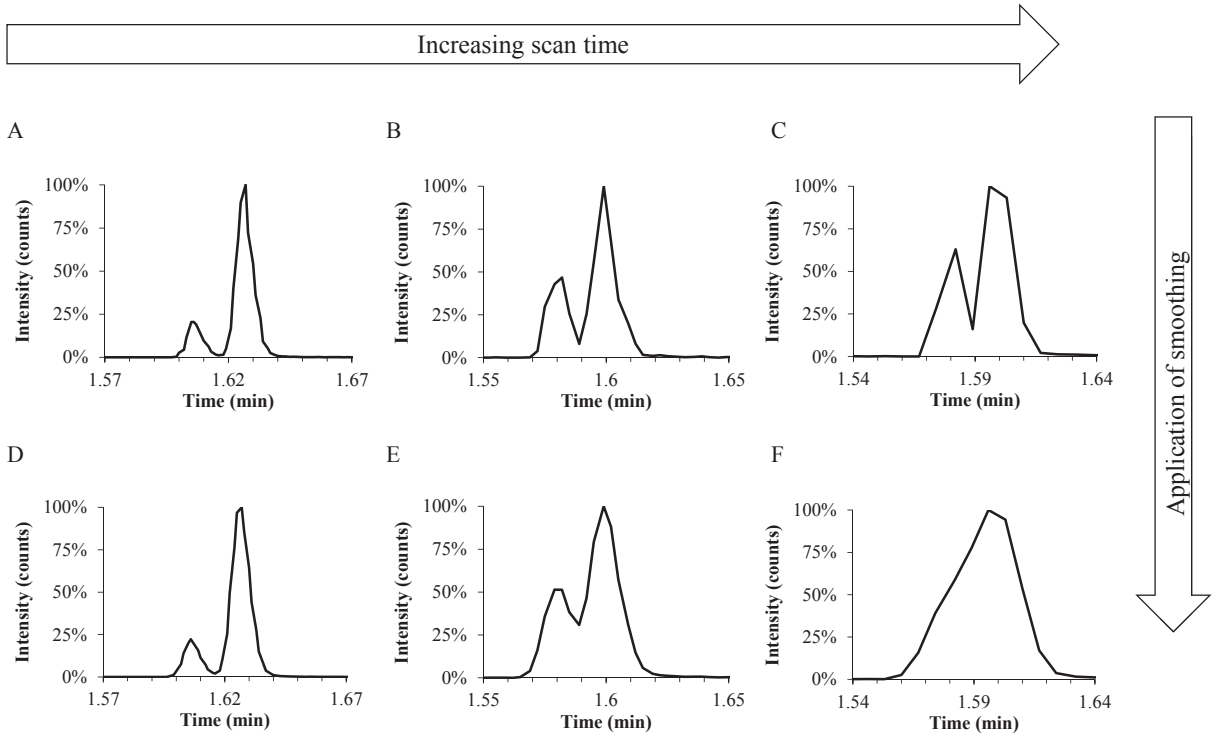

Fig. 2. Effect of the mass spectrometric scan time and smoothing on the separation of close-eluting isomers. Extracted ion chromatogram of rilpivirine E and Z isomers, measured with different scan times, before (upper panel) and after (lower panel) application of a Savitzky-Golay smoothing factor of two iterations over a single data point window. The scan time increases from left to right from 30 ms (A,D) to 86 ms (B,E) and 200 ms (C,F).

The use of smoothing in quantitative analysis is required to level out the noise: in general we observed that application of even the smallest possible smoothing factor resulted in a lower peak intensity, but roughly in a two-fold increase of the sensitivity in terms of signal-to-noise ratio. The amount of smoothing that is adequate for a chromatographic separation is related to the number of data points per peak. In Fig. 2 panel A, B and C, the main peak (above 10% of the peak height) contains 11, 7 and 4 data points, respectively, explaining the difference in appearance after smoothing in D, E, and F. Of note, even though it is common practice to use 15–20 data points per peak, the next section demonstrates that also these sparsely described peaks (with 11, even down to 7 points) can give acceptable quantitative results. To make this even more visible, the precision of the peak area of rilpivirine is plotted against the number of data points across the peak in Supplementary Fig. 2. Although this shows a general trend of decreasing CVs with an increasing number of data points per peak, it also shows that even with the scan time of 100 ms, which gave only 4 data points on the peak at a concentration of 36 ng mL^{-1}, the CV of the peak area is only 4.9%. The good precision of sparsely described peaks was also observed by Henry et al. when they compared an Orbitrap MS and a QqQ MS for quantitative analysis [15].

Both the smoothing window and the number of iterations were optimized. Increasing the window generally resulted in broadening of the peak with limited additional improvement of the signal-to-noise ratio. Also the effect of increasing the number of iterations on the signal-to-noise ratio was small, but it did improve the fluency of the peak shape. A Savitzky-Golay smoothing factor of two iterations at a window of one scan was considered optimal for the Quan/Qual method, based on manual inspection of the peaks and comparison of the chromatographic resolution.

3.2. Evaluation of scan modes for quantitative analysis

The mass protocols TOF-MS, MS^E and $HDMS^E$ were compared for their quantitative performance. In the TOF-MS mode a scan time of both 30 ms and 100 ms was tested to investigate whether the quantitation is compromised by the short scan time. The resulting linear ranges and theoretical detection limits are visualized in Fig. 3 and the accuracies and precisions in Fig. 4. Before actually comparing these results from the different modes, we will shortly discuss the methods applied to obtain them.

