MetaboQuant: a tool combining individual peak calibration and outlier detection for accurate metabolite quantification in 1D $^1$H and $^1$H-$^13$C HSQC NMR spectra

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Solution nuclear magnetic resonance (NMR) spectroscopy is widely used to analyze complex mixtures of organic compounds such as biological fluids and tissue extracts. Targeted profiling approaches with reliable compound quantification are hampered, however, by signal overlap and other interferences. Here, we present a tool named MetaboQuant for automated compound quantification from pre-processed 1D and 2D heteronuclear single quantum coherence (HSQC) NMR spectral data and concomitant validation of results. Performance of MetaboQuant was tested on a urinary spike-in data set and compared with other quantification strategies. The use of individual calibration factors in combination with the validation algorithms of MetaboQuant raises the reliability of the quantification results. MetaboQuant can be downloaded at http://genomics.uni-regensburg.de/site/institute/software/metaboquant/ as stand-alone software for Windows or run on other operating systems from within Matlab. Separate software for peak fitting and integration is necessary in order to use MetaboQuant.

Metabolomic analyses of biological fluids and tissue extracts have become a major application of nuclear magnetic resonance (NMR) (1). Targeted profiling approaches are used to quantify predefined compounds (2), yet signal overlap due to the large number of endogenous and exogenous compounds present in biological specimens makes this process difficult (3). One way to address this problem is by fitting expected line shapes to an observed signal (4), although this approach is less useful in heavily overlapped regions. One may exclude strongly overlapped signals and integrate only signals that appear free of overlap by eye, but an automatic routine would present a better solution since it would not rely on the skill and experience of the researcher inspecting the spectra. In addition, automation saves time when quantifying large spectra sets containing hundreds of signals as is often the case in metabolomic studies.

Another problem encountered when performing quantitative NMR analyses is that signal intensities of equally concentrated compounds—even different signals from the same compound—might vary considerably. Different strategies have been proposed to circumvent this issue in HSQC spectra, for example, using experimentally determined (3,5) or theoretically calculated (6) calibration factors, or reconstruction of time-zero HSQC spectra (7). With the exception of time-zero HSQC spectra, these approaches rely on the multiplication of peak integrals by individual calibration factors.

Here, we describe a software tool named MetaboQuant that calculates accurate compound concentration values from 1D and 2D NMR peak intensities using individual calibration factors and different outlier detection algorithms. MetaboQuant performs the quantification procedure automatically once the required parameters have been set.

MetaboQuant does not perform peak picking, fitting, or integration on NMR spectra, steps for which other software such as the proprietary Amix (Bruker BioSpin, Rheinstetten, Germany) or the free ACD/NMR Processor Academic Edition (www.acdlabs.com/resources/freeware/nmr_proc) is available.

Materials and methods

Test data
For performance tests, a latin square spike-in experiment was prepared where the sum of the molar amounts of spike-ins added to aliquots of a biological specimen is the same (Table 1). Eight metabolites commonly found in human urine, namely alanine, creatinine, histidine, betaine, acetate, phosphocreatine, $\beta$-hydroxybutyrate (BHBA), and lactate (all purchased from Sigma-Aldrich, Steinheim, Germany) were added in varying amounts to aliquots of a urine specimen collected from a healthy volunteer. Briefly, each added metabolite was dissolved in pure water and these solutions were diluted to yield eight different concentrations of each metabolite. The urine specimen was split into eight 200 µL aliquots and 25 µL each of the previously prepared metabolite solutions (all with different

Method summary:
MetaboQuant offers a novel approach for the accurate quantification of metabolites from NMR data by using individual calibration factors and by automatically excluding unreliable and overlapped signals.
The intuitive graphical user interface displays all configuration parameters and provides easy access to all files.

**Results and discussion**

Functions and data flows within MetaboQuant are visualized in Figure 2A following a structured analysis design (9). Each circle in Figure 2A corresponds to one panel in the main program window (Figure 1). In the following paragraphs, the work-flow of MetaboQuant is described briefly.

**Data input**

A file containing annotated peak integrals is used as data input. The file may be a tab delimited text file, an Excel file, or an Amix output file.

Adjusting integrals of individual signals

First, peak integrals are divided by the number of nuclei contributing to each peak. The number of nuclei is taken from a peak information file provided by the user. A peak integral may be scaled to a reference substance and given as the fraction of the integral (FI) of the reference substance. The FIs may then be multiplied by a correction factor, for instance for differences in reference substance concentration across samples.

Next, each FI may be scaled by an individual calibration factor taken from the peak information file. A set of calibration factors for commonly observed compounds is included in the software package. However, these values are only applicable to measurements performed using exactly the same measurement and sample parameters. The parameters used can be found at http://genomics.uni-regensburg.de/site/institute/software/NMR/Latin_Square.zip. Statistical analyses were performed using Excel 2007 (Microsoft, Redmond, WA).

