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A proposal for comparing methods of quantitative analysis of endogenous compounds in biological systems by using the relative lower limit of quantification (rLLOQ)☆

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ABSTRACT

Accuracy, precision and lower limit of quantification (LLOQ) are experimentally achievable key analytical factors by which the quality of analytical methods can be ascribed and objectively evaluated. Endogenous substances (endobiotica) are physiologically present in biological fluids and tissues at varying basal concentration ($C_{0,Ln}$). Formally, the definition of *accuracy* and *LLOQ* is same for xenobiotica and endobiotica. However, these analytical factors must be determined differently, notably by considering the $C_{0,Ln}$ value of endobiotica. Often, the impact of the endogeneity on the analytical method is underestimated. This especially applies to the LLOQ, because the LLOQ values for endobiotica are regularly not fixed measures due to the varying $C_{0,Ln}$ value in biological samples. In order to circumvent these difficulties and for a more reliable and objective evaluation and comparison of analytical methods for endobiotica, this work proposes the use of the *relative lower limit of quantification*, i.e., *rLLOQ*. The rLLOQ is defined as the percentage ratio of the LLOQ value, i.e., C_{LLOQ} to $C_{0,Ln}$: $rLLOQ = (C_{LLOQ}:C_{0,Ln}) \times 100$. Thus, the rLLOQ describes that fraction of $C_{0,Ln}$ that can be still determined with acceptable values for accuracy (e.g., recovery of $100 \pm 20\%$) and precision (e.g., RSD $\leq 20\%$) or with a total error (i.e., recovery + precision) of $\leq 30\%$. Examples from the quantitative analysis of selected endogenous compounds by previously validated GC–MS, GC–MS/MS and LC–MS/MS methods support the appropriateness and expressiveness of the rLLOQ in the quantitative analysis of endobiotica.

1. Introduction—accuracy and lower limit of quantification

Interestingly, most published work on analytical quantitative measurement and on regulation and harmonization of analytical techniques refer to pharmaceuticals, and definitions, recommendations and regulations have been originally drafted for xenobiotica. Although these issues can be and have been partially adapted for endogenous compounds, the endobiotica, this important class of physiological substances has not been in the focus of analysts and administrations so far. A very large part of published analytical work refers to substances that occur by nature in the body of human beings, animals and plants. However, endobiotica have not received the analytical attention they actually deserve. This especially applies to the analytical factors accuracy and lower limit of quantification (LLOQ) for endogenous substances and makes analysts unsure. Moreover, non-regulation of these important analytical issues hampers reliable comparison of analytical methods for the quantitative determination of endobiotica in relevant biological media. Thus, many very different concentrations measured in healthy humans may be regarded as valid and the analytical methods that yielded these results may be assumed as analytically equivalent.

Requirements for initial validation and publication in and implications for reviewers of the Journal of Chromatography B have been addressed by the journal's editors [1–3]. Key aspects of analytical method validation have been recently reviewed and discussed by Rozet et al. in the Journal of Chromatography A [4] and by Arajuo in the current issue of the Journal of Chromatography B [5]. Due to the particular importance in quantitative methods for endogenous compounds, the present work focuses on methods accuracy and lower limit of quantification. Important aspects of method validation in the quantitative determination of endogenous compounds in biological samples using chromatographic techniques have been recently discussed by van de Merbel [6].

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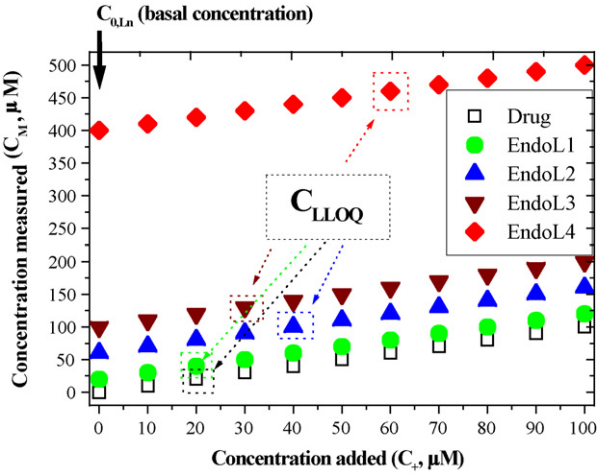