First, the acceptance criteria: for a Quan/Qual workflow to be adopted on a regular basis in pharmaceutical research and

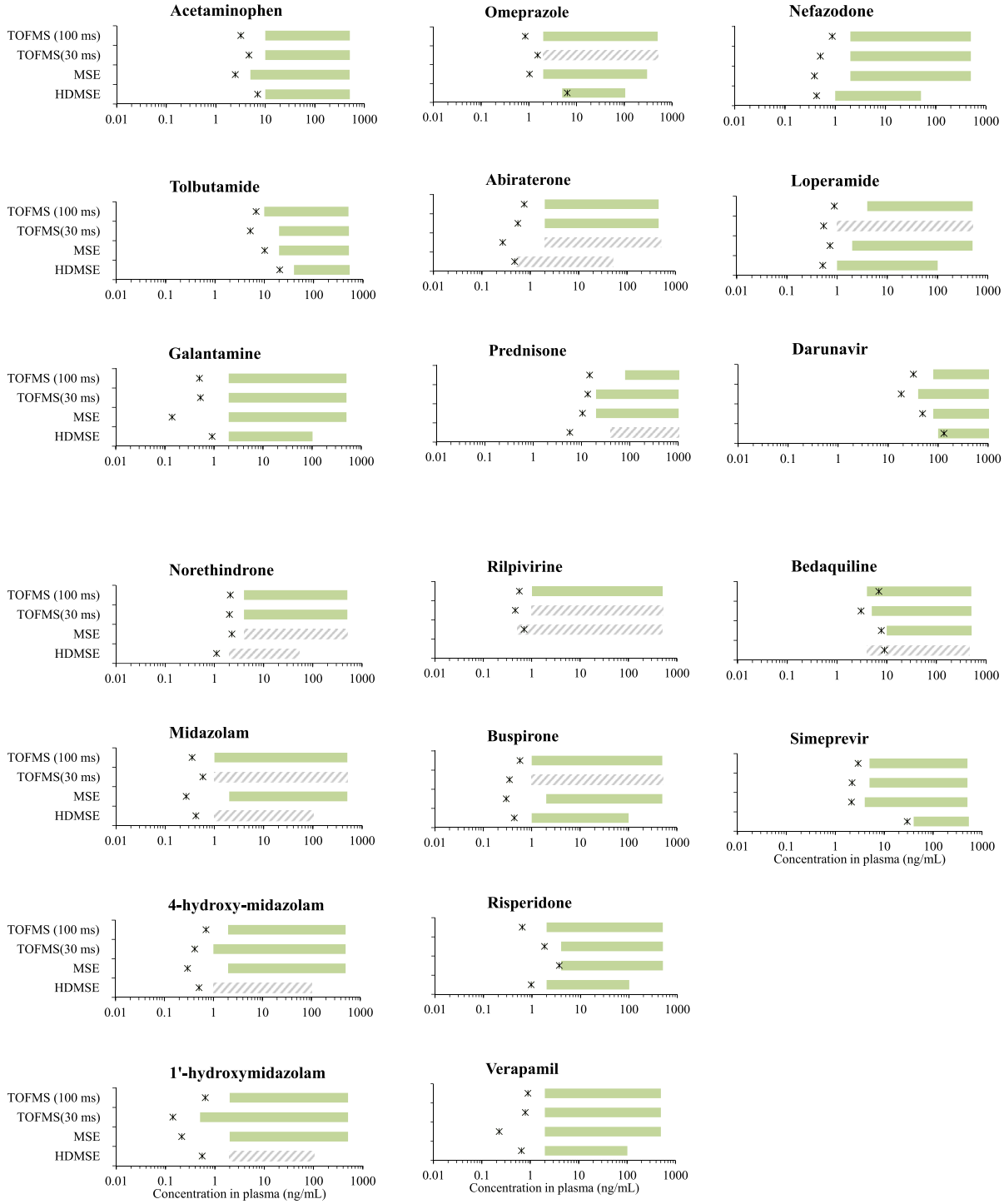

Fig. 3. Experimental linear dynamic ranges and calculated limits of detection (LoDs) in various mass spectrometric modes. The green bars show the linear calibration ranges of the 19 test compounds for each of the analysis modes. A striped bar indicates that one or more QC levels within the linear range have an accuracy deviating more than 20%. The asterisks indicate the theoretical LoDs (due to their calculation as 3 times the standard deviation of the lowest accepted QC level and the availability of only 5 QC levels throughout the tested dynamic range, this theoretical value can be too high in cases where the lowest QC level(s) were outside acceptance criteria). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

