relementR package

elementR¹ is an R package facilitating the reduction of elemental microchemistry data from solid-phase LA-ICPMS analysis (laser ablation inductive coupled plasma mass spectrometry). The **elementR** package provides a reactive and user friendly interface for conducting all steps needed for an optimal data reduction while leaving maximum control for users.

Documentation Summary

- 1. How to start?
- 2. What is the structure of the elementR application?
- 3. What data are required?
- 4. What do the different steps of the element reduction procedure contain?
 - Step 1: Building the project -
 - Step 2: Filtration of the standard replicates -
 - Step 3: Verification of the machine drift -
 - Step 4: Filtration of the sample replicates -
 - Step 5: Averaging of the sample replicates -
 - Additional step: Optional settings -
- 5. What is the exact process when elementR performs the machine drift verification and correction?
- 6. What are the exported data from elementR?
- 7. More questions?

¹ To cite this package:

Charlotte Sirot, Francois Guilhaumon, Franck Ferraton, Audrey Darnaude, Jacques Panfili and Amber-Robyn Childs (2016). elementR: A shiny application for reducing elemental LA-ICPMS data from solid structures. R package version 1.0. https://CRAN.R-project.org/package=elementR

1. How to start?

To install and load the **elementR** package:

- Prerequisites -

- 1. Have access to internet
- 2. To be installed and run properly, the **elementR** package needs an up-to-date version of the R software (at least \geq 3.2.3, to update your version click <u>here</u>).
- 3. For Macintosh platform and during its installation, <u>elementR</u> will ask you to install "Xcode/otool"packages², accept the installation. Moreover, the <u>software needs XQuartz</u>. <u>Please check that XQuartz is installed and runs properly on your computer</u>. If not, please visit here.

- Through the Cran website -

Open your usual R environment (R, Rstudio...) and run the following instructions:

```
install.packages("elementR", dependencies = T)
library(elementR)
```

- Through the Github website -

1. Install **elementR**'s dependencies by running the following commands in your R console:

```
pkg <- c("gdata", "shiny", "devtools", "shinyjs", "gnumeric", "R6", "shinydashboard",
"abind", "stringr", "lmtest", "tcltk2", "reader", "readODS", "readxl")
lapply(1:length(pkg), function(x) {install.packages(pkg[x], dependencies = T)})</pre>
```

2. Install and source **elementR** package from GitHub by running the following commands in your R console:

```
library(devtools)
install_github("charlottesirot/elementR", ref = "master", force = T, dependencies = T)
library(elementR)
```

The only function required to perform the data reduction is the runElementR() function. To launch the data reduction procedure, just run:

```
runElementR()
```

At this point, the web browser opens a new page with the operational application based on the **elementR** code.

Note: <u>elementR</u> runs on any web browser. However, as the graphic of the user interface has been developed based on Firefox, authors highly recommend to run <u>elementR</u> through this last web browser for an optimal design. Click here to download Firefox.

² If the installation is stuck at the installation of the tcltk package, this probably means that XQuartz does not run properly. Check its validity.

2. What is the structure of the elementR application?

The data reduction process conducted by the **elementR** package is organized in a session framework including five main steps (indicated on the left side bar of the opened page of the web browser):

- \rightarrow Step 1. The setting of the main parameters of the procedure (see <u>step1</u>)
- \rightarrow Step 2. The filtration of the standards (see step2)
- → Step 3. The machine drift verification & correction (see <u>step3</u>)
- \rightarrow Step 4. The filtration of the samples (see step4)
- \rightarrow Step 5. The sample replicate averaging (see <u>step5</u>)

Note that these steps have to be carried out in the order mentioned above, the third step, for instance, being not available until the first and second one are validated.

The left side bar allows to navigate between these steps and to know exactly which part of the procedure is currently running, being validated or remains to be completed.

The two last tabs "Configuration" and "Source code for app" are not part of the data reduction procedure but provide additional information detailed later in this document (see <u>additional settings</u>).

3. What data are required?

Running **elementR** code requires the appropriate data to be in a specified format and organized in a session framework.

- What data? -

- → Standard data a set of data from standard analysis (at least one). **elementR** is compatible with all standard types (NIST 612, NIST 610, MACS...). However, standards of a single session <u>must all be the same type of standard</u> (that is the reason we call them **standard replicates** and that they are stored **in the same folder**).
- → Calibration data a calibration file providing for each investigated chemical element:

$$Calib_{X} = \frac{[X]_{calibType}}{[Internal standard]_{calibType}}$$

where:

- Calib_x is the value to include in the calibration file for the chemical element X
- $[X]_{calibType}$ is the concentration of the chemical element X contained in the calibration material
- [Internal standard]_{calibType} is the concentration of the internal standard element contained in the calibration material
- \rightarrow Sample data a set of data from sample analysis (at least one, obviously :D). Sample may have one or more replicates, these replicates being averaged in the last step.

