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## **Anti Citrullinated Protein Antibodies-IgG variable domain glycosylation in rheumatoid arthritis**

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# Chapter 5

## HappyTools: A software for high-throughput HPLC data processing and quantitation



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## ABSTRACT

High-performance liquid chromatography (HPLC) is widely used for absolute quantitation. The advent of new columns and HPLC technology has enabled higher sample throughput, and hence, larger scale studies that perform quantitation on different sample types (*e.g.* healthy controls vs. patients with rheumatoid arthritis) using HPLC are becoming feasible. However, there remains a lack of methods that can analyse the increased number of HPLC samples. To address this in part, the modular toolkit HappyTools has been developed for the high-throughput targeted quantitation of HPLC measurements. HappyTools enables the user to create an automated workflow that includes retention time ( $t_r$ ) calibration, data extraction and the calculation of several quality criteria for data curation. HappyTools has been tested on a biopharmaceutical standard and previously published clinical samples. The results show comparable accuracy between HappyTools, Waters Empower and ThermoFisher Chromeleon. However, HappyTools offered superior precision and throughput when compared with Waters Empower and ThermoFisher Chromeleon. HappyTools is released under the Apache 2.0 license, both the source code and a Windows binary can be freely downloaded from <https://github.com/Tarskin/HappyTools>.

## INTRODUCTION

High-performance liquid chromatography (HPLC) with fluorescence detection (FD) is a widely used technique for the analysis of biological samples, *e.g.* proteins, metabolites and glycans. Glycans are a class of post-translational modifications that can be added to a protein, thereby modifying the structure and function of the protein (1,2). One of the main advantages of HPLC-FD in glycan analysis is its ability to separate isomers, *e.g.* enabling the differentiation of the two isomers of one of the main glycans of immunoglobulin G (IgG) (3). Furthermore, the relatively low cost of an HPLC-FD instrument compared to a matrix-assisted laser desorption/ionization (MALDI)-time of flight (TOF)-mass spectrometry (MS) or liquid chromatography (LC)-electrospray ionization (ESI)-MS setups means that an HPLC-FD setup is very cost-effective for profiling biological and biopharmaceutical samples. Traditionally, sample analysis on the HPLC is time consuming due to gradient lengths. However, in recent years column manufacturers and researchers have been working on improving the throughput of HPLC (4–6). Therefore, the bottleneck is slowly shifting from the experimental side (*e.g.* glycan release and sample clean-up) to the data analysis side. Consequently, many HPLC-FD studies use a limited number of samples and tend to rely on commercially available software packages for the data analysis, *e.g.* Waters Empower or ThermoFisher Chromeleon (7,8).

There are several software tools available to assist with the identification and annotation of HPLC-FD data, such as GlycoDigest and GlycoStore (9–11). However, despite the widespread availability of HPLC-FD equipment there has been a lack of novel quantitative software tools for data processing being developed compared to other equipment, such as MALDI-TOF-MS or LC-ESI-MS (12–14). Such novel software tools have enabled the largest glycomics study to date, a study comprising the acquisition and comparison of over 20,000 MALDI-Fourier-transform ion cyclotron resonance (FTICR)-MS spectra (15). The lack of such freely available high-throughput (HT) data processing tools for HPLC-FD has hindered its application in large scale studies. To enable larger scale studies, a software tool dedicated to the data processing of HPLC-FD data should contain retention time ( $t_r$ ) calibration, peak detection, peak quantitation and should facilitate data curation.

To address the deficiency of software tools for HPLC-FD data processing, a modular toolkit called HappyTools has been developed that can perform all the steps of a completely automated data analysis workflow, including automated peak detection,  $t_r$  calibration and peak quantitation. To facilitate rapid development and reduce the overall source code complexity, HappyTools uses standardized source code from our previous work (13,14). Key improvements include automated peak detection, a Gaussian Peak Quality (GPQ)

