eurofins | BioPharma Product Testing

Compounds Relative Response Factor, a Reliable Quantification Within Extractable Testing

by Marco Giulio Rozio, Antonio Rosato, Lucia Iadarola and Simone Carrara - Eurofins BioPharma Product Testing Italy

Introduction

Extractables and Leachables (E&L) studies are critical for the identification and quantification of potential harmful compounds which could contaminate pharmaceutical products and medical devices by migrating from the container closure systems and from the equipment [1,2].

The E&L is an evolving field of the analytical chemistry where more accurate methods in recognizing and quantifying the migrating substances are mandatory to assess their presence within acceptable limits. The knowledge of the chemical structure of these compounds is essential to evaluate their toxicological implications through a reliable Permitted Daily Exposure (PDE) [3,4,5].

The quantification of the extractables candidates is also crucial since, due to the screening nature of the extractables studies, a semi-quantitation in the chromatographic techniques is usually performed against one or few reference standards which could show very different instrumental responses with respect to the investigated compound. The result is an approximated quantification which could lead to a rough or even wrong toxicological evaluation which results in unfounded concerns or, in a worst-case scenario, to the lack of detection of the compounds.

Current regulatory and industry guidelines on extractables quantitation

Guidelines on E&L testing (USP chapter <1663>, ISO 10993-18, PQRI) advise to apply when possible quantitative techniques which should be typically based on the instrumental response of 'authentic reference compounds [3,4]. It is nevertheless difficult to apply this approach since the pattern of extractables migrating from a material is not known until the analytical testing is performed. Under conditions of uncertainty, also considering the screening nature of extractables studies, the semi-quantitatation against one of few reference standards for all the migrating compounds is allowed but a more precise assessment is the optimal strategy to overcome all related drawbacks [10, 11, 12, 13].

Several publications on this topic demonstrated the large variations in the Response Factors (RF) of known extractables, through the evaluation of different antioxidants, slip agents, plasticizers and monomers which are common released compounds from the plastic items. The choice of one or few reference standards for the quantification of all the extractables detected in the chromatographic techniques has significant implications on the total number of extractables reported above the Analytical Evaluation Threshold (AET) as well as an impact on their semi-quantitation, leading to a double analytical uncertainty on the compounds which have to be considered toxicologically relevant [14,15,16].

Study goals

Taking into consideration the above mentioned implications, this whitepaper describes a useful workflow for a new Extractables quantitation analytical model, able to provide more precise and reliable data by the application of the compounds Relative Response Factor (RRF) in the concentration rescaling during the extractables assessment.

This study is focused on HPLC/MS analysis that showed higher rates of RRFs variation, although overall implications are reflected in all E&L techniques based on semi-quantitative analysis.

While the primary goal is to establish a more precise quantification, this approach is also able to improve the detection capability of traditional analytical extractables testing offering a new screening methodology.

Definitions

- **Extractables:** The chemical entities that can be released from an item (packaging system, process component, medical device) under laboratory conditions applying specific extraction solvents, temperature and time of contact.

- **Leachables:** The chemical entities that are leached into a product from a packaging/delivery system, process component or medical device, under normal conditions of storage and use, or during accelerated drug product stability studies.

• **Permitted Daily Exposure (PDE):** A substance-specific dose that is unlikely to cause an adverse effect if an individual is exposed at or below this dose every day for a lifetime.

• **Response Factor (RF):**The ratio between the concentration of a compound and its corresponding analytical response.

• **Relative Response Factor (RRF):** The ratio between the Response Factor of a compound and the Response Factor of a chosen reference standard.