- How should the data be formatted? -

- \rightarrow The format of data compatible with the **elementR** application include Excel worksheets (.xls, .xlsx), LibreOffice (.ods) or text (.csv) files.
 - For the text format, the separator must be the semi-colon
 - For any format, the decimal have to be indicated by a point
 - For Excel format, **elementR** reads the first worksheet of the file. For OpenOffice format (.ods), only the first sheet will be imported (whatever its name).
- → Sample and standard data should conform to a standard layout (Fig. 1):
 - In column 1, the time of successive analysis
 - In the following columns, the chemical elements with element names as column heads
 - All data must contain the same chemical elements in the same order
 - Data must contain only numerical characters (except for the names of the columns)

Note:

When you upload your data, **elementR** checks the validity of these points and indicates if there are any problems.

Examples of compatible format of standard and sample data are provided with the **elementR** package, take a look (in the folder where R packages are installed³, open the **elementR** folder and look at the "Example_Session", you will find sample and standard examples of data format).

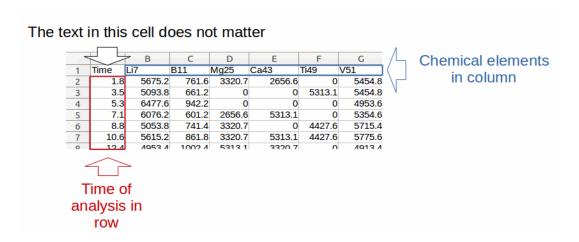


Figure 1: Example of compatible format of sample and standard replicate data for **elementR** application: chemical elements in columns (in blue) with their name at the top of each column, time of analysis as rows (in red). The text included in the cell at the top of the analysis time does not matter.

³ If you do not manage to find this folder on your computer, you can find its path by running:

install.packages("devtools", dependencies = T)
library(devtools)
filePath <- system.file("", package="elementR")</pre>

- \rightarrow Calibration data the file comprising the calibration data must have its own organization (Fig. 2):
 - In columns, the chemical elements with their name at the head of each column
 - The text in the cells of the first column does not matter
 - The data of the calibration file must contain the same chemical elements in the same order than in the standard and sample files
 - Data must contain only numerical characters (except for the first column and the names of the chemical elements)

Note:

When you upload your data, **elementR** checks the validity of these points and indicates if there are any problems.

Examples of compatible format of calibration data are provided with the elementR package, take a look (in the folder where R packages are installed⁴, open the **elementR** folder and look at the "Example_Session", you will find the file called "Calibration File NIST612.csv").

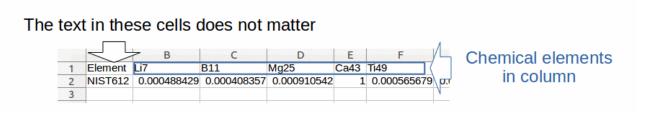


Figure 2: Example of data structure compatible with **elementR** application for calibration data: chemical elements in columns (in blue) with their name at the top of each column. The text included in the cells of the first column does not matter.

- How must the data be organized? -

- → The data must be organized in a session framework (Fig 3):
 - All files corresponding to standard replicates must be included in the same folder called "standards", and each file labeled according to each replicate (= batch of standard replicates)
 - All samples must be included in the same folder called "samples", and each subfolder labeled according to each sample
 - All sample replicates of the same sample (even if there is only a single replicate) must be included in the sub-folder with the name of the considered sample (= a batch of sample replicates)
 - The "standards" and the "samples" sub-folders must be included in a folder labeled according to the name of the project

Note:

When you upload your data, **elementR** checks the validity of these points and indicates if there are any problems.

⁴ if you do not manage to find this folder on your computer, see here

An example of compatible format session is provided with the **elementR** package, have a look (in the folder where R packages are installed, open the **elementR** folder⁵ and look at how the "Example Session" folder and its contents is organized).

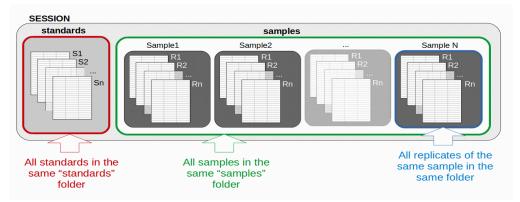


Figure 3: Example of session organization compatible with **elementR** application: all standards (S1, S2, Sn) are in a "standards" folder (in red), all samples are in a "samples" folder (in green). Each sample replicate R1, R2, Rn (even if there is only a single replicate) must be included in a sub-folder with the name of the sample (in blue). The name of the sample sub-folders and of the standard and sample replicates do not matter.