Fig. 1. Relationship between measured concentration (C_M) and added concentration (C_+) for a pharmaceutical *Drug* representing an exogenous compound with a very low basal concentration ($C_{0,Ln}$) close to zero and for a physiological compound *Endo* which may occur in a biological sample at four different levels (L1, L2, L3 and L4) corresponding to four different basal concentrations ($C_{0,Ln}$, $n = 1, 2, 3, 4$). Symbols set in dotted squares indicate the values for the LLOQ (C_{LLOQ}), which was defined as the lowest added concentration that can be measured with recovery and precision (RSD) values of 100 ± 20% and ≤20% [7]. All data used to construct this figure are theoretical and are summarized in Table 1. This figure shows that the LLOQ value of the endogenous substance *Endo* depends upon its basal concentration $C_{0,Ln}$.

1.1. Accuracy

Accuracy describes "The degree of closeness of the determined value to the nominal or known true value under prescribed conditions. This is sometimes termed trueness" [7]. This rather general definition may lead to misinterpretations and to the use of different formulas to calculate the accuracy of a method. In the present work, the accuracy of an analytical method is meant and expressed as recovery in percent, i.e., recovery is the numerical value of accuracy. For clarity, it is explicitly stated that in this work the term *recovery* does not apply to the term *yield*. The precision or more correctly the imprecision of an analytical method is expressed as relative standard deviation (RSD) in percent. Also, because the "true" basal concentration of an endogenous substance in its relevant biological system is by definition not known and reference materials for the majority of endobiotica are not available, the term "trueness" is not used in the present work. The reader is referred to recent articles [4–6].

Commonly, the accuracy (recovery, %) of an analytical method for a given analyte in a certain biological sample (e.g., plasma) is calculated by using the general Formula (F1):

$$Rec = (C_M : C_+) \times 100 \quad \text{(F1)}$$

whereas *Rec* is the recovery value (in %), C_M is the analyte concentration measured by the analytical method in the biological sample, and C_+ is the known nominal concentration of the analyte added to the sample.

Formula (F1) is valid only for xenobiotica, such as most drugs, that do not physiologically occur in biological samples or they are not present as contaminants therein, i.e., their basal concentration $C_{0,Ln}$ is zero: $C_{0,Ln} = 0\ \mu M$ (Fig. 1). If the analyte is physiologically present in the biological sample at a basal concentration $C_{0,Ln}$, i.e., $C_{0,Ln} \neq 0\ \mu M$ (Fig. 1), the value of $C_{0,Ln}$ must be considered appropriately, i.e., it must be subtracted from the measured concentration C_M, when calculating the methods recovery for the analyte for each added concentration. To the knowledge of this author, Formula (F2) (or equivalent formulas) is most frequently used for calculating the recovery of endogenous substances in their biological matrices:

$$Rec = [(C_M - C_{0,Ln}) : C_+] \times 100 \quad \text{(F2)}$$

It is very important to point out that endogenous substances occur in biological systems at many different basal concentrations or levels (Ln), with $n = 1, 2, 3, \ldots$, whereas $C_{0,L1} < C_{0,L2} < C_{0,L3} \cdots$. It follows from Formula (F2) that the recovery of an analytical method – as calculated from data generated in validation experiments from replicate analyses – for an endogenous substance may depend upon the extent of the basal concentration $C_{0,Ln}$ measured in the matrix being investigated. Formula (F1) is derived from Formula (F2), if $C_{0,Ln}$ is set equal to zero. It is worth mentioning that Formula (F2) can also be applied to xenobiotica present in a sample, for instance due to preceding drug administration. Erroneously, Formula (F3) is used by some authors for determining the methods recovery (accuracy) for endogenous analytes:

$$Rec = [C_M : (C_{0,Ln} + C_+)] \times 100 \quad \text{(F3)}$$

Formulas (F2) and (F3) describe the degree of closeness of the measured C_M value to the nominal value C_+, but these formulas express different things (see next section). Thus, proper consideration of $C_{0,Ln}$ and use of the correct formula, i.e., Formula (F2), are essential in validation experiments and quality control. Data from the author's group presented in this article are based exclusively on the use of Formula (F2).