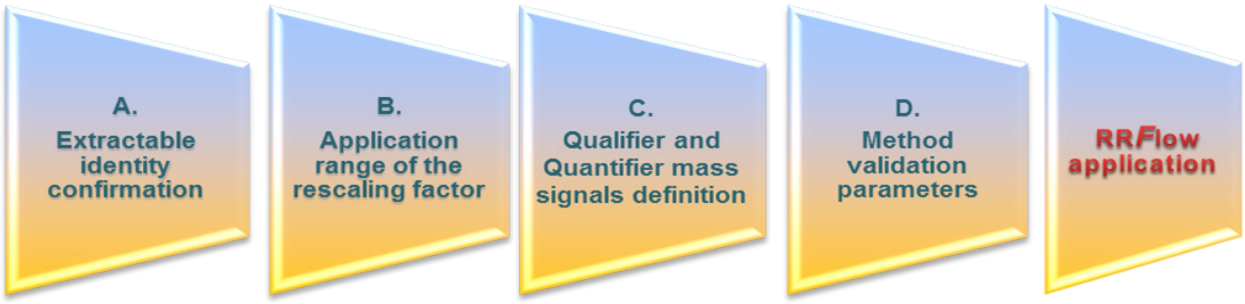
• **Qualifier mass signal (QLF):** The most intense and/or representative mass signal present in the compound mass spectrum.

• **Quantifier mass signal (QTF):** The mass signal which significantly contribute to the definition of the peak area of the compound.

The RR*F*low approach

First step on the determination of a reliable extractables Relative Response Factor (RRF) that can be applied within the extractables study data assessment is to reduce analytical variables by setting specific keypoints as showed in figure 1.

Figure 1. RR*F*low keypoints



A. Extractable identity confirmation

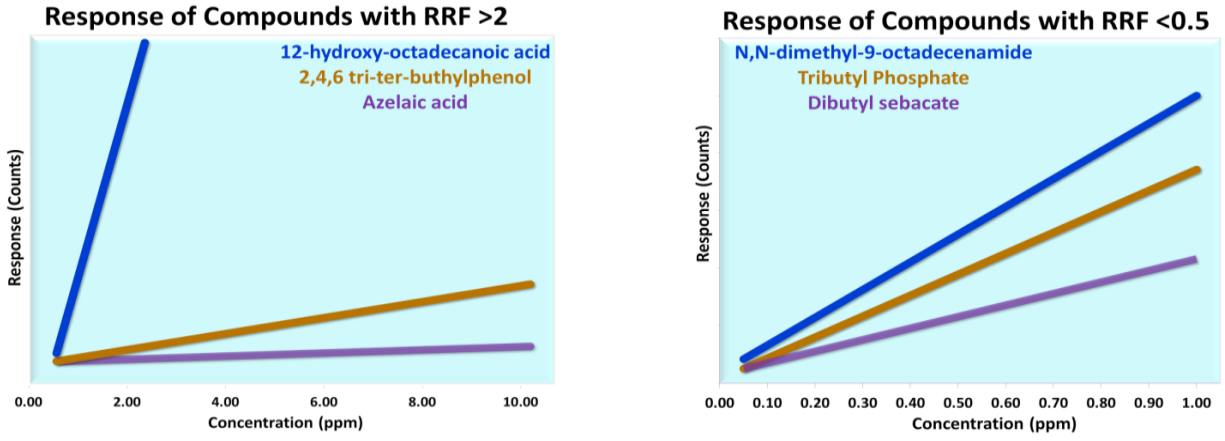
The characterization of a new extractable requires usually a first mass spectra check into the proprietary extractables databases or in on-line database (e.g. Chemspider, mzCloud, NIST, Wiley) trying to get a tentative of identification. The subsequently extractable identity confirmation is based on the reference standard purchasing, if commercially available, and its analysis in the most used extraction solution to simulate water-based products for the determination of the organic release in extractables testing (Isopropanol/water 50:50 v/v solution). The aim of the analysis is to compare the retention time and mass spectra for identity confirmation.

After the identification, the information regarding relative retention time (RRT), RRF determined at one level of concentration, and other useful data are reported in the EED (Eurofins Extractables Database).

B. Application range of the rescaling factor

The application of the rescaling factor should be focused only to those compounds which exhibits RRF< 0.5 and RRF>2 since for those which fall between these values, the approximation obtained in the traditional approach, based on the semi-quantification assessment, can be considered acceptable.
Figure 2 gives an exemplification of the Response variability in the two different sets of compounds.

Figure 2. Exemplificative response curves of extractables with RRF>2 and RRF<0.5.