4. What do the different steps of the elementR reduction procedure contain?

- Step 1: Building the project -

In this first step, you build the project and set its parameters (Fig. 4).

Here, you have the choice to upload a new session (choose "New project") or to load an existing project for checking, finishing or editing results (choose "Load Project"). You can then upload the project or any other file thanks to dialog boxes which will be opened during these steps.

At any step of the session procedure, you can change the running project by going back to this first step and by clicking on the "Starting another project" button.

→ In the "New project" mode

By choosing this mode, you can select the directory containing the data to be filtered (see their organization in step 3.) and set the main information regarding the running session:

- The chemical element considered as internal standard (by default, Ca)
- The calibration data corresponding to the type of standard
- The rank of each standard and sample in ICPMS analysis. These inputs let *elementR* know the sequence of the standard and sample analysis in order to detect and correct the machine drift (for more details about this procedure and see <u>Step 3</u>. and <u>5</u>.)(the rank is an open choice. However, make sure that you complete all fields with unique numeric figures).
- When all is completed, you can validate your choice and proceed to the next step of the data reduction procedure.

⁵ if you do not manage to find this folder on your computer, see here

Note:

The **elementR** package provides an example of a session to run. To this aim, launch the runElementR() function and press the "Run Example" button.

To fill in the rank, you can click on the corresponding box and type in the rank of your replicate. You can then go to the next replicate by pressing "tab" on your keyboard.

→ In the "Load project" mode

By choosing this mode, you have to select the .RData file corresponding to the project to open and all data of this session will be automatically uploaded. You can validate your choice and proceed to the next step of the data reduction procedure.

Note:

The **elementR** package provides an example of a session to load. To this aim, run the runElementR() function and press the "Load Example" button .

When this first step is over, an "Export project" button appear at the bottom of the left side bar. It allows users to save their results by exporting the final data and saving the project (for more details on this procedure see step <u>6.</u>). Moreover, a brief summary of the session running is then displayed.

VERY IMPORTANT:

- → You must close all dialog boxes to be able to continue the filtration process. Moreover, if you run elementR from Windows, the dialog boxes can be hidden behind the others application dialog boxes. However, an icon will pop up in your taskbar to indicate that the dialog box is opened; click on it to see the dialog box.
- → The project and associated data are exported only when you have pressed the "Export Project" button. There is no automatic saving.

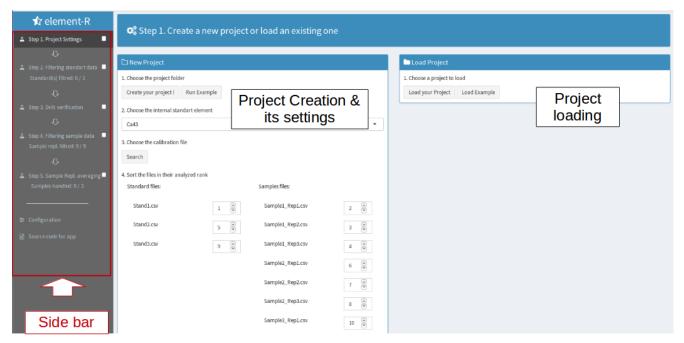


Figure 4: Standard filtration tab functionalities

Examples of project to run (to create or to load) are also provided ("Run example" or "load example" respectively) for users to have an overview of the reduction procedure. If you decide to run them, remember that exported data will be located in the folder of the installed package (to locate the package see here).

- Step 2: Filtration of the standard replicates -

In this second step, you proceed to filtering the standard replicates.

To this aim, you must first select the standard replicate to filter (Fig. 5). Once the replicate is chosen, raw and reduced data of the considered replicate appear in two boxes (Fig. 5). The left box allows you to to select the background (blank) and plateau thresholds using the slide selectors below the plot. The right box plots calculate the data in order to check the validity of your selected thresholds (i.e. reduced and intermediate data, see below).

elementR allows the user to check all the intermediate and final data of the filtration procedure. To do this, go to the right box and select the required data (see <u>Appendix 1</u> for more details about rendered data):

- "Blank" for the blank values
- "Raw" for raw data
- "Plateau" for plateau data
- "Blank removed" for data_SuppBlank.
- ">LOD" for data_SupLOD
- "Normalized" for data Norm
- "Outlier free" for the data OutlierFree

Once all thresholds are approved, all information can be saved by clicking on the "Save" button. You can delete this validation at any step of the procedure by going back on this tab and by clicking on the "Delete" button. You can repeat the procedure as many times as required until you obtain satisfactory results.