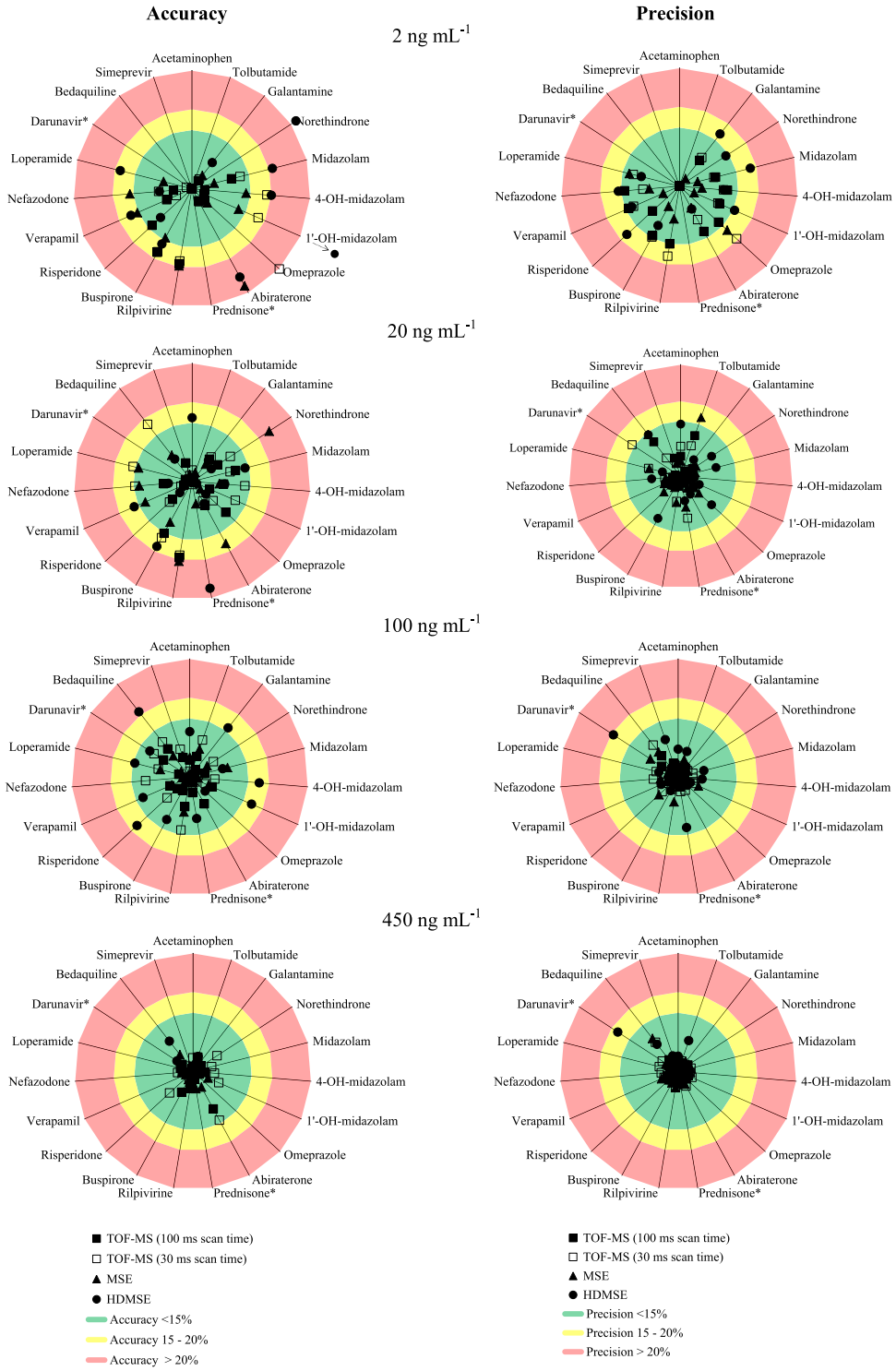

Fig. 4. Accuracy and precision per QC level in various mass spectrometric modes. The radar plots on the left visualize per QC level the accuracies (n = 5, absolute deviations) of the 19 test compounds for each of the MS protocols. Data points within the first green ring have a high accuracy (<15% deviation) and data points in the outer red ring have a low accuracy (>20% deviation). The radar plots on the right visualize the precision (n = 5) of the 19 test compounds for each analysis mode. Only QC levels within the calibration range are included. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

development, the quantitative capacity should be comparable to traditional quantitative bioanalytical assays. In non-regulatory exploratory bioanalysis, acceptance criteria of 20%–25% for calibration points, accuracy and precision are commonly applied. In a regulated setting, more strict criteria of 15% and 20% at LLoQ level are prescribed in the FDA guidelines for Bioanalytical assay validation [16]. In the current study, we fixed the acceptance criteria to 20% at all concentration levels to demonstrate the differences in performance of the tested quantitative MS modes.

Second, the use of internal standards: for quantitative drug

analysis it is widely agreed that the use of a stable isotope labeled IS for each analyte to be quantified is preferred over any other type of standardization [17]. However, these are often not available in early drug discovery, so we selected a set of internal standards (stable isotopes of drugs in the standard set) covering a wide chemical space. Each analyte was matched with the internal standard giving the best precision of the peak response ratios in the QC samples. Generally, the differences in precision between the internal standards were very small, except for the bedaquiline-d6 isomers A and B, which showed high variability in peak response and did not improve the precision as an internal standard for any compound but bedaquiline. This could be explained by the observation of severe matrix effect at the retention time of bedaquiline (2.57 min) due to co-elution of phospholipids and indicates that a stable isotope IS indeed compensates for this matrix effect. For most other analytes a good precision was even obtained without internal standard correction, supporting that the used sample preparation method is repeatable.

Third, the HDMS^E data analysis: the quantitative data included in Figs. 3 and 4 were analysed using Targetlynx software, which only uses the retention time, intensity and *m/z* value for 2D peak integration, ignoring the ion mobility data. The software UNIFI does offer possibilities to quantify peaks taking into account the ion mobility data, using 3D or 4D peak detection and 2D or 3D peak integration (see Supplementary Fig. 3). The use of the additional degree of separation for quantitative analysis can be useful for compounds with common masses, e.g. of endogenous metabolites with many (possibly co-eluting) isomers. In addition, using 4D peak detection, the exact top of a peak can be found more accurately, and quantification is therefore expected to give a more precise result than when using pre-set windows for *m/z*, retention time and drift time. The three quantification methods were tested for the current targeted analysis, but showed similar accuracy and precision values. The potential benefits of using the drift time did in this case not outweigh the short processing times and user-friendliness of Targetlynx as compared to UNIFI.