C. Qualifier and Quantifier mass signals definition

The RRF determination is based on the sum of the Quantifier signals (QTFs), the mass signals which significantly contribute to the definition of the peak area of the compound (charged molecule, adducts, dimer, fragments), but nevertheless sometimes can be more than ten per each candidate. Considering the high number of the analytes that are usually detected in the extractables studies, the targeted searching of these signals would lead to a huge data generation. A useful expedient to reduce the impact of this assessment is the choice of the Qualifier signal (QLF), among the QTFs, which is the most intense and/or representative mass signal in the compound mass spectrum and it will be always present in the chromatogram if the extractable compound is contained into the analyzed samples. For this reason its detection at a specific RRT is used as triggering step before proceeding with the concentration rescaling which is instead based on the sum of the all QTFs. This gimmick highly decrease the number of data that need to be elaborated by the analytical software and permits to focus the attention on those compounds which need actually a deeper investigation. The QLF and QTFs are evaluated by Extraction Ion Currents (EIC) which permits to obtain a specific and sensitive data assessment, based on the high resolved m/z signals at the 3rd decimal place obtained through the QTOF detectors.

D. Method validation parameters

For each compound subjected to the RRFlow the following parameters should be tested in order to verify data reliability:

- **Specificity of the method:** the signals for the RRF determination should be chosen considering the sum of the most representative ions of the compound (QTFs); only the not-interfered mass signals have to be used for the compounds quantification.
- **Linearity of the response:** the setting of a concentration range in which the analytical response of the compound is linear is a crucial step to permit the application of a unique reliable RRF for the concentration compound rescaling. The range for the linearity assessment should be set from 0.05 µg/mL to 10 µg/mL, which represents by Eurofins expertise the range of concentration in which falls the most of the compounds detected in the extractables studies.
- **Limit of Quantitation (LOQ):** the determination of the method sensitivity is important to set the lowest concentration on which the rescaling factor can be applied. For those compounds which presented a low analytical response, the LOQ should be used as the lowest point of the range for the linearity determination.
- **RRF (average):** it is the mean value of the RRFs calculated at each reference standard level of the tested linearity range.

RRFlow application: from theory to practice

To demonstrate the presented workflow, a set of 120 compounds have been chosen among the most common extractables found in the E&L studies, which were analyzed by the HPLC/MS instrumental method described in Table 1 obtaining information about their relative retention time, relative response factor and other useful data that were reported in the EED (Eurofins Extractables Database).

Table 1. HPLC/MS instrumental settings

Instrument Parameter	HPLC/MS Settings
Instrument	Agilent: 1260 (G1312) HPLC – 6530 Q-TOF
Column	Agilent Zorbax Eclipse XDB-C18, 2.1 mm x 50 mm 1.8 µm
Column temperature	65°C
Mobile phase	A) 5mM Ammonium acetate in Water B) 50:50 Acetonitrile:Methanol v/v
MS detection	ESI+ and ESI-
MS scan range	50 - 1500 amu

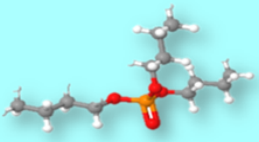
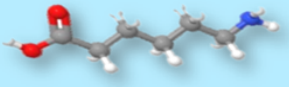
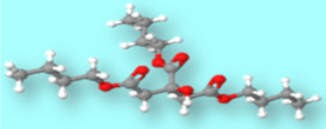
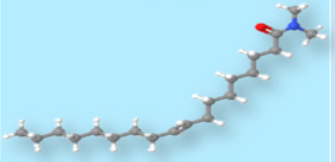
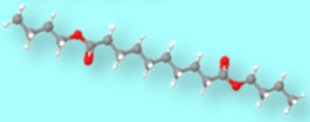
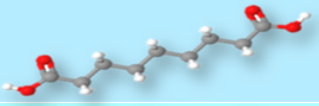
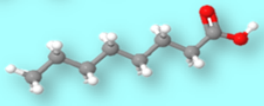
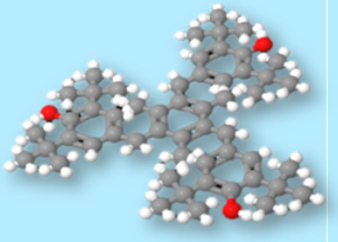
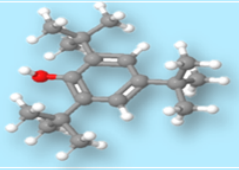
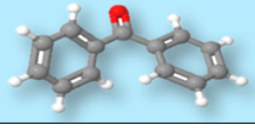
A perspective evaluation of the RRF was performed by comparing the response of target compounds and the usual reference compounds used by Eurofins in the semi-quantitative approach. The concentration for this first assessment was set to 1 µg/mL for all compounds.