When step 2 is running, a "graphic export" box (collapsed by default, enlarged by clicking on the white cross) allows you to export graphic displayed on this page with the export format sets in the "Configuration" tab. This procedure could last depending on the number of graphics and chemical elements to export. Be patient or reduce the number of graphics or elements to export ©.

VERY IMPORTANT: The project and associated data are exported <u>only when you have pressed the "Export Project" button. There is no automatic saving.</u>

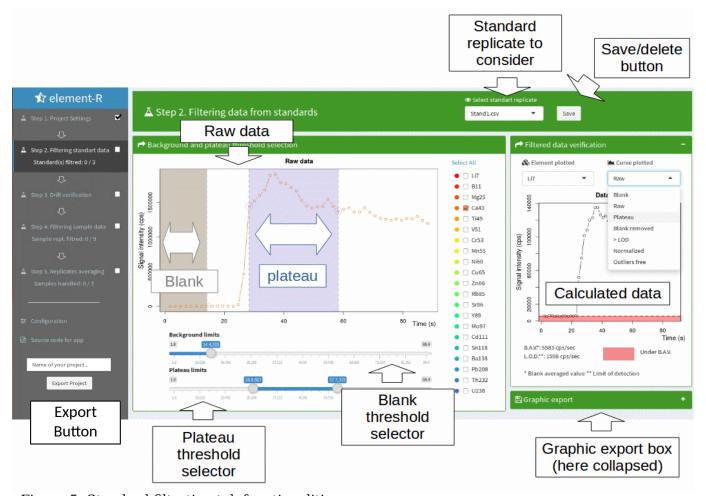


Figure 5: Standard filtration tab functionalities

- Step 3: Verification of the machine drift -

In this third step, you can check if machine drift occurs during the ICPMS session.

Machine drift is evaluated using linear regression (for more details about the regression, its parameters and the correction of the machine drift performed by **elementR**, see step 5.). If a machine drift occurs (as indicated by a *pvalue* of the slope < 0.05 written in red), all the chemical element(s) affected by this drift and all the parameters of the linear regression performed on their values are displayed. If no machine drift occurs, six elements are displayed randomly. However, you can display more elements by adding them in the "element to plot" widget (Fig. 6).

At this step, you have the choice to correct the machine drift for some elements (by clicking on the "Correction" check box of these considered elements) or for all of them (by clicking on the "Correct all" check box beside the names of the elements to display). Note that the chemical elements with a non-significant slope cannot be corrected.

Once approved, the information regarding machine drift correction can be saved by clicking on the "Save machine drift" button. The user can also delete the validation at any step of the procedure by returning to this tab and by clicking on the "Change machine drift" button. You can repeat the procedure as many times as required until you

obtain satisfactory results.

When step 3 is running, the "graphic export" box (collapsed by default, enlarged by clicking on the white cross) allows users to export the graphics displayed on this page with the export format set in the "Configuration" tab.

VERY IMPORTANT: The project and associated data are exported <u>only when the user</u> <u>has pressed the "Export Project" button. There is no automatic saving.</u>

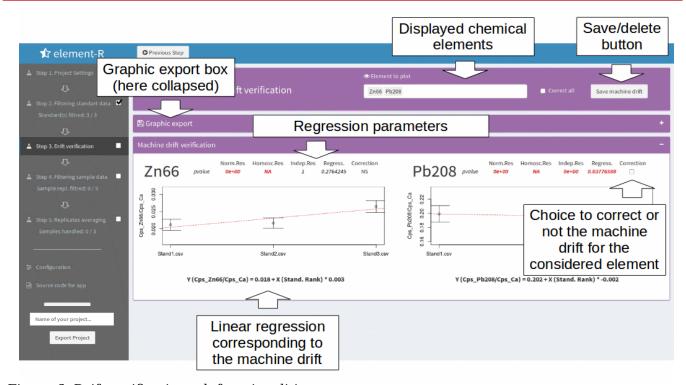


Figure 6: Drift verification tab functionalities

- Step 4: Filtration of the sample replicates -

In this forth step, you proceed to filtering the sample replicates.