Comparing the four scan protocols in Figs. 3 and 4, it was observed that they have comparable detection limits, with some outliers with a high detection limit in the HDMS^E mode. As expected from Fig. 1C, the difference in sensitivity between the scan time of 30 ms and 100 ms is marginal. The accuracy and precision of the lowest CS and QC levels were however for some compounds affected by carry-over, which resulted in an apparent low sensitivity/high LOD/LOQ for many compounds as compared to literature data of compound-specific LC-QqQ MS methods. An example is galantamine, for which, irrespective of the Synapt G2S scan protocol, the linear range started at 2 ng mL^{-1}, while others reported an LLoQ of 0.12 ng mL^{-1} for galantamine in (rat) plasma using QqQ MS [18]. At 1 ng mL^{-1} galantamine could already be detected, but the carry-over induced variation was too high. Attempts to decrease the carry-over by including the additional sharktooth-shaped gradient in the cleaning phase of the chromatogram and additional switches of the injection valve only partially solved the problem. Since the main cause appeared to be in the autosampler, it is expected that the carry-over would be less of a problem (and even lower concentrations could be quantified) if a UHPLC-system with a flow-through needle would be used [19].

All accuracy and precision values are tabulated in Supplementary Table 2 and Supplementary Table 3, respectively. The TOF-MS mode with a scan time of 100 ms was most accurate with a median accuracy level (absolute deviation) of 4% (see Fig. 4) and most precise with a median coefficient of variation of 3% (only for 2 compounds >15%, at the lowest QC level of 1 ng mL^{-1}). These values are in the range of what is expected from a quantitative bioanalytical assay from the FDA guidelines [16], demonstrating the quantitative applicability of this Quan/Qual method for a wide range of compounds, as represented by the 19 test compounds. A scan time of 30 ms gave a similar precision (median of 4%) as the scan time of 100 ms, but was less accurate (the absolute deviation had a median of 7% and exceeded 20% for 5 compounds at the lowest QC level).

To increase the amount of qualitative data, other MS protocols can be used, but as Figs. 3 and 4 implicate, this comes at the cost of a reduced quantitative performance. The performance of the MS^E mode is almost as good as the TOF-MS mode, with accuracy and precision values $\leq$ 20% at the lowest QC level and $\leq$15% at the higher QC levels for all analytes except norethindrone, abiraterone and rilpivirine. The HDMS^E mode gives less accurate results and a smaller linear dynamic range (up to 1 order of magnitude) than the other modes. This can be explained by the ions focusing in the helium cell and the ion mobility cell before they enter the TOF section, thereby more easily leading to saturation of the detector. Especially for non-targeted analysis, this limited dynamic range can be a problem. The Masslynx software does offer solutions to enhance the dynamic range, whereby alternately all ions or only a part of them reach the detector, but this halves the number of data points. For targeted analysis, a post-acquisition possibility to overcome problems with detector saturation is using less abundant natural isotopes for quantitative analysis [20].

An advantage of the MS^E and HDMS^E modes over the TOF-MS mode is that they can give additional specificity. Although not tested in this investigation, quantification can be performed on the product ion of a targeted compound, which is collected in the high energy trace.

3.3. Evaluation of scan modes for drug metabolite analysis

The two most important aspects of a qualitative LC-MS method are (i) the number of compounds (peaks) that can be detected and (ii) the amount of structural information that is collected for these compounds. From the MS scan modes available on the Synapt G2S it is theoretically the HDMS^E mode that would score best on these aspects. This mode gives an additional dimension in separation (ion mobility), allowing more peaks to be discovered (e.g. by separation of coeluting isomers). Additionally, it gives the collisional cross section (which can be calculated from the drift time) as an additional feature of the detected compound, next to the accurate mass of both parent and fragment ions.

For a Quan/Qual workflow, however, the applied MS protocol should perform well in both quantitative and qualitative analysis and, as demonstrated in the previous section, the HDMS^E mode has some disadvantages in quantitative analysis, e.g. a shorter dynamic range, and a lower accuracy and precision compared to the TOF-MS and MS^E modes. Since the TOF-MS mode has the clear qualitative limitation of only providing the parent mass, here only the MS^E and HDMS^E mode were evaluated.

Binary comparison of the 120-min and 0-min buspirone incubated rat hepatocyte samples with UNIFI resulted in a peak list of 19590 features found in the MS^E mode and 11967 features in the HDMS^E mode. Filtering these lists is required to look at the more relevant metabolites of buspirone and to eliminate false positives. After filtering, only 54 features were left on the MS^E list and 204 on the HDMS^E list. An additional useful filter would be to remove all tentatively identified features with a mass error >5 ppm. This would leave 36 compounds on the MS^E list and 79 on that of HDMS^E, but due to inaccurate mass detection of some compounds (possibly related to their high abundance and detector saturation) in HDMS^E mode this led to false negatives. Although the number of tentatively identified metabolites was in favor of the HDMS^E mode, a closer look at the tentative identities, chromatograms and spectra

learned that on the MS^E peak list relatively many more compounds were likely to be real metabolites than on the $HDMS^E$ peak list. Therefore, it may be better to evaluate the two modes based upon some examples than on the numbers only.