30 compounds out of the 120 analyzed showed a RRF which fell out of the range of the 'no-rescaling factor application' (0.5>RRF>2) and for this reason they were subjected to the steps of the RRFlow approach.

A six point calibration curve was therefore performed for each compound in the chosen range (0.05 - 10 µg/mL) and an average RRF was defined. A maximum RSD of 20% and a correlation coefficient of the curve R^2>0.98 were set as acceptance criteria. In the case the system suitability was not satisfied, the range of linearity was reduced (for compounds which showed a low instrumental response) or split in two (for compounds which showed instrumental response saturation) to fulfill the precision requirements.

Table 2 summarizes data from 10 compounds subjected to this assessment; it is interesting to see how different can be the RRF and the range of linearity in which this rescaling factor can be applied.

Table 2. RRFlow application on 10 selected compounds

Compound	CAS	Formula	Structure	RRF	Linearity Range (ppm)
Tributyl phosphate	126-73-8	C12H27O4P		0.33	0.05 - 1.00
6-Aminocaproic acid	60-32-2	C6H13NO2		13.67	0.05 - 5.00
Citroflex tributylcitrate	77-94-1	C18H32O7		0.21	0.05 - 1.00
N,N-dimethyl-9-octadecenamide	3886-90-6	C20H39NO		0.14	0.05 - 1.00
Dibutyl sebacate	109-43-3	C18H34O4		0.30	0.05 - 1.00
Azelaic acid	123-99-9	C9H16O4		32.69	1.00 - 10.00
Octanoic acid	124-07-2	C8H16O2		9.48	0.10 - 10.00
Irganox1330	1709-70-2	C54H78O3		8.41	0.05 - 1.00
2,4,6 tri-tert-butylphenol	732-26-3	C18H30O		39.86	0.05 - 10.00
Benzophenone	119-61-9	C13H10O		15.24	0.10 - 10.00

RR*F*low application in extractables amount rescaling

The average RRF determined by the RR*F*low will be used to re-calculate the amount of the corresponding compound detected in the extractables study, by applying the following formula:

Actual extractable concentration = **Experimental concentration** x **Average RRF**

Where:

- Experimental concentration: is the extractable compound concentration calculated by the semi-quantitative screening against a fixed reference standard.

- Average RRF: is the average value of the RRF calculated in the linearity range as result of the RR*F*low.

The above formula have to be applied only when the actual Extractable concentration results above the Analytical Evaluation Threshold set. In the case the actual extractables concentration falls outside the range of linearity the sample should be reanalyzed after analytical treatment (concentration or dilution) to permit a reliable rescaling. An example of the RRF application in the data analysis of real extractables studies output is reported in Table 3 below.

Table 3. RRF application in extractables concentration rescaling

Extractable Compound	CAS	Expected RT (min)	Polarity	RRF	Linearity Range (ppm)	Experimental RT (min)	Semi-Quantitation (µg/mL)	RRF-Quantitation (µg/mL)	>AET	Inside Linear range	Final amount (µg/mL)
Tributyl phosphate	126-73-8	5.630	ESI+	0.33	0.05 - 1.00	5.65	0.16	0.05	FAIL	PASS	< AET
Dibutyl Sebacate	109-43-3	6.570	ESI+	0.30	0.05 - 1.00	6.47	1.10	0.33	FAIL	FAIL	< AET
Octanoic acid	124-07-2	4.230	ESI-	9.48	0.1 - 10.00	4.24	0.09	0.85	PASS	FAIL	SCR*
Irganox 1330	1709-70-2	9.895	ESI-	8.41	0.05 - 1.00	9.91	0.11	0.93	PASS	PASS	0.93
Cytroflex tributylcitrate	77-94-1	5.760	ESI+	0.21	0.05 - 1.00	5.79	0.99	0.21	PASS	PASS	0.21
6-Aminocaproic acid	60-32-2	0.402	ESI+	13.67	0.05 - 5.00	0.41	**0.006** →	**0.08**	PASS	PASS	0.08