To this aim, you must first select the sample and the replicate to filter (Fig. 7). Once the replicates are chosen, raw and reduced data of the considered replicate appear in two boxes (Fig. 7). The left box allows you to select the background (blank) and plateau thresholds using the slide selectors below the plot. The right box plots calculates the data in order to check the validity of your selected thresholds (i.e. reduced and intermediate data, see below).

elementR allows the user to check all the intermediate and final data of the filtration procedure. To do this, go to the right box and select the required data:

- "Blank", "Raw", "Plateau", "Blank removed", ">LOD" and "Normalized" have the same results as for standard filtration (see here)
- "Outlier free" is not performed for sample filtration as all sample values matter

- "Concentration" for data_Norm converted into concentrations (data_Conc)
- "Conc. corrected" for data_Norm converted into concentrations by taking in account the machine drift for the chemical element(s) chosen to be corrected (data_ConcCorr)

Once all thresholds are approved, all information can be saved by clicking on the "Save" button. Users can delete this validation at any step of the procedure by returning to this tab and by clicking on the "Delete" button. You can repeat the procedure as many times as required until you obtain satisfactory results.

When step 4 is running, a "graphic export" box (collapsed by default, enlarged by clicking on the white cross) allows users to export graphics displayed on this page with the export format set in the "Configuration" tab.

VERY IMPORTANT: The project and associated data are exported only when user have <u>pressed the</u> <u>"Export Project" button. There is no automatic saving.</u>

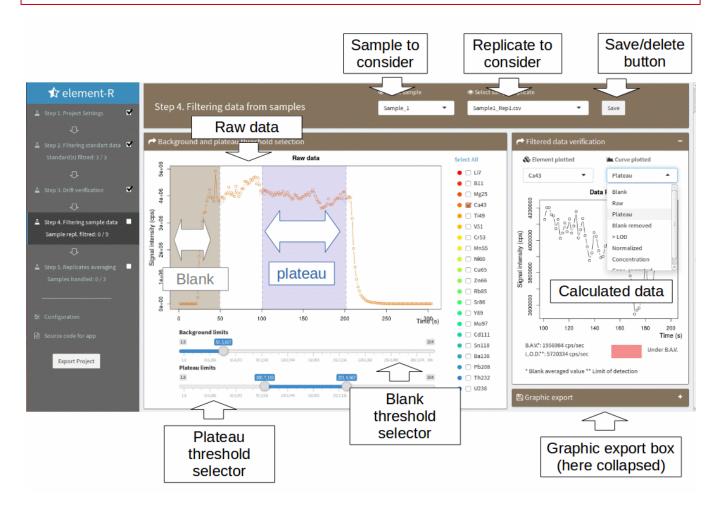


Figure 7: Sample filtration tab functionalities

- Step 5: Averaging the sample replicates -

In this last step, you proceed to averaging the sample replicates according to two modes: "spot" and "raster", depending on which mode you used during the ICPMS analysis

To this aim, you have to first select the sample to average and indicate its mode of averaging (Fig. 8).

 \rightarrow the "Spot" mode (Fig. 8):

A reactive table displays the average and standard deviation (SD) per chemical element for each replicate of the considered sample and for all replicates (total mean and total SD). You can modify which replicates to keep for the final calculation, the total average and SD being automatically recalculated.

Once approved, all information can be saved by clicking on the "Save averaging" button. You can delete the validation by going back on this tab and by clicking on the "Delete averaging" button. You can repeat the procedure as many times as required until you obtain satisfactory results.

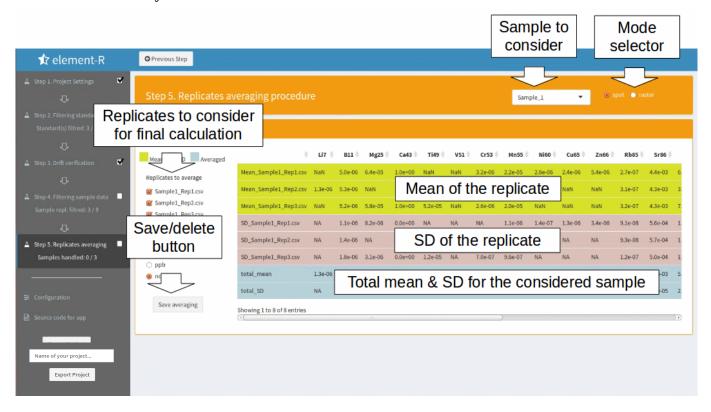


Figure 8: Sample replicate averaging tab functionalities in spot mode

 \rightarrow The "Raster" mode (Fig. 9 & 10):

A reactive plot displays the reduced data (i.e. in concentration, corrected or not from the machine drift according to your instructions in step 3) for each replicate of the considered sample. You can choose to select which replicates are part of the final calculation and can visually realign their data by moving the replicate curves back and forth (using the "realign" numerical inputs). Once all curves are realigned, you

can proceed to data averaging by clicking on the "Mean" button. At this point, a black curve appears corresponding to the final averaged value of the considered sample (see Fig.10).