score and a plugin functionality. The availability of automated peak detection addresses a problem that was present in our previous work, namely that a pre-defined analyte list is required to quantify a sample (13,14). The automatically determined analyte list significantly reduces the time required to generate the final analyte list for quantitation. The GPQ is part of several HPLC-FD specific quality control (QC) criteria that HappyTools can calculate to facilitate data curation. It is calculated as the percentage of total peak area ( $A_p$ ) that is explained by a fitted Gaussian peak, *i.e.* total  $A_p$  divided by the Gaussian  $A_p$ . The GPQ parameter can help to identify overlapping peaks, peak tailing and peak fronting. The current features of the HappyTools toolkit should facilitate most HPLC-FD based research, however some studies will require highly customized features. Therefore, a plugin system was developed for HappyTools that enables any externally developed Python package to utilize all the functions of HappyTools. Key benefits of this approach are that the development time for a custom feature is significantly reduced and that the developers only have to maintain their own codebase as the HappyTools codebase will be maintained by the HappyTools development team. For example, an affiliated group is currently developing a highly specialized plugin for automated peak detection algorithms of specific sample types (16).

While HappyTools can be used on any 2D chromatographic or electrophoretic data, it was developed and tested using chromatographic glycomics data. Specifically, the performance and applicability of HappyTools has been demonstrated on two sample sets, namely a set of 9 replicates of a biopharmaceutical monoclonal antibody (mAb) reference standard and a set of previously published anti-citrullinated protein antibodies (ACPA)-IgG samples (17). The results show that HappyTools enabled a highly automated data processing workflow that yielded comparable accuracy but improved precision and throughput when compared to either Waters Empower or ThermoFisher Chromeleon.

## DESIGN AND IMPLEMENTATION

### *General Design and Dependencies*

HappyTools has been developed as a graphical application for Python 2.7 (18). The program requires several external Python libraries to function, *i.e.* SciPy, numpy and matplotlib (19–21). The GitHub repository for HappyTools contains a pip freeze output, to facilitate the deployment of the package. A Windows binary of HappyTools is also available for each release on the GitHub repository. A modular design was used to facilitate further development and to enable the use of some of the HappyTools functions in other programs. For example, Python plugins can be added to HappyTools by placing

them in the plugin folder, which includes an example called “Demo”. The source code and all related HappyTools files have been released under the Apache 2.0 License (22).

### *Data Import*

HappyTools is designed to use non-proprietary data formats, *e.g.* a text format that can be exported from ThermoFisher Chromeleon. The currently supported formats are ThermoFisher Chromeleon “.txt” and Waters Empower “.arw” files. HappyTools will implement support for additional data formats in future releases, until all current data formats are supported.

