Software comparison for metabolomics by NMR

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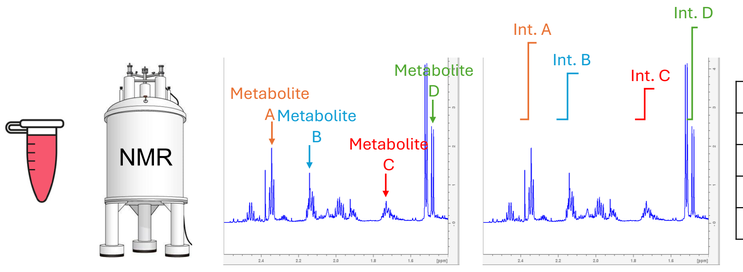
I. Introduction

METABOLOMICS = Identification / Quantification of METABOLITES (< 3.5 kDa) in biofluids



Concentration table

Metabolite	A	B	C	D	...
Sample 1	0.53	0.31	0.26	0.67	...
Sample 2	0.55	0.60	0.55	0.12	...
...	...	...	...	...	...
Sample n	Int_n A	Int_n B	Int_n C	Int_n D	...

METABOLOMICS STUDY STEPS

SAMPLE PREPARATION + ACQUISITION

SOFTWARE

MUST BE:

Reproducible · Accurate · User-Friendly · Supported by a Database · Fast · Available · Adaptable

Numerous applications are developed for metabolomics purpose. These are designed to perform multiple actions (**processing/identification/quantification**) while dealing with **large datasets**.

These software are expected to present some characteristics (listed on the right). We will compare five software based on these characteristics.

II. Material & Methods

SAMPLE PREPARATION — 3 x (540 µL DMEM + 60µL pH buffer $H_2PO_4^-$ / HPO_4^{2-} [DSS] = 5.31 mM [Formate] = 48.94 mM) → NMR

ACQUISITION
- 5 different pulse sequences (with solvent suppression and/or macromolecule suppression ...)
- NB: ChenomX & MagMet recommend specific pulse sequences and acquisition parameters

PROCESSING[1-4] — ChenomX NMR Mixture Analysis · COLMAR Complex Mixture Analysis by NMR · MagMet · CCPN · NOVA

The applications were selected based on their **availability** and straightforward **installation**. Some laboratories use an in-house protocol, which is not always available for outsiders.

GOAL

Reproducibility Name	3 spectra uploaded: Spectra acquired	3 spectra uploaded: Tube	Variability including: Software	Variability including: Experiment Acquisition	Variability including: Sample Preparation
SOFTWARE	Same	Same	V	-	-
INSTRUMENTAL	Different	Same	V	V	-
ANALYTICAL	Different	Different	V	V	V

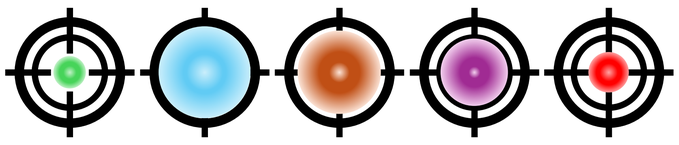
III. Results

VARIABILITY (in CV(%))

CV(%) calculation
All spectra were acquired with an identical acquisition protocol, and the concentrations of 18 metabolites shared by all databases were normalised by the formate concentration.
For each triplicate, the concentration's **CV(%)** were calculated **per metabolite**.
The table shows the **average** and **variability** of the CV(%) values **per software** analysis.

Outliers
Metabolites were ranked by CV(%) values. Metabolites at the extremities were considered as outliers when the **CV-difference** with the nearest metabolite **exceeded the standard deviation**. The outliers were removed, and the average and variability across the CV values were adapted.
Automatic analyses presented outliers coming from misfitting (COLMAR: CV(histidine) = 95%; MagMet: CV(histidine) = 65%) or superpositions (MestreNova: CV(arginine) = 124%).

Targets – Maximum variability on the concentrations for each software analysis (after outlier removal => lower rows in the table)
The darkening of the colour represents the metabolites distribution.