Averaged data can be saved by clicking on the "Save averaging" button or deleted if the realignment is not optimal by clicking on the "Delete averaging" or "Delete Realignment" button. You can repeat the procedure as many times as required until you obtain satisfactory results.

When step 5 is running in raster mode, a "graphic export" box (collapsed by default, enlarged by clicking on the white cross) allows users to export any graphics displayed on this page with the export format set in the "Configuration" tab.

<u>VERY IMPORTANT: The project and associated data are exported only when the user has pressed the "Export Project" button. There is no automatic saving.</u>

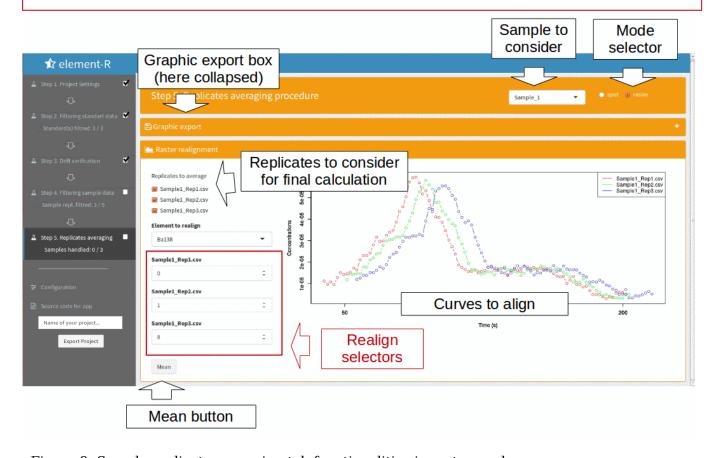


Figure 9: Sample replicate averaging tab functionalities in raster mode

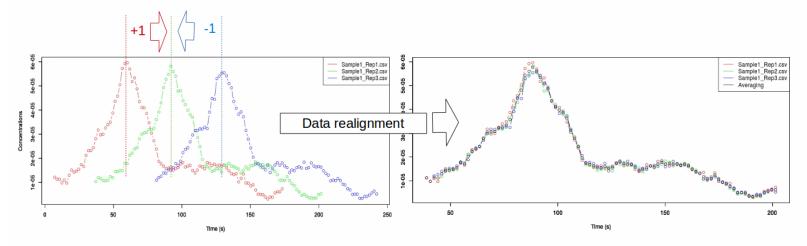


Figure 10: Sample replicate realignment and averaging in raster mode

- Additional step: Optional settings -

This tab displays optional settings:

- The value to input when data are below the limit of detection (step 2 & 4)
- The color of the plotted chemical element (as some colors are not properly seen, you can change the color of a particular chemical element or all colors by clicking on the colored rectangle of the considered element). This option is only available after you have validated the running project.
- Graphic and data export parameters

Note:

These parameter settings are an optional step and its validation is optional.

You can set these parameters at any step of the procedure.

5. What is the exact process when elementR performs the machine drift verification and correction?

The verification of the ICPMS drift performed by the **elementR** package is conducted by evaluating the evolution of the standard signal of each chemical element throughout the session.

→ Machine drift verification (Fig. 11)

In the case of having more than two standard replicates, a linear regression of the standard signal as a function of their rank in ICPMS analysis is processed (R function lm of the stats package). All parameters of the regression are statistically tested and displayed (normality, homoscedasticity and independence of the residuals, determined by the shapiro.wilks function of the stats package, hmctest and dwtest functions of the lmtest package (Hothorn et al. 2015), p.value of the regression and coefficients (slope and intercept)).

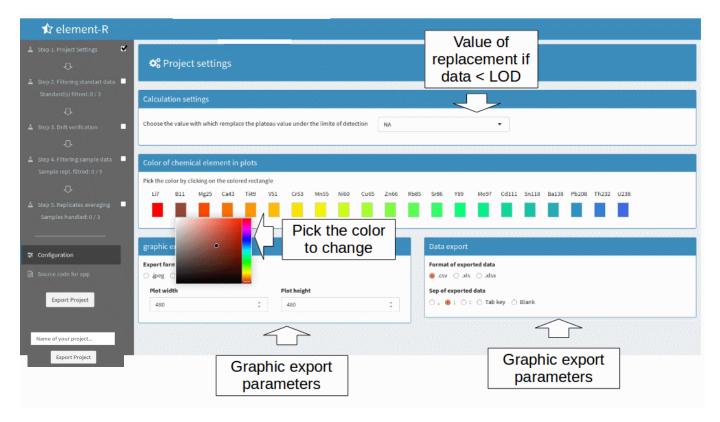


Figure 11: Configuration tab functionalities

In the case of only two standards available, the linear regression is performed without testing its significance. The intercept and slope are then displayed and you will be able to correct the drift for this (ese) chemical element(s).