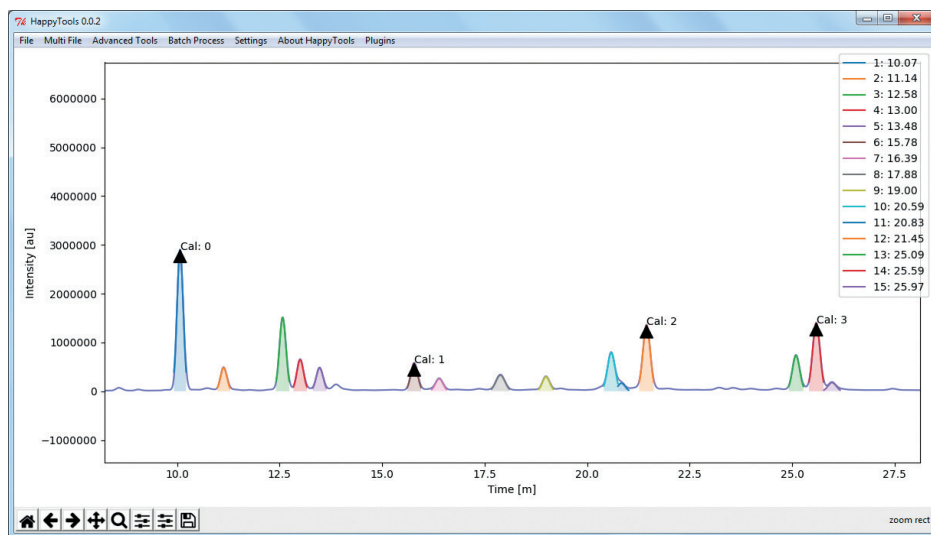
### *Automated Peak Detection*

HappyTools performs  $t_r$  calibration and quantitation based on a pre-defined peak list, which is a tab separated text file with the peak name, peak  $t_r$  and the  $t_r$  window ( $\Delta t_r$ ) per line. A peak file can either be provided by the user or generated by the automated peak detection algorithm of HappyTools. Many methods exist for automated peak detection in HPLC, ranging from using the 1<sup>st</sup> and 2<sup>nd</sup> order derivative of locally smoothed signals to bi-Gaussian mixture models (16,23,24). HappyTools uses an approach that uses the local maxima and minima of a 1<sup>st</sup> order derivative of the piecewise polynomial fitted raw data, which we have dubbed the first order derivative – Gaussian peak detection (FOD-GPD) algorithm. First, the overall background and noise are determined using a modification of the MassyTools method, *i.e.* taking the average and standard deviation of a set of sequential data points that yields the lowest average (13). Subsequently, the algorithm fits a univariate spline through a user-defined region of the raw data. The 1<sup>st</sup> order derivative ( $f'(x)$ ) of the univariate spline is determined and used to identify the local maxima and minima of  $f'(x)$ , as this offered superior performance over identifying where the second order derivative ( $f''(x)$ ) is 0. The highest intensity data point that falls between a neighbouring maximum and minimum of  $f'(x)$  is used to determine the intensity cut-off for the subsequent loop. Specifically, a setting of 1% tells the next step of the algorithm to continue until the current highest intensity data point falls below 1% of the initial highest data point.

The main part of the algorithm is a loop that continues until the highest intensity falls below the user-specified cut-off value. A new univariate spline is fitted for each iteration of the loop, from which the local maxima and minima are determined by using the 1<sup>st</sup> order derivative. The data points between a neighbouring maximum and minimum of  $f'(x)$  that yield the highest intensity data point in  $f(x)$  are used to fit a Gaussian peak. The last step in the loop is to subtract the Gaussian peak from the overall data. A schematic representation of the algorithm is presented in **S1 Fig**. The FOD-GPD algorithm will show



the raw data and the automatically detected peaks in the graphical user interface (GUI) of the program for the user to review (**Fig 1**). The automatically detected peaks can then be stored using the HappyTools peak list format, where each line contains an arbitrary peak number, the  $t_r$  of each Gaussian peak and a  $t_r$  window based on the full width at half maximum (FWHM). Furthermore, a set of potential calibrant peaks is automatically determined by taking a set of the four highest intensity data points that the algorithm attempts to space evenly in  $t_r$ .



**Fig 1.** Automated peak detection using HappyTools. An HPLC measurement of 2-aa AC-PA-IgG glycans was used to perform automated peak detection, with a threshold of 5% relative abundance of the initial main peak at around 10 min. Automated peak detection using a method that fits Gaussian peaks identified 15 peaks, which includes a peak that partially overlaps with another peak at 20.85 min. However, it is important to note that using a Gaussian function on non-Gaussian data that is the result of chromatographic problems will identify additional peaks (**S2 Fig**).

### Retention Time Calibration

High quality quantitation requires that all peaks for quantitation are well defined. Therefore,  $t_r$  calibration of chromatograms is an essential step in HPLC data processing. A set of  $t_r$  calibrated chromatograms enables the consistent quantitation of a low abundant analyte that is not present in all chromatograms. Furthermore, a stringent  $t_r$  calibration also enables integration using the minimum  $\Delta t_r$  to match the actual peak width. Whereas, a non-stringent  $t_r$  calibration forces the  $\Delta t_r$  to be set wider than the actual peak width. HappyTools performs  $t_r$  calibration based on a user defined calibrant peak list instead of the commonly used alignment based on automatically detected features (25). The

format of the calibrant peak list is similar to the quantitation peak list, specifically it is a tab separated text file with the peak name,  $t_r$  and  $\Delta t_r$ . The algorithm takes the highest intensity data point within each calibrant peak  $t_r \pm \Delta t_r$  as the observed  $t_r$ . The signal-to-noise ratio (S/N) of each calibrant peak is then calculated, and only calibrant peaks passing a user specified S/N threshold are retained. A 2<sup>nd</sup> degree polynomial ( $f_p(x)$ ) is fitted through the retained  $t_r$  coordinates ( $t_r(\text{Obs.})$ ,  $t_r(\text{Exp.})$ ), only if the number of retained  $t_r$  coordinates surpasses the user defined minimum number of calibrants. Finally, the  $t_r$  calibration is performed using the formula  $t_r(\text{new}) = f_p(t_r(\text{original}))$ .