* SCR = Sample Concentration Required

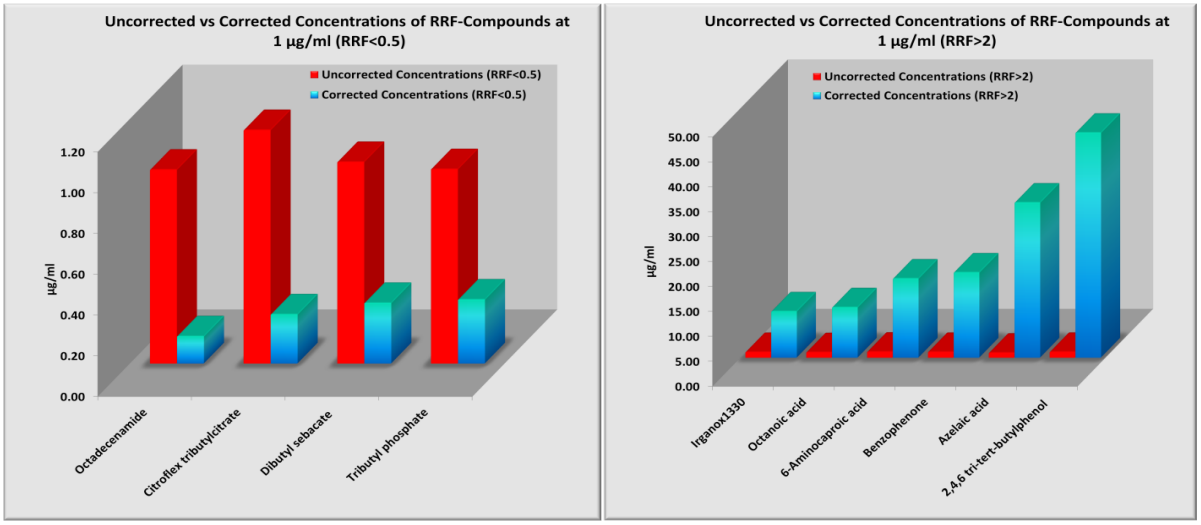
The enhanced detection capacity

It is important to underline how the detection by Extraction Ion Currents (EIC) permits to increase several times the detection capacity in the extractables screening for low responsive compounds. As an example case, the 6-Aminocaproic acid reported in table 3 was detected through the RR*F*low approach at a concentration of 0.006 µg/mL which would have not been found by applying the usual semi-quantitative evaluation because hidden in the noise of the chromatographic baseline.

The re-calculation based on the specific RRF gives therefore the possibility to reveal and to quantify the 6-aminocaproic acid as real extractable. Uncorrected responses versus corrected responses related to 10 compounds subjected to RR*F*low are reported in Figure 3.

The observed high level of difference between the real concentration and the semi-quantitative one is impressive.

Figure 3. Corrected/uncorrected concentrations of extractables with RRF>2 and RRF<0.5.



Conclusion

This whitepaper illustrates a new analytical workflow (RR*F*low) which allows a more precise and reliable extractables estimation able to overcome all the disadvantages of the semi-quantitative extractables determination employed during the past 2 decades. This new approach is based on the average compounds Relative Response Factor (RRF), properly determined through the analysis of calibration curves of standard reference compounds in a specific range of linearity of the response.

This new concept of screening permits to combine the common non-targeted extractables screening with a new targeted assessment that is specifically focused on known compounds that have been incorrectly estimated during the semi-quantitative evaluation.

The proposed workflow provides a solid starting point able to increase precision in extractables data generation avoiding the overestimation/underestimation of extractables levels and consequently to permit a more accurate toxicological evaluation.

References

[1] DR. Jenke, Linking extractables and leachables in container/closure applications, J Pharm Sci Technol. 59(4) (2005) 265-281.