In the case of onlyone standard available, the value of the single standard is used for converting signal intensity into concentration. No correction is possible.

If no standard is available (for some element, for instance), the data to be converted into concentration are replaced by NA. No correction is possible.

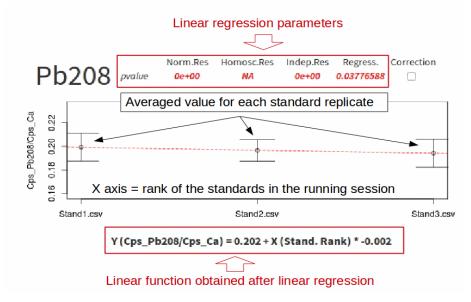


Figure 12: Machine drift verification performed by **elementR**

→ Machine drift correction (Fig. 12)

Note that you have the choice to correct or not the temporal drift of the machine if you consider this drift relevant or not.

If chemical element(s) are chosen to be corrected, **elementR** proceeds to an additional step after the concentration conversion as followed:

Example of Pb208 in the "Example Session":

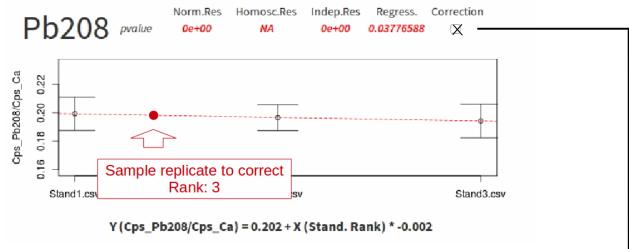


Figure 12: Machine drift correction performed by **elementR** for sample replicate at rank 3

Without drift correction

VS.

With drift correction

Conversion of the intensity signal into concentration for Pb208:

$$[Conc.] = \frac{Calib_{Pb\,208} \times IntenSignal_{sample.Pb\,208}}{IntenSignal_{standard.Pb\,208}}$$

Where:

- \rightarrow [Conc.] is the final concentration
- \rightarrow *Calib*_{Pb208} is the Pb208 value of the considered calibration type (here, NIST 612)
- \rightarrow *IntenSignal*_{sample.Pb208} is the Pb208 intensity for the considered sample replicate
- \rightarrow IntenSignal_{standard.Pb208} is the average intensity for all standard replicates within the running session

Conversion of the intensity signal into concentration for Pb208:

Calculation of the theoretical value (*TheoretStand*) of the standard intensity at the rank 3 (according to the equation above):

TheoretStand_{Rank 3. Pb 208} = $-0.02 \times 3 + 0.202$

Conversion of the signal intensity into concentration by taking into account the machine drift correction

$$[Conc.] = \frac{TheoStand_{Rank 3.Pb 208} \times IntenSignal_{sample.Pb 208}}{IntenSignal_{standard.Pb 208}}$$

Where:

- \rightarrow [Conc.] is the final concentration
- \rightarrow *TheoretStand*_{Rank3.pb208} is the theoretical Pb208 value at rank 3 of the calibration type (here, NIST 612)
- \rightarrow IntenSignal_{sample.Pb208} is the Pb208 intensity for the considered sample replicate
- \rightarrow IntenSignal_{standard.Pb208} is the average intensity for all standard replicates within the running session

6. How to export data from elementR?

As soon as data are loaded, an "Export project" button appears at the bottom of the left sidebar. You can name your project and press it at any step of the data reduction procedure to export:

- The entire project and its settings in an .Rdata file. This file is the one to load for re-opening an existing project (see step 1 and Fig.5)
- All the intermediate and final data in worksheet format. Note that only validated data are exported.

All these files are exported in the sub-folder called "Results" located in the running project folder.

Note:

The **elementR** package provides an example of exported data in the sub-folder called "**Results**" from the **elementR** folder⁶

By default, **elementR** exports data in .csv. If you want to change this parameter, go to the "Configuration" tab.