As a first example we show the extracted ion chromatogram of *m/z* 418.2454, corresponding to buspirone + 2O, without smoothing (Fig. 5A). In the MS^E chromatogram, 13 peaks of buspirone + 2O isomers can be distinguished; in the $HDMS^E$ chromatogram two of the isomers are combined into one peak due to the low number of data points on the peak. This phenomenon gets worse when smoothing is applied as shown in Fig. 5B, wherein only 10 or 11 metabolites seem to be left, as opposed to still 13 in the smoothed MS^E chromatogram. Looking at the ion mobility separation of the $HDMS^E$ analysis, Fig. 5C, the peaks are again all distinguishable and even the detection of an additional 14th peak in the squared section may be argued. The difference in drift time between the metabolites is in this case that small that the additional structural information in the $HDMS^E$ mode will be limited to non-existent. Altogether, the qualitative information that can be obtained with MS^E is in this example very similar to that with $HDMS^E$.

A more pronounced difference in ion mobility was found for two isomeric glucuronic acid conjugates of buspirone in a second example. Here, it was observed again that the two peaks that can be distinguished in the non-smoothed EICs collected in MS^E mode and $HDMS^E$ mode melted into one peak after smoothing in the $HDMS^E$ mode (see Fig. 5D and E). In the ion mobility plot (Fig. 5F) the two peaks are however clearly resolved and, although not further investigated for this study, the experimental collisional cross section may help in their structural identification. However, in practice it is pretty difficult to elucidate metabolites based on their collisional cross section unless applied in a relative mode [21] and IMS is more often applied for its additional dimension in separation and confirmation of identity as opposed to structural elucidation.

These two examples show that due to the high degree of separation obtained in the chromatographic method, the qualitative advantage of the additional orthogonal separation in $HDMS^E$ analysis is limited for this Quan/Qual method. Additionally, they stress the importance of critically controlling and evaluating the effect of data smoothing in qualitative analysis.

3.4. Application of the Quan/Qual method to a non-targeted study

3.4.1. Quality control (Quan)

The Quan/Qual UHPLC-MS^E method was used to explore the

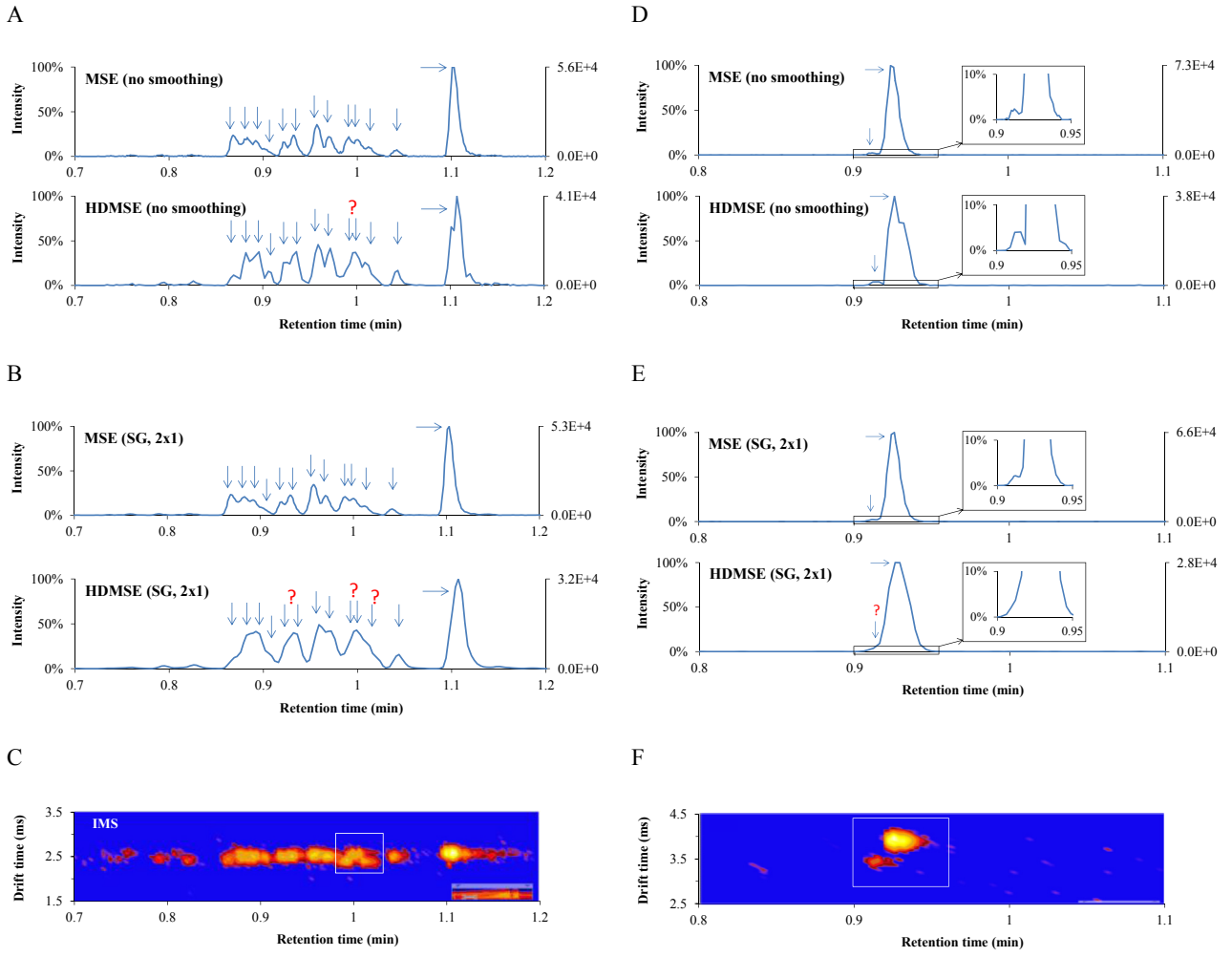

Fig. 5. Effect of mass spectrometric mode and settings on real-life samples. Extracted ion chromatograms of buspirone + O_2 (*m/z* 418.2454, panel A, B and C) and buspirone + O + Glucuronide (*m/z* 578.2826, panel D, E and F) in a rat hepatocyte sample after 120 min incubation with buspirone, collected with MS^E and $HDMS^E$. Panels A and D shows the raw chromatograms, panels B and E the chromatograms with a Savitzky-Golay smoothing factor of two iterations at a window of one scan and panels C and F the ion mobility plot. The arrows indicate the distinguishable peaks.