### Peak Quantitation

There are several methods to quantify a peak, a commonly utilised technique is to fit a peak shape on the raw data and report the area of the fitted peak shape (24,26). This method works well for high abundant analytes that generally show symmetric peak shapes. However, for low abundant analytes that may not have a clear peak shape the peak fitting method yields poor results (14). An alternative method to determine the  $A_p$  is peak integration of the raw data as follows:

$$A_p = \sum_{i=1}^n t_m I_i$$

where  $t_m$  is the time that it takes to measure a single data point and  $I_1, I_2, \dots, I_n$  are the signal intensities. Recent research using LC-MS data has shown that peak integration provides comparable results for high abundant analytes and yields superior results for low abundant analytes using MALDI-TOF-MS data (13). Therefore, HappyTools performs peak quantitation using an adaptation of the peak integration method previously used in both MassyTools and LaCyTools (**Equation 1**) (13,14).

### Quality Control Criteria

HappyTools calculates three QC criteria for each analyte to facilitate data curation. The first criterion calculates the residual retention time ( $r_t$ ) by comparing the expected and observed  $t_r$  of each signal. The observed  $t_r$  is determined by taking the function maximum of a fitted interpolated univariate spline through all the data points of a signal. The  $r_t$  is then calculated for each analyte as described below (**Equation 2**).

$$r_t = |t_r(\text{Obs.}) - t_r(\text{Exp.})|$$

HappyTools can also determine the S/N of an analyte, based on a implementation used in both MassyTools and LaCyTools (13,14). Briefly, the  $t_r$  region around an analyte that has the lowest average intensity is identified, from which the average intensity is used as

background and the standard deviation is used as noise. Subsequently, the background is subtracted from the maximum intensity prior to dividing the remainder by the noise. GPQ is the third criterion offered by HappyTools, which determines how well a single Gaussian peak matches the quantified signal (27). The background value is subtracted from each data point, after which a Gaussian is fitted to all background subtracted data points. The GPQ is then calculated by dividing the  $A_p$  of the fitted Gaussian peak with the background subtracted  $A_p$  (**S3 Fig**).

## MATERIALS AND METHODS

### *Samples*

This study used previously measured and published clinical samples, to assess the performance of HappyTools (17). Permission for conduct of the study was in compliance with the Helsinki Declaration, and was approved by the Ethics Review Board at the Leiden University Medical Center.

### *Chemicals, Reagents and Enzymes*

Immunoglobulin G 1 from human myeloma (I5154) and acetic acid were acquired from Sigma-Aldrich (Dorset, UK). Trypsin Gold (V5280) was obtained from Promega (Madison, USA). Phosphate-buffered saline (PBS), trifluoroacetic acid (TFA) and hydrophilic interaction amide cartridges (LudgerClean LC-A-24) are all components in the LudgerTag™ V-tag Glycopeptide Labeling and Enrichment Kit (LT-VTAG-24) which is sourced from Ludger Ltd (Abingdon, UK). Acetonitrile was acquired from Romil (Cambridge, UK).

### *Sample Preparation*

A total of 10  $\mu$ g of IgG was digested using trypsin Gold. Samples were buffer exchanged into PBS to a concentration of 1 mg/mL. 10  $\mu$ L of the protein solution was transferred into an Eppendorf vial and 4  $\mu$ L of 0.5 mg/mL trypsin in 0.1 M acetic acid in water was added. The mixture was incubated for 1 h at 55°C with sonication. The sample pots were cooled to room temperature and 5  $\mu$ L of the V-Tag labelling reagent was added directly to each digested sample. The samples were vortexed and briefly centrifuged. The labelling reaction was allowed to proceed for 1 hour at 37 °C. The V-Tag labelled samples were cleaned up using hydrophilic interaction amide LC-A cartridges. Each sample was loaded onto a primed cartridge in 76% aqueous acetonitrile. The cartridge was washed with 76% acetonitrile, 0.1% TFA in water solution. The purified V-Tag labelled glycopeptides were eluted from the amide cartridge in 0.5 mL of a solution containing 40 % ACN and 0.1% TFA in water.