- Exports for replicates -

For each replicate filtered (here, named X), **elementR** exports (name of data in Annexe 1):

- "data Blank X" corresponding to data Blank
- "data plateau X" corresponding to data_Plateau
- "data SuppBlank X" corresponding to data_SuppBlank
- "data SupLOD X" corresponding to data_SupLOD
- "data Norm X" corresponding to data_Norm
- "data OutlierFree X" corresponds to data_OutlierFree
- "data Conc X" corresponds to data_Conc
- "data ConcCorr X" corresponds to data ConcCorr

- Exports for sample batch -

When the last step (step 5) has been performed on at least one sample (here, called Y), **elementR** exports:

- \rightarrow "finalReplicates_X" corresponding to the data_ConcCorr taking into account the realignment (export only in raster mode)
- \rightarrow "final_Y" corresponding to the final data of the sample (i.e. the averaged value for all chosen replicates).

⁶ if you do not manage to find this folder on your computer, see here

- Overview tables -

Some data summarizing session settings or recapitulating the main results of steps are also exported in order to provide as many details as possible on the running procedure:

- "SummarySettings" summarizes the main settings for each filtered replicate (rank in ICPMS analysis, blank and plateau thresholds, blank averaged value & limit of detection for each chemical element)
- "regression_parameters" indicates the parameters of the linear regression corresponding to the machine drift (residuals normality, homoscedasticity, independence, *pvalue* of the slope, intersect and slope)
- "SummaryStandard" displays the average and the standard deviation per chemical element for each standard replicate and for all standards of the session

VERY IMPORTANT:

You can export as many times as required at any step of the data reduction procedure. Be careful, the overwritten data will be lost and unrecoverable.

The project and associated data are exported <u>only when the user has pressed the "Export Project" button. There is no automatic saving.</u>

7. More questions?

If, despite the care devoted to programming this package and write this documentation, you have difficulties to install or run **elementR**, if you have questions about the procedures or calculations, or if you want to report bugs, do not hesitate to consult the official **elementR** documentation provided by the CRAN, ask questions on GitHub (click on the "Source code" tab) or send us an email to charlott.sirot@gmail.com.

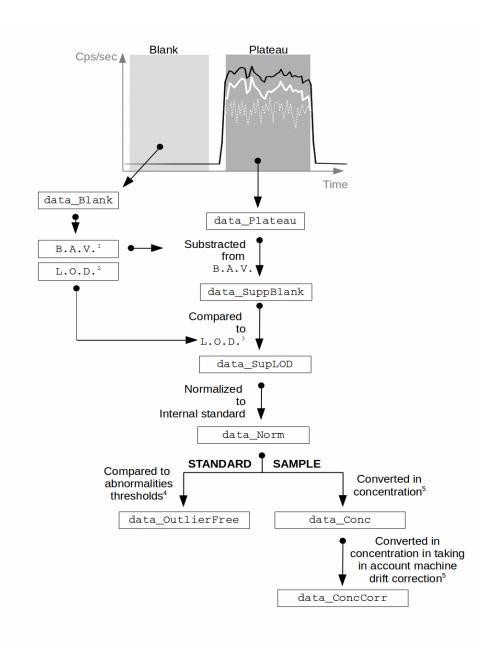
8. References

Elsdon & Gillanders. Interactive effects of temperature and salinity on otolith chemistry: challenges for determining environmental histories of fish. Can. J. Fish. Aquat. Sci. Vol. 59, 2002.

Fowler et al. Experimental assessment of the effect of temperature and salinity on elemental composition of otoliths using laser ablation ICPMS. J. Fish. Aquat. Sci. Vol. 52, 1995.

Milton & Chenery. The effect of otolith storage methods on the concentrations of elements detected by laser-ablation ICPMS. J. of Fish Biology, Vol. 53, 1998.

Thorrold et al. 1998. Accurate classification of juvenile weakfish *Cynoscion regalis* to estuarine nursery areas based on chemical signatures in otoliths. Marine Ecology Press Series, Vol. 173, 1998.



Appendix 1. Calculations included in step 2 and step 4 of the data reduction procedure performed by **elementR**:

- 1. Blank averaged value (B.A.V.): averaged values for each chemical element of data_Blank
- 2. Limit of detection (L.O.D.): 3 times standard_deviation of $data_Blank$
- 3. Comparison of data_SuppBlank to L.O.D.: data_SuppBlank values below the L.O.D. are replaced by NA, 0 or B.A.V. (according to the user's choice)
- 4. Comparison of $data_Norm$ to abnormalities thresholds: $data_Norm$ values below the LimitMin or up to LimitMax are replaced by NA where:

```
LimitMin = mean(data_Norm) - 2 x standard_deviation(data_Norm)
LimitMax = mean(data_Norm) + 2 x standard_deviation(data_Norm)
```

5. See step <u>5.</u>