**Software development**

MetaboQuant was developed using Matlab 2007b (MathWorks, Natick, MA). Executable files for Windows (Microsoft), a Matlab script 2007b (MathWorks, Natick, MA). Executable files for Windows (Microsoft), a Matlab script and a script for use on other operating systems and a detailed manual are available at http://genomics.uni-regensburg.de/site/institute/software/metaboquant. MetaboQuant is accessed via an intuitive graphical user interface (Figure 1) providing access to all parameters and files from one program window.

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### Table 1: Latin square spike-in experiment.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Lactate</th>
<th>Alanine</th>
<th>Creatinine</th>
<th>3-Hydroxybutyrylrate</th>
<th>Histidine</th>
<th>Phosphocreatine</th>
<th>Betaine</th>
<th>Acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>6000</td>
<td>47</td>
<td>94</td>
<td>168</td>
<td>375</td>
<td>750</td>
<td>1500</td>
<td>3000</td>
</tr>
<tr>
<td>Sample 2</td>
<td>3000</td>
<td>6000</td>
<td>47</td>
<td>94</td>
<td>188</td>
<td>375</td>
<td>750</td>
<td>1500</td>
</tr>
<tr>
<td>Sample 3</td>
<td>1500</td>
<td>3000</td>
<td>6000</td>
<td>47</td>
<td>94</td>
<td>188</td>
<td>375</td>
<td>750</td>
</tr>
<tr>
<td>Sample 4</td>
<td>750</td>
<td>1500</td>
<td>3000</td>
<td>6000</td>
<td>47</td>
<td>94</td>
<td>188</td>
<td>375</td>
</tr>
<tr>
<td>Sample 5</td>
<td>375</td>
<td>750</td>
<td>1500</td>
<td>3000</td>
<td>6000</td>
<td>47</td>
<td>94</td>
<td>188</td>
</tr>
<tr>
<td>Sample 6</td>
<td>188</td>
<td>375</td>
<td>750</td>
<td>1500</td>
<td>3000</td>
<td>6000</td>
<td>47</td>
<td>94</td>
</tr>
<tr>
<td>Sample 7</td>
<td>94</td>
<td>188</td>
<td>375</td>
<td>750</td>
<td>1500</td>
<td>3000</td>
<td>6000</td>
<td>47</td>
</tr>
<tr>
<td>Sample 8</td>
<td>47</td>
<td>94</td>
<td>188</td>
<td>375</td>
<td>750</td>
<td>1500</td>
<td>3000</td>
<td>6000</td>
</tr>
</tbody>
</table>

Concentrations of the spiked-in compounds are given in µmol/L. The sum of spiked-in concentrations is the same for each sample.
of each signal belonging to a compound. The next bundle of algorithms (the third circle in Figure 2A) contains several checks for this purpose. Details are shown in Figure 2B as an UML activity diagram (10).

For a given sample matrix, some of the observed signals are prone to overlap. Therefore, the user can specify the signals to be used for quantification of each compound. Imagine a metabolite signal overlapped by another signal of low intensity. If the concentration of the compound in question is high, this may not pose a problem, as the low-intensity overlap will not change the peak integral too much. Conversely, when the compound’s concentration is too low to yield a visible NMR signal, the overlapping signal might still be visible, leading to an incorrect interpretation of the results. Consequently, a presumably non-overlapped signal of this compound may be marked as obligatory. If this signal is not observed in the spectrum because it is below the noise level, the compound is marked as not detected. Obligatory signals are prone to overlap. In such cases, the overlapped peaks should be excluded from quantification or a reliability check might cause quantification failure taken from a file provided by the user and scaling and normalization of calculated concentrations.

Scaling and normalization of calculated concentrations

Scaling and normalization of calculated concentrations. Dilution factors may be used to correct concentration values in those instances where limited amounts of specimen require dilution to achieve the volume needed for NMR measurements. This correction is achieved either by multiplying all values with the same dilution factor or by using individual dilution factors provided in the spectrum file. The obtained quantitative values may be normalized to a chosen compound (i.e., divided by the concentration of this compound). For replicate samples, means and technical errors may be calculated. Replicate samples have to be marked in the spectrum title.
Report of results
The calculated concentrations are saved to an output file and the values of all excluded peaks are stored for manual inspection. All parameters, file names, and the program version are stored in the results file. These data allow the exact reproduction of the quantification procedure at a later time point and enable reproducible results.

Evaluation
To test the performance of MetaboQuant, a latin square spike-in experiment was performed. First, H-13C HSQC spectra were generated as these generally yield excellent quantification results upon correction of each peak integral with an individual calibration factor (3). If the quantification works well, a slope of 1 should be observed for each compound when performing a linear regression of measured concentrations against spiked-in concentrations. In addition, the standard deviation of the 8 slopes should be close to 0.