effect of a dosing vehicle on the metabolite profile of a rat. Targeted analysis using Targetlynx was applied to assess the quality of the measurements. The precision (%CV) of the 19 drugs across the 9 pooled QC samples was between 4% and 14%, except for bedaquiline which had a precision of 30%. As observed before, the variability at this point in the chromatogram is more severe, probably due to co-elution with matrix interferences.

When calculated across all 27 samples (18 study samples and 9 QC samples), the precision of the spiked analytes varied between 12% for tolbutamide and 104% for bedaquiline. It appeared that acetaminophen and galantamine had systematically lower peak areas in the sample groups of PEG-dosed rats than in the other groups. For acetaminophen this is shown in the upper pane of Fig. 6. Herein, the open dots represent the relative peak area as compared to the mean peak area of acetaminophen and they are systematically lower for the PEG-dosed rat groups. Further investigation of the chromatogram and spectra at the retention time of acetaminophen (0.49 min) and galantamine (0.7 min) learned that several high-intensity PEG polymer chains coelute in this area and are most probably responsible for this major ion suppression. Therefore, also the precision was calculated excluding all samples containing plasma of PEG-dosed rats (i.e. also the QC sample pools). This resulted in acceptable (<30%) precision for the 12 compounds eluting up to 1.51 min (midazolam), except for darunavir. Darunavir had a precision of 53% even though it eluted at 1.47 min, but this can be attributed to the overall very low response that was observed for this compound. The 7 compounds eluting after midazolam suffered from more matrix effect-related variability and had precisions up to 97% (for bedaquiline without IS compensation).

To investigate if reliable quantification of these later-eluting compounds is still possible, the stable-isotope labeled internal standards of verapamil and bedaquiline were used to calculate the precision of the analyte − internal standard peak area ratio. Across all 27 samples this resulted in an improvement of the precision of verapamil from 50% to 10% and bedaquiline from 104% to 10%, indicating that reliable quantification is indeed possible in this region of the chromatogram if a(n) (stable-isotope labeled) internal standard is applied. Remarkably, the signal of acetaminophen and galantamine was so much suppressed in the PEG-dosed rat plasma samples, that even their stable-isotope labeled internal standards could not completely correct for this, as can be seen from the black dots in the upper pane of Fig. 6. The lower pane of Fig. 6 shows an example of a compound (risperidone) eluting between the region disturbed by PEG and the region where other endogenous matrix constituents cause signal variability. Even without any correction the peak area deviation from the mean is within 20% for most samples and after correction with the stable isotope internal standard both accuracy and precision improve.

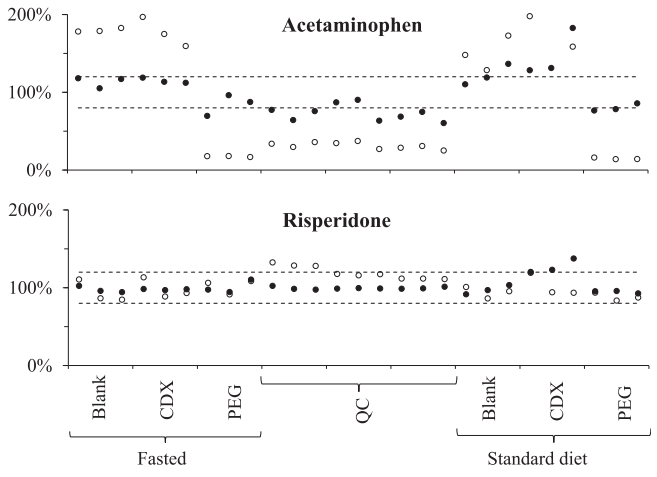

Fig. 6. Quality control of the Quan/Qual analysis method in a non-targeted application. Quantitative analysis results of two representative spiked analytes after applying the Quan/Qual method in a metabolomics study exploring the effect of a dosing vehicle and fasting state on the rat endogenous metabolic profile. The dots represent the peak area of the spiked analyte compared to the average peak area of that analyte, whereby no internal standard correction is applied for the open dots (°) and stable isotope labeled internal standard correction is applied for the black dots (•).

Although in this situation the use of HDMS^E instead of MS^E could be applied to clean fragmentation spectra from co-eluting PEG-related fragments [22], its use would not solve the ion suppression, which is a process taking place in the ion-source.

3.4.2. Exploratory non-targeted analysis (Qual)

Exploratory non-targeted analysis was performed on rat plasma samples, aiming to investigate if the fasting state and the use of a dosing vehicle influence the rat metabolomic profile and whether the Quan/Qual method is capable to provide biologically relevant information. Profound statistical analysis or biological interpretation is beyond the scope of this paper.