### Data Acquisition

V-Tag labelled samples were analysed by HILIC-UPLC using an ACQUITY UPLC® BEH-Glycan 1.7  $\mu\text{m}$ , 2.1 x 150 mm column from Waters at 40 °C on an ACQUITY UPLC H Class instrument with a fluorescence detector ( $\lambda_{\text{ex}}$  = 250 nm,  $\lambda_{\text{em}}$  = 360 nm), controlled by Empower version 3.0 build 3471. The solvents used for the HILIC-UPLC were 50 mM ammonium formate (A) made from Ludger stock buffer LS-BUFAMMFORM-2M-50ML (Ludger) and Acetonitrile 190 SpS (B). The gradient used for HILIC-UPLC was run as follows: 0.0 min, 28% solvent A; 54.0 min, 48% solvent A; 57.0 min, 100% solvent A; 60 min, 28% solvent A. The flow rate was 0.4mL/min from 0.0 to 57.min, from 57.0 to 60.0 min the flow rate was 0.2mL/min.

### Data Processing

V-Tag labelled samples were processed using Waters Empower, ThermoFisher Chromeleon and HappyTools. The Waters Empower processing included automated peak detection and integration, using the ApexTrack algorithm. The ThermoFisher Chromeleon processing of the V-Tag labelled glycopeptides included automated peak detection and integration, using the default method. ThermoFisher Chromeleon could not reliably quantify two peaks that were partially overlapping, therefore these two peaks were quantified as a single peak. The results were exported to excel, with each measurement producing a single excel file from which the absolute  $A_p$ s were taken to calculate the average relative  $A_p$  and coefficient of variation (CV).

V-Tag labelled glycopeptide samples were first  $t_r$  calibrated using HappyTools using four calibrant peaks (**S1 Table**). The mean  $t_r$  for all seven glycopeptide peaks was acquired by overlaying the calibrated chromatograms using HappyTools' normalized batch plot functionality. HappyTools was used to quantify all parameters for the seven glycopeptide peaks (**S2 Table**). The following settings were used for HappyTools; start  $t_r$ : 11.0 min, end  $t_r$ : 25.0 min, background window: 1.0 min, minimum number of peaks for calibration: 4, minimum signal-to-noise for calibration: 9, minimum relative abundance for peak detection: 1%, order of baseline function: 1 and number of data points for the determination of the baseline: 100.

HappyTools was also used to process a set of previously published ACPA-IgG samples that were previously processed using ThermoFisher Chromeleon (17). HappyTools was run without  $t_r$  calibration as the measurements were already calibrated. Subsequently, the quantitation was performed using the same peaks as were used in the original study with a quantitation width of 0.2 min. The following settings were used for HappyTools; start  $t_r$ : 5.0 min, end  $t_r$ : 30.0 min (ACPA-IgG Fab and ACPA-IgG part 2) or 35.0 min (ACPA-

IgG part 1), background window: 1.0 min, minimum number of peaks for calibration: 4, minimum signal-to-noise for calibration: 27 (ACPA-IgG part 1 and ACPA-IgG part 2) or 9 (ACPA-IgG Fab), order of baseline function: 1 and number of data points for the determination of the baseline: 100.