For comparison, quantification was performed using both Amix 3.9.13 and MetaboQuant 1.2 (using peak integrals calculated by Amix). Five different quantification approaches were used, as listed in Table 2, and the results are presented and discussed in the following paragraphs. For a list of the retrieved concentration values, see Supplemental Table S2.

Quantitative results using Amix
To retrieve absolute concentrations, the mean of the peak integrals for a compound calculated by Amix has to be manually multiplied by the reference concentration. When using the exact reference concentration to calculate quantitative results, the mean of the observed slopes deviates significantly from the expected slope of 1 (Table 2). In addition, the slopes vary strongly as indicated by the high standard deviation. For most compounds, this indicates a poor correlation between spiked-in and measured concentrations. This deviation is explained by the fact that in HSQC spectra signal intensities are influenced by compound-dependent factors such as different INEPT transfer efficiencies. The considerable deviations between measured and expected values are also evidenced by the Bland-Altman plot (Figure 3A).

As noted above, an individual calibration factor may be calculated for each C-H group. The calibration factors of the eight spiked-in metabolites were averaged to obtain a mean calibration factor. As expected, using a mean calibration factor brings the slopes of the regression lines (Table 2) closer to 1 with a relative standard deviation of 14.2%. The Bland-Altman plot (Figure 3B) shows improved quantification results as compared with the Amix exact reference approach. However, a trumpet-shaped distribution of the residuals indicates high variances for large concentrations. The correlation of variance to metabolite concentration value is a well-known phenomenon of NMR data (13). Of 64 possible concentration values (8 compounds in 8 samples), 48 values were determined. Amix automatically excluded compounds for which one or more peaks were not found in the spectrum, affecting mostly histidine. Due to a bad line shape for the histidine peak at 8.01/1379 ppm (H-13C), Amix did not integrate this peak in 5 out of 8 spectra. In the remaining spectra, the peak was integrated but with drastically decreased intensities.

Quantitative results using MetaboQuant
The basic mode of MetaboQuant offers several outlier detection algorithms, but requires no compound-specific values. When using the basic mode in combination with a mean calibration factor, 59 values were calculated, yielding a mean slope close to 1 with a relative standard deviation of 16.5% (Table 2). The resulting Bland-Altman plot (Figure 3C) is similar to that obtained with Amix using a mean calibration factor, although more concentration values were obtained. The four lowest BHBA values and the lowest acetate value were not determined. For the lowest concentration of BHBA and acetate, no peaks were observed above noise level. The three other missing BHBA values were excluded as the number of observed peaks was below the threshold. The histidine peak at 8.01/1379 ppm was automatically excluded in all samples by outlier detection. Thus, quantitative results rested on the remaining histidine signals. It is not surprising that the standard deviation is higher in this case than in the Amix results mentioned above, as more low-abundance compounds were quantified.

Next, the advanced mode of MetaboQuant without individual lower limits of quantification was applied. This mode includes scaling peak integrals by individual calibration factors and reliability checks. The number of calculated concentration values rose to 62 and the relative standard deviation of the calculated slopes (Table 2) was 6.87%. The Bland-Altman plot in Figure 3D shows considerably smaller deviations between measured and expected values when compared with the previously discussed approaches. Additionally, the trumpet-shape (indicating stronger deviations) starts at higher concentration levels and is less pronounced than for the basic mode and Amix, showing improved quantification results. Figure 3D shows that all strongly deviating values were over-quantified. This is probably the result of peak overlap still present in the data even after employing all reliability checks. The lowest concentration values of acetate and BHBA were missing since no peaks were observed above the noise level. The standard deviation is lower compared with both Amix and basic mode MetaboQuant results, indicating high reliability of the quantification results.

Finally, concentration values falling below predefined individual lower limits of quantification were excluded automatically. This yielded 61 concentration values, excluding the lowest acetate value and the 2 lowest BHBA values. The other performance values (Table 2) and the Bland-Altman plot (Figure 3E) were similar to advanced mode without checking quantification limits.

The fact that the reliability checks removed badly integrated signals of histidine indicates that the routines are robust in excluding outliers due to both signal overlap (causing increased integrals) and technical reasons such as imperfect peak picking or integration (causing decreased integrals).

Quantification was repeated on 1D 1H spectra of the same samples, using Amix peak shape analysis. Results are similar to the results for HSQC spectra (Supplemental Tables S3 and S4; Supplemental Figure 1). The slightly worse performance is probably due to the fact that 1D 1H spectra are more prone to peak overlap than HSQC spectra. Using line shape analysis methods reduces this issue to some extent. Therefore, we recommend line shape analysis to create peak integrals for use in MetaboQuant.

As a note of caution, a thorough investigation of all peaks used for quantification should be performed on test samples to identify peaks that are prone to creating non-reliable values. Such peaks should be excluded from quantitative analysis. When setting up a quantification protocol, comparison to an orthogonal
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Competing interests
The authors declare no competing interests.

References

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