[2] W. Ding, G. Madsen, E. Mahajan, S. O'Connor, K. Wong, Standardized extractables testing protocol for single-use systems in biomanufacturing,Pharm. Eng. 34 (2014) 1–11.

[3] ISO 10993- Part 18: Chemical characterization of materials (Last version 2020-01).

[4] USP <1663> in USP 41 General Chapter :Assessment of Extractables Associated withPharmaceutical Packaging/Delivery Systems _1663_, United StatesPharmacopeial Convention, Rockville, MD, 2017, 7910–7924.

[5] USP <1664> in USP 41 General Chapter :Assessment of Drug Product Leachables Associated withPharmaceutical Packaging/Delivery Systems _1664_, United StatesPharmacopeial Convention, Rockville, MD, 2017, 7924–7937.

[6] D. Jenke , T. Carlson, A compilation of safety impact information for extractables associated with materials used in pharmaceutical packaging, delivery, administration, and manufacturing systems J Pharm Sci Technol. 68(5) (2014) 407-455.

[7] TH. Broschard, S. Glowienke, US. Bruen, LM. Nagao, A. Teasdale, CL. Stult, KL. Li, LA. Iciek, G. Erexson, EA. Martin, DJ. Ball, Assessing safety of extractables from materials and leachables in pharmaceuticals and biologics - Current challenges and approaches. Regul Toxicol Pharmacol. 8(2016) 201-211.

[8] D. Jenke, Development and Justification of a Risk Evaluation Matrix To Guide Chemical Testing Necessary To Select and Qualify Plastic Components Used in Production Systems for Pharmaceutical Products. J Pharm Sci Technol. 69(6) (2015) 677-712.

[9] D. Jenke, Moving forward towards standardized analytical methods forextractables and leachables profiling studies, PDA J. Pharm. Sci. Technol. 69(2015) 471–476.

[10] D. Jenke, MJ. Garber, Y. Hua, M.Gill, Development and validation of an HPLC-MS-MS method for quantitating bis(2,2,6,6-tetramethyl-4-piperidyl) sebacate (Tinuvin 770) and a related substance in aqueous extracts of plastic materials, J. Chromatogr. Sci. 48(3) (2010) 200-207.

[11] SA. Zdravkovic , MD. Bruss, RF. Piccoli, DM. Wood, A Method Utilizing Ultra-High Performance Liquid Chromatography with Ultraviolet and Mass Spectrometric Detection for the Analysis of Material Extracts Produced during a Controlled Extraction Study. J Pharm Sci Technol. 68(5) (2014) 504-526.

[12] C. Ta, J. Bones, Development and validation of an ultra-performance liquid chromatography method for the determination of bis(2,4-di-tert-butylphenyl)phosphate and related extractable compounds from single-use plastic films. J Chromatogr A. 1492 (2017) 49-54.

[13]P. Legrand, A. Desdion, G. Boccadifuoco, A. Dufaÿ Wojcicki, A. Worsley, V. Boudy, SG. Dufay, Development of an HPLC/UV method for the evaluation of extractables and leachables in plastic: Application to a plastic-packaged calcium gluconate glucoheptonate solution, J Pharm Biomed Anal. 155 (2018) 298-305.

[14] D. Jenke, A. Odufu, Utilization of internal standard response factors to estimate the concentration of organic compounds leached from pharmaceutical packaging systems and application of such estimated concentrations to safety assessment, J. Chromatogr. Sci. 50(3) (2012) 206-212.

[15] MA. Jordi, S. Khera, K. Roland, L. Jiang, P. Solomon, J. Nelson, SS. Lateef, J. Woods, L. Martin, S. Martin, F. Aiello, N. Chen, Qualitative assessment of extractables from single-use components and the impact of reference standard selection,. J Pharm Biomed Anal. 150 (2018) 368-376.

[16] SA. Zdravkovic, CT. Duong, AA. Hellenbran, SR. Duff, AL. Dreger, Establishment of a reference standard database for use in the qualitative and semi-quantitative analysis of pharmaceutical contact materials within an extractables survey by GC-MS. J Pharm Biomed Anal. 151 (2018) 49-60.