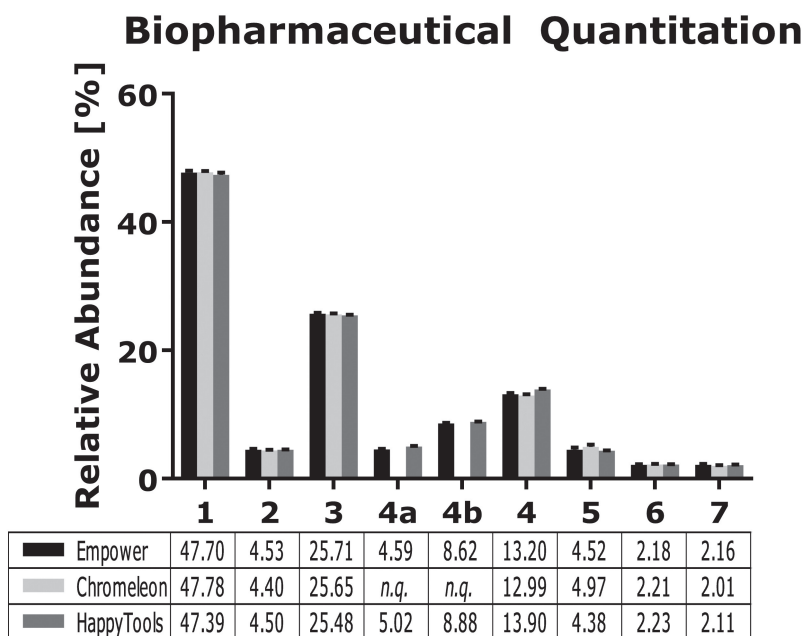
## RESULTS

HappyTools was tested using data from a biopharmaceutical standard and previously published clinical samples to test the accuracy, precision and throughput of the quantitation. The accuracy achieved by HappyTools is comparable to both Waters Empower and ThermoFisher Chromeleon, while the precision of HappyTools was superior to both Waters Empower and ThermoFisher Chromeleon. Furthermore, the processing time using HappyTools was significantly shorter than the total processing time using both Waters Empower and ThermoFisher Chromeleon. The complete datasets, a visual tutorial of HappyTools and a document describing how to reproduce the results used in this study have been included as supplementary material (**S1 Data**). The results below demonstrate the application of HappyTools on relatively simple samples. Notably, preliminary data on more complex samples, *e.g.* full plasma *N*-glycome, are promising and indicate a broad applicability of the tool (data not shown).

### *Biopharmaceutical mAb Reference Standard*

A set of 9 replicates of V-Tag labelled tryptic glycopeptides from a mAb reference standard was measured by hydrophilic interaction liquid (HILIC) chromatography and processed using Waters Empower, ThermoFisher Chromeleon and HappyTools. HappyTools' automated peak identification was used to detect all peaks above 1% relative abundance of the initially highest peak, which yielded 20 peaks (**S4 Fig**). The number of peaks that were used for the comparison was reduced to 7 to match the number of peaks that could be quantified using both Waters Empower and ThermoFisher Chromeleon. The results show that the relative  $A_p$ s reported by HappyTools are comparable to both Waters Empower and ThermoFisher Chromeleon (**Fig 2 and S3 Table**). The precision of HappyTools was superior to both Waters Empower and ThermoFisher Chromeleon, with all peaks except one showing a lower CV (**S3 Table**). HappyTools showed an average 2.22- and 2.26-fold change improvement of the CVs when compared to Waters Empower and ThermoFisher Chromeleon, respectively (**S4 Table**). It is possible to further improve the accuracy and precision by adjusting the quantitation window per glycopeptide, *e.g.* lowering the quantitation window for all analytes from  $\pm 0.15$  min to  $\pm 0.10$  min lowered the CV from 0.67% to 0.53% for the most abundant glycopeptide (data not shown).

Lastly, the throughput of HappyTools was superior to both Waters Empower and ThermoFisher Chromeleon. The total processing time using both ThermoFisher Chromeleon and Waters Empower included manual adjustment of the peak edges within each run, to ensure that the results were comparable. The average relative abundance and variation were acquired by either processing the individual results in Excel (ThermoFisher Chromeleon) or by creating several templates (Waters Empower). The processing time using HappyTools included performing automated peak detection and manual curation of the automatically detected peaks. The resulting total processing time using HappyTools was 1 hour, whereas the total processing time using ThermoFisher Chromeleon or Waters Empower was 3 hours.