	Coefficient of Variation (%)				
	ChenomX	**COLMAR**	**MagMet**	**Ccpnmr**	**MestreNova**
Software	2 (± 1)	0 (± 0)	0 (± 0)	5 (± 6)	0 (± 0)
Instrumental	3 (± 2)	6 (± 6)	5 (± 9)	5 (± 3)	10 (± 28)
Analytical	2 (± 1)	12 (± 22)	10 (± 15)	2 (± 1)	11 (± 27)
Outliers *Cause*	/	Histidine *Wrong fit*	Histidine *Wrong fit*	/	Arginine *Superposition*
Instrumental	/	6 (± 6)	3 (±4)	/	3 (± 3)
Analytical	/	7 (± 8)	7 (± 7)	/	4 (± 3)

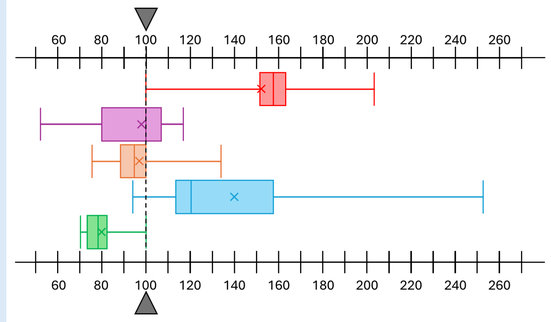
Table – Average of the concentration CVs per software analysis and reproducibility type. The values between parentheses represent the variation of the CV values along the metabolites.

ACCURACY (in %)

Ratio (%) calculation
"ANALYTICAL" spectra were used (*acquired with the same protocol on 3 different tubes*) and for each dataset extracted from a software, the concentrations of the 18 metabolites shared by all databases were normalised by the formate concentration.
For each triplicate, the **ratio (%) [measured] / [real]** was calculated **per metabolite**. The table shows the **average** and **variability** of the ratio values **per software** analysis.

Outliers
Metabolites were ranked by ratio values. Metabolites at the extremities were considered as outliers when the **ratio-difference** with the nearest metabolite **exceeded the standard deviation**
The outliers were removed, and the average and variability of the ratio values were adapted.

Box & Whisker plot – Distribution of [measured] / [real] (%) along metabolites for each software analysis. The average value is represented by x mark.



	ChenomX	**COLMAR**	**MagMet**	**Ccpnmr**	**MestreNova**
[measured] / [real]	80 ± 8	140 ± 44	97 ± 14	98 ± 26	152 ± 43
Outliers *Cause*	/	/	/	Methionine *Human error?*	Arginine, Choline *Superpositions*
[measured] / [real]	/	/	/	94 ± 19	152 ± 26

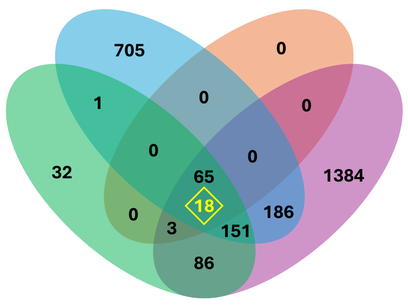
Table – Average and variability of the [measured] / [real] ratio (%) per software analysis.

DATABASE

The total number of metabolites per database is indicated in the table below, and their repartition is shown in the Venn diagram on the right.
The number in yellow indicates the metabolites present in the sample and shared by all database. These metabolites were used to assess the reproducibility and accuracy of each software.

MestreNova does not provide a database, but contains a data management system and allows to connect to an external database for which a licence is required.

Venn diagram – Repartition of the metabolites between the software databases



	ChenomX	COLMAR	MagMet	Ccpnmr	MestreNova
Total	337	1107	68	1874	-

Table – Total number of metabolites present in each software's database.