Based on the quality control and in order to minimize the number of false positive features that discriminate between the groups it was decided to exclude the PEG-dosed rat plasma samples and only analyze the chromatographic window of 0.2–1.51 min. This means that 4 groups with 3 biological replicates were included in the analysis. An alternative approach would be to include the PEG dosed rat plasma samples and analyze only the window between 0.7 and 1.51 min. Fig. 7 shows the resulting PCA plot of the features with a p-value < 0.05 (144 of the original 1996 features) based on one-way ANOVA. There is a clear distinction between the four groups, suggesting that indeed features are influenced by fasting state and/or by the administration of cyclodextrin as a dosing vehicle. The five most important discriminative features identified by PLS-DA (see Table 2) differed significantly between the fasted and fed rats, and were upregulated in the fasted rat. A comparison of their *m/z* values against the Scripps Center for Metabolomics database revealed potential identities for three of them, all acylcarnitines. This would be in line with previous literature, wherein acylcarnitines were reported as plasma biomarkers of fed versus fasted rats, being most abundant in fasted rats [23].

The separation in the metabolic profile observed in Fig. 7 between both fasting state and administration of cyclodextrin warrants the selection of not only fasting state-matched, but also dosing vehicle-matched blank samples in for example pharmaco-metabolomic studies. Obviously, more information could be extracted from this data, e.g. by comparing only two groups simultaneously, but already this example clearly demonstrates the applicability of the Quan/Qual method in a non-targeted metabolomics study.

4. Conclusions

Quan/Qual method development requires balancing between the amount of qualitative data, the quality of the quantitative data and the analysis time. In this study, we investigated the effect of the mass resolution, scan mode, scan rate, smoothing, and peak integration in Quan/Qual method development and analysis, using a 4.25 min widely-applicable UHPLC chromatography. Based on the results it is proposed that the optimal balance can be obtained using the normal resolution mode with MS^E analysis, a scan time of 30 ms, and a Savitzky-Golay smoothing factor of two iterations at a window of one scan. In a targeted quantitative application, applying 2D peak integration in Targetlynx software, these settings enabled quantification with accuracy and precision values ≤ 20% at the lowest QC level and ≤15% at higher QC levels for 16 out of 19

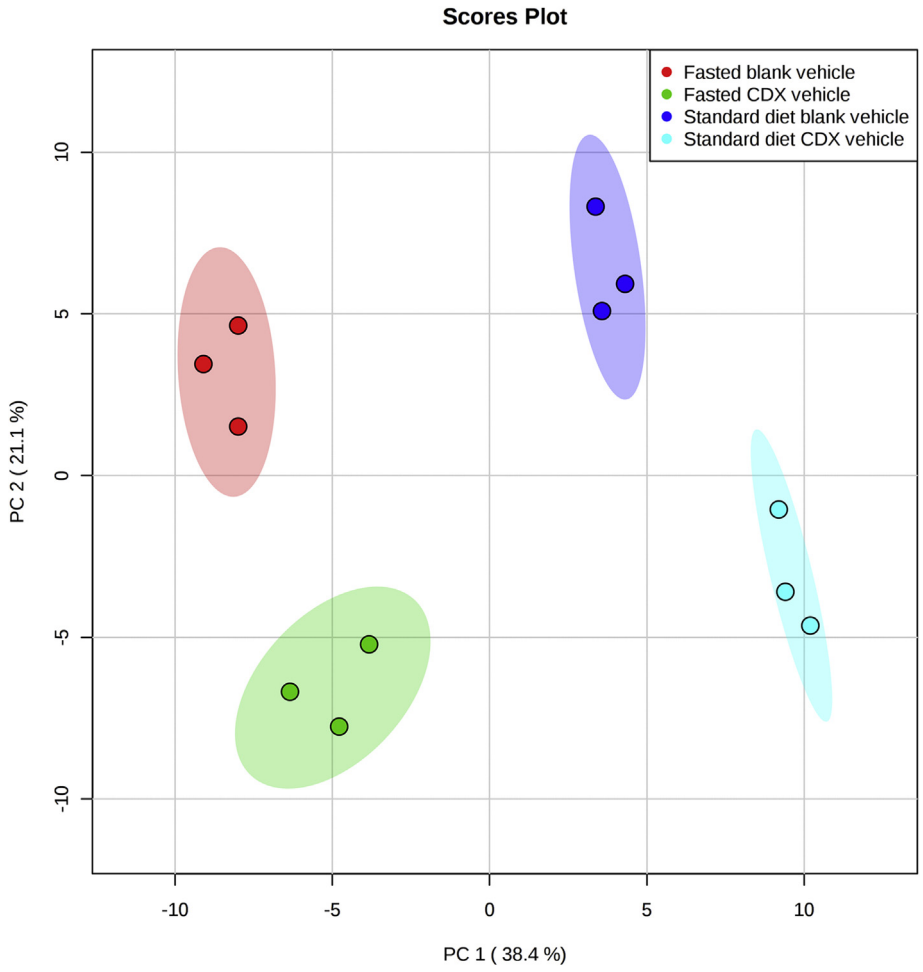

Fig. 7. Example of results of the Quan/Qual analysis method in a non-targeted application. Principal component analysis plot of the features with an ANOVA p-value > 0.05 upon comparison between plasma samples of fasted and fed (standard diet) rats, dosed with cyclodextrin (CDX) or nothing (blank).

Table 2
Use of the Quan/Qual analysis method to find discriminative features in plasma of fed, fasted and/or cyclodextrin-dosed rats. Summary of the top 5 most discriminative features identified by PLS-DA. This top 5 is based on the features with an ANOVA p-value > 0.05 upon comparison between plasma samples of fasted (F, n = 3) and fed (SD, standard diet, n = 3) rats, dosed with either cyclodextrin (CDX, n = 3) or nothing (BLK, blank, n = 3).