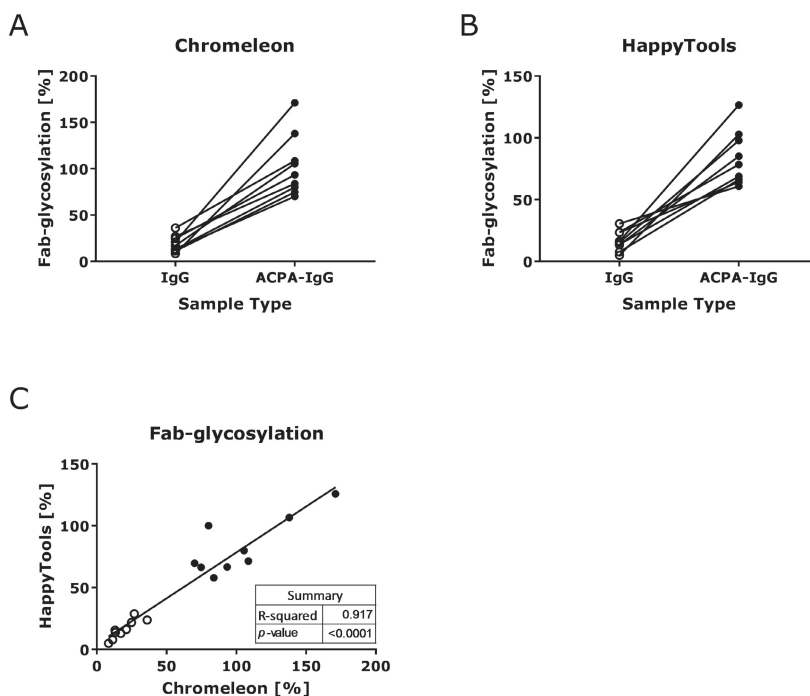
**Fig 2.** Biopharmaceutical quantitation using Waters Empower, ThermoFisher Chromeleon and HappyTools. A set of 9 replicates of V-Tag labelled tryptic glycopeptides were used to compare the three different software tools. The results show that all methods yield comparable accuracy, while HappyTools yields superior precision. Peak 4a and peak 4b could not be quantified separately using ThermoFisher Chromeleon but was instead quantified as a singular peak. The individual values for peaks 4a and peak 4b obtained from Waters Empower and HappyTools were summed to compare with ThermoFisher Chromeleon.

### Clinical Samples

A total of 36 measurements of 2-aminobenzoic acid labelled ACPA-IgG, ACPA-IgG Fc and ACPA-IgG Fab glycans were previously prepared, measured using HILIC-ultra high performance liquid chromatography (UHPLC) and exported to .txt format using ThermoFisher Chromeleon (17). The ACPA-IgG and ACPA-IgG Fab measurements were used to assess if HappyTools produces comparable results to the previously used ThermoFisher Chromeleon in a clinical setting. Specifically, Fab glycosylation in IgG has been found to be vastly different between ACPA-IgG and normal IgG, with ACPA-IgG showing on average five times higher levels of Fab glycosylation (17). The observed difference in Fab glycosylation suggests that ACPA-IgG may mediate novel immunological activities (17). The study compared IgG and ACPA-IgG Fab glycosylation (**S5 Fig**), hereby the glycans of the F(ab')<sub>2</sub> fragments and Fc glycopeptides were compared to the glycan profile of the total antibody and the percentage of Fab glycosylation was calculated. The following formula was used to calculate the percentage Fab-glycosylation:

$$Glycosylation_{Fab}[\%] = \frac{\left(\frac{G2S2_{Total}}{G2S2_{Fab}}\right)}{\left(\frac{G1F_{Total}}{G1F_{Fc}}\right)}$$

where G2S2 consists of GP21, GP22, GP23 and GP24, G1F<sub>Total</sub> consists of GP8 and GP9 and G1F<sub>Fc</sub> is taken from glycopeptide measurements of the original publication (17). The results for both Chromeleon and HappyTools show a higher percentage of Fab-glycosylation in ACPA samples than IgG samples, with the values reported by ThermoFisher Chromeleon and HappyTools showing a significant correlation (**Fig 3 and S5-S7 Tables**). This result shows that the same clinical finding, namely an increase in Fab-glycosylation in ACPA-IgG samples, can be observed with either ThermoFisher Chromeleon or HappyTools. Finally, the total throughput using HappyTools was far superior than what was achieved during the original study. Specifically, during the original study the processing took around 10 hours while the re-analysis using HappyTools took only 1 hour.



**Fig 3.** ThermoFisher Chromeleon vs. HappyTools comparison of total IgG and ACPA-IgG. A total of 36 UHPLC measurements was used to compare the quantitation as performed by HappyTools with the original quantitation performed using ThermoFisher Chromeleon. Data points that derived from IgG are indicated by an open circle, while data points that derive from ACPA-IgG are portrayed by closed circles. The results show that both programs yield a similar result and more importantly that there is a significant ( $p < 0.0001$ ) correlation between the two data sets.