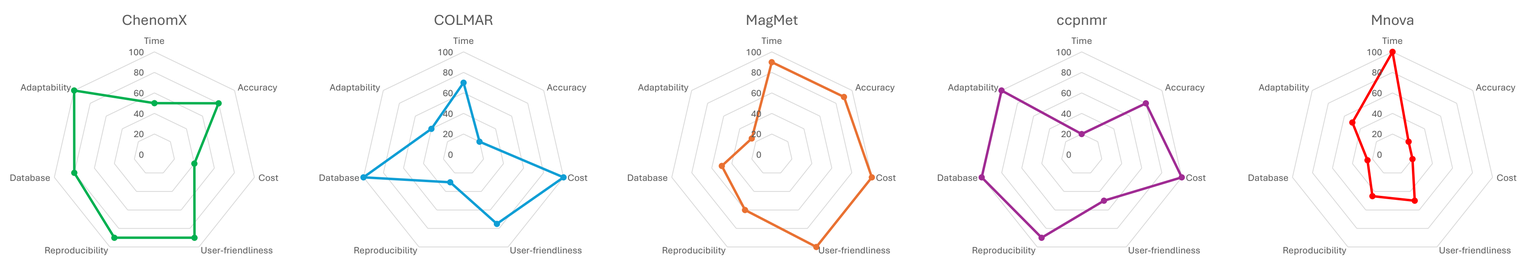
OTHER SOFTWARE FEATURES

The time required for an analysis, the price of the software, and a personal appreciation for the user-friendliness and adaptability of the software are summarised in this table. Some key points to justify the appreciations are mentioned on the lower row, with icons of the referred feature.
A colour gradient indicates which software is more advantageous than another software.

	ChenomX	**COLMAR**	**MagMet**	**Ccpnmr**	**MestreNova**
Time for 1 spectrum	48 min	6 min	11 min	2h20	1 min
Time for 25 spectra	14h	2h30	5h30	> 17h30	24 min
Cost	~500 € / year	Free	Free	Free	~825 € / year
User - Friendliness	+	+	++	--	-
Adaptability	++	-	--	+	-
Justifications	- Manual -Clear visualisation	- Semi-automatic - "click" protocol with parameters - Multi-analyses in parallel	- Full-automatic - "click" protocol - Poor database	- Manual - Poor visualisation - Crashes with large datasets	- Semi-automatic - No "click" protocol - Integration and no fit - Need to create "templates"

IV. Discussion / Conclusion

Radar graphs summarise the characteristics of the five applications. The axes were ordered to **minimise potential inter-axis correlations**. The values on the graph are based on a personal **APPRECIATION** based on all results obtained with each software and each experiment type.



1 – **Averages and variabilities calculated on groups of three spectra.** – These results illustrate **general tendencies** but should be interpreted with **caution**. For instance, the variability of Ccpnmr is likely closer to 5% rather than 2% as depicted by the "analytical variability". A human bias probably explains the difference between these "instrumental" and "analytical" variabilities.

2 – **MestreNova appears underperforming.** – Whereas the four other applications which deconvolute and fit all signals, **MestreNova integrates** only **one deconvoluted signal** per metabolite. This becomes limiting in overcrowded spectra, for metabolites lacking isolated signals or for pH-dependent protons. This limitations explain the **lower reproducibility, accuracy** and **adaptability**. The **absence** of **integrated database** in the software is justified by the quantification strategy of MestreNova.

3 – **Manual analyses show better reproducibility and adaptability.** – Reproducibility decreases with the number of metabolites considered for the calculations, particularly for automatic software. However, in untargeted metabolomics, as many metabolites must be quantified to extract maximal information. When all quantified metabolites are considered, the analytical CV(%) values are: ChenomX: 2.7 ± 2.8, COLMAR: 24.1 ± 37.6, MagMet: No changes, Ccpnmr: 2.4 ± 1.8, MestreNova: 34.8 ± 128.9. It is therefore obvious that **manual** software **outperforms automatic** software, likely due to their ability to **adapt** to crowded regions, low-concentration metabolites, pH-dependent signals and spectra defaults.

4 – **Experiment type bias** – All results presented are based on a **single experiment type** (*^1H + presaturation*). Some software are **more accurate** and **reproducible with alternative NMR experiments**. We can cite the *^1H + noe mixing + presaturation + gradient pulses* experiment that improves accuracy for ChenomX , Magmet and MestreNova analyses, or the *^1H + t_2-filter* experiment that enhances reproducibility for MagMet and MestreNova analyses. These effects are software-dependent, and experiment choice should match the sample type and study goal.

5 – **Best software?** – The optimal software depends on the objective of the metabolomics study. **Automatic** analyses show promise for **well-defined** and **standardises samples**, with MagMet appearing suited for such contexts. Conversely, **manual** approaches remain **more relevant for untargeted analyses**, where adaptability and accuracy are key.

6 – **Future investigations?** – A: Assess the **accuracy of relative quantification** across samples with different known concentrations. B: Introduce a "**validation step**" to correct errors like false-positive or misassignments. This was intentionally omitted here, but error sources were identified. Such additional step could improve reproducibility and accuracy in automatic analyses, though with a severe backlash in terms of time, possibly approaching manual performances. C: Assess software adaptability on **more complex samples**, e.g. by adding macromolecules.

V. Reference & acknowledgements

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There were **no financial affiliation** to the software providers.

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