	Feature		Average raw peak area (n = 3) (Coefficient of Variation)				Proposed identity		
	Observed *m/z*	T_R (min)	F blank	F CDX	SD blank	SD CDX	Name	*m/z*	ppm error
1	260.1861	0.75	2.3E+05 (14%)	1.8E+05 (26%)	9.7E+04 (22%)	6.2E+04 (4%)	hexanoylcarnitine	260.1856	1
2	288.2171	1.07	7.9E+04 (9%)	6.5E+04 (28%)	3.8E+04 (19%)	2.6E+04 (8%)	octanoylcarnitine	288.2169	0
3	384.2743	1.43	3.0E+04 (8%)	2.4E+04 (32%)	1.1E+04 (21%)	8.0E+03 (8%)	Unknown	Unknown	Unknown
4	316.2491	1.35	7.3E+04 (12%)	5.9E+04 (27%)	3.5E+04 (20%)	2.4E+04 (6%)	decanoylcarnitine	316.2482	2
5	360.2728	1.39	1.8E+04 (4%)	1.4E+04 (35%)	7.0E+03 (9%)	5.4E+03 (25%)	Unknown	Unknown	Unknown

T_R: Retention time.

tested analytes. In a drug metabolite application, applying 3D peak detection in UNIFI software, these settings resulted in both parent *m/z* values and fragmentation spectra for compound identification with limited loss of chromatographic resolution. Finally, in a non-targeted metabolomics application, applying Progenesis QI for peak detection, alignment over sample groups and integration, these settings revealed several acylcarnitines as biologically relevant metabolites to distinguish fasting from fed rats.

Since the above mentioned HRMS settings are optimized on a specific (although widely applicable) UHPLC method and because each Quan/Qual method can have its own (main) purpose we will here recommend on the optimization of the mass spectrometric parameters for future Quan/Qual method development.

4.1. Mass resolution

The Synapt G2S mass spectrometer has three mass resolution modes: high sensitivity, normal resolution and high resolution.

Although the performance of each mode highly depends on instrument tuning, the high resolution mode is not recommended for Quan/Qual analysis because of the extreme loss in sensitivity inherent to the TOF-section of the instrument running in W-mode instead of V-mode. The difference between the high sensitivity and normal resolution modes in terms of signal-to-noise ratio, mass resolution and file size were not found to be significant in this study, making both modes evenly applicable for Quan/Qual analysis. Contrary to ToF-based high-resolution mass spectrometers like the tested Synapt G2S, Orbitrap-based MS systems show an inverse relation between scan time and mass resolution. For high-throughput Quan/Qual applications with narrow UPLC peaks, this means that the mass resolution should be optimized to prevent compromising the chromatographic resolution.

4.2. Scan mode

The scan mode selection should be based on the objective of the Quan/Qual method. The TOF-MS, MS^E and $HDMS^E$ scan modes, respectively, provide an increasing amount of qualitative information. Their quantitative performance in terms of accuracy, precision and linear dynamic range showed an opposite trend, although the performance of the MS^E mode came close to that in the TOF-MS mode. For generic high-throughput Quan/Qual method development the MS^E mode would therefore be advised, but if the focus is more on the qualitative aspect of the method then $HDMS^E$ could give some extra information.

4.3. Scan time

The present study showed with several examples how the scan time can affect the chromatographic resolution. If the scan time is too long, the number of data points across the chromatographic peak is insufficient to adequately describe it, which, especially after smoothing, results in a lower chromatographic resolution. Scan time should therefore be adapted to the peak width. The presented Quan/Qual MS^E method with 30 ms scan time and peaks of around 11 data points shows that the common standards of a scan time of at least 100 ms and at least 15–20 data points per peak can be pushed.

4.4. Smoothing

Also the amount of smoothing was demonstrated to impact the chromatographic resolution. It is advised to apply a smoothing factor to increase the signal-to-noise ratio, but to keep it as low as possible to prevent peak broadening and masking of partially resolved peaks.

4.5. Software

Only using UNIFI software sounds attractive for Quan/Qual analysis as it can do both targeted quantitative and non-targeted qualitative analysis and can use all dimensions of the collected data (retention time, *m/z*, drift time and intensity) to precisely detect peaks. While this software was indeed found to be very useful for drug metabolite profiling and processing of ion mobility enabled analyses, we considered other software packages more user-friendly for other specific tasks. It must be mentioned however that UNIFI software is still continuously being further developed, to optimize e.g. speed, capabilities and user-friendliness. Targetlynx can perform fast targeted quantitative analysis, which is why we, at least for now, recommend using this software unless the drift time is crucial to separate the target compounds. For non-targeted comparison of sample groups, Progenesis QI software was found to be very intuitive and much faster when compared to UNIFI.

4.6. Other recommendations

One of the observations in e.g. the application of the developed Quan/Qual method on the rat plasma samples was the major influence of matrix effect on the quantification of targeted and non-targeted compounds. For targeted quantitative analysis, stable isotope labeled internal standards can correct well for the quantification of the target, but for non-targeted analysis more sophisticated approaches may be required to limit or correct for matrix effects [23]. This should be a point of consideration in future Quan/Qual study design.

In summary, this paper describes the possibilities and limitations of UHPLC-HRMS Quan/Qual analysis. In addition, it provides both a ready-to-use accurate, precise and sensitive high-throughput Quan/Qual UHPLC-MS^E method, widely applicable in e.g. drug metabolism and (pharmaco)metabolomics studies, and recommendations on the development of future Quan/Qual methods.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.aca.2018.02.055.

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