## DISCUSSION

High performance liquid chromatography (HPLC) has long been considered the gold standard for quantitation of carbohydrates, specifically when combined with HILIC and 2-aminobenzamide (2-AB)-labelled glycans (28). While the number of samples that is measured by HPLC has been increasing, *e.g.* a study into the Immunoglobulin G glycome measured 2298 individuals, the data analysis is mostly achieved using a high amount of manual processing using the manufacturers software (3). Therefore, the main goal of HappyTools was to provide a framework independent of the manufacturer that would enable high-throughput processing of HPLC data, which includes  $t_r$  calibration, quantitation and the determination of various quality criteria. The application of the software on biopharmaceutical and clinical samples showed similar or better performance than either Waters Empower or ThermoFisher Chromeleon. The main improvements were



observed in the precision and throughput. Specifically, the biopharmaceutical samples showed a 2.22- or 2.26-fold change improvement when compared to Waters Empower and ThermoFisher Chromeleon, respectively. Furthermore, the total processing time showed a 10-fold reduction for the biopharmaceutical samples and a 3-fold reduction for the clinical samples.

However, HappyTools was primarily designed as a targeted data processing package and therefore requires knowledge of the analytes that can be present in each sample. For instance, when comparing samples from a healthy and an immunocompromised source, the software requires a list of analytes for quantitation. To address this issue HappyTools includes a basic automated peak detection functionality, which identifies the  $t_r$  and  $\Delta t_r$  of all signals above a user-defined threshold. The implemented peak detection algorithm assumes that signals give a Gaussian peak shape. However, the algorithm will have difficulties with chromatograms that contain significant peak tailing. Therefore, it is important to curate the results when an automatically detected peak list is used for quantitation. HappyTools can also calculate several quality criteria that facilitate easy results curation, such as the Gaussian Peak Quality (GPQ) and the signal-to-noise (S/N) value. For example, the GPQ will yield a poor value for the tail of a peak if it is quantified based on an automatically determined peak list.

There are also some limitations to HappyTools, which include a lack of good manufacturing practice (GMP) functionality and the dependency on Python. The manufacturer software packages generally include options to protect data integrity and prevent data manipulation (29,30). HappyTools currently does not contain any method to guarantee data integrity, *e.g.* by verifying if results match with a given data set. However, there are plans to include such a functionality by calculating a number representing the raw data (checksum) for each processed chromatogram and including it with the results. Furthermore, HappyTools will then also include the option to calculate the checksum for any chromatogram which will allow the researcher to validate that the results match the data set. The second limitation is that HappyTools requires Python 2.7 with several external libraries meaning that it requires some IT knowledge to deploy and use HappyTools. Therefore, a Windows binary will also be released for every major version/release of HappyTools which should enable researchers to easily try the software. Furthermore, we are also in the process of developing a web application-based version of HappyTools, which will allow researchers to perform automated  $t_r$  calibration, quantitation and the calculation of chromatographic quality criteria on a limited number of samples.

In summary, HappyTools provides a fully open-source and transparent toolkit for the high throughput data processing of HPLC data. HappyTools enables  $t_r$  calibration, quantitation and the calculation of various quality criteria. HappyTools has been shown to offer similar precision and superior throughput when compared to currently available software such as ThermoFisher Chromeleon. The source code for HappyTools and a Windows binary can be freely downloaded from [www.github.com/Tarskin/HappyTools](http://www.github.com/Tarskin/HappyTools).

### *Acknowledgements*

We would like to thank Genadij Razdorov and Felix Agakov for helpful discussions related to the automated peak detection. Furthermore, we would like to thank Louise Royle for helpful discussions about good manufacturing practice and the use of manufacturer software.

### *Competing Interests*

BCJ, RAG, JLH and DIRS work for Ludger Ltd – a commercial company that specialises in the development and validation of glycoprofiling technology for biologic therapeutics and biological tissues. Some of the Ludger products include glycan purification kits and glycan release kits. There are no patents and products in development. This does not alter the authors' adherence to PLOS ONE policies on sharing data and materials.

### *Supporting information available*

Additional information is available as stated in the text. This information is available via: <https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0200280#sec019>

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