1.2. Lower limit of quantification

The lower limit of quantification is "The lowest amount of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy" [7]. Analogous to the definition of accuracy, the above definition for LLOQ is rather very general and may also lead to distinctly different interpretations and results with regard to the analysis of endogenous substances. In addition, the author understands the LLOQ value as an experimentally measured value but not as a calculated value, for instance on the basis of the lower limit of detection (LLOD) from analyses of matrix-free analyte solutions of the method and the signal-to-noise (S/N) ratio (see below and next section).

Usually, the lowest added concentration point, i.e., C_{LOW+}, of the calibration curve is reported in the literature as the LLOQ value of the method. However, the concentration C_{LOW+} is rather arbitrary and must not represent the actual LLOQ value of the method (Table 1 and Fig. 2). In the opinion of this author, the actual LLOQ of a method for endogenous substances is the lowest added analyte

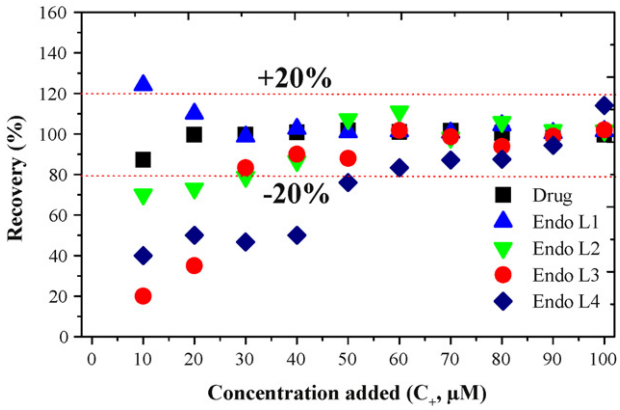

Fig. 2. Relationship between recovery and added concentration for a hypothetical pharmaceutical *Drug* and for a hypothetical physiological compound *Endo* occurring at four different basal concentrations ($C_{0,Ln}$, $n = 1, 2, 3, 4$) in a biological sample. Recovery values were calculated by using Formula (F2). See the text as well as Fig. 1 and Table 1 for more details.

Table 1
Theoretical data on added and measured concentrations and calculated[a] values for rLLOQ and $rLLOQ_{Rec}$ for a hypothetical pharmaceutical *Drug* and a hypothetical physiological compound *Endo* present at four different basal concentrations (L1, L2, L3, and L4) in a biological sample used in validation experiments.

Added (μM)	Measured (μM)				
	Drug	Endo L1	Endo L2	Endo L3	Endo L4
0	0.08 ± 0.08	20.6 ± 4.0	61.4 ± 9.7	104 ± 16	396 ± 27
RSD	100	19.4	15.8	15.4	6.8
10	8.8 ± 2.4	33.0 ± 4.7	68.4 ± 14.8	106 ± 19	400 ± 33
RSD	*27.3*[b]	14.2	*21.6*	17.9	8.3
Recovery (%)	87.3	*124*	*70*	*20*	*40*
LLOQ (μM)	–	–	–	–	–
rLLOQ (%)	N.A.	–	–	–	–
$LLOQ_{Rec}$ (μM)	N.A.	–	–	–	–
$rLLOQ_{Rec}$ (%)	N.A.	–	–	–	–
P[c]	3.8E−5	0.002	0.4029	0.85936	0.83961
20	20.0 ± 3.2	42.6 ± 5.1	76.0 ± 11.9	111 ± 11	406 ± 36
RSD	16	12	15.7	10	8.9
Recovery (%)	99.6	110	*73*	*35*	*50*
LLOQ (μM)	**20**[d]	**20**	–	–	–
rLLOQ (%)	N.A.	103	–	–	–
$LLOQ_{Rec}$ (μM)	N.A.	18.2	–	–	–
$rLLOQ_{Rec}$ (%)	N.A.	93.6	–	–	–
P	6.3E−7	6.7E−5	0.06643	0.44082	0.63137
30	30.0 ± 2.7	50.2 ± 3.7	85.0 ± 9.4	129 ± 8.2	410 ± 35
RSD	9	7.4	11.1	6.4	8.5
Recovery (%)	99.7	98.7	*78.7*	83.3	*46.7*
LOQ (μM)	–	–	–	**30**	–
rLLOQ (%)	N.A.	–	–	29	–
$LLOQ_{Rec}$ (μM)	N.A.	–	–	36	–
$rLLOQ_{Rec}$ (%)	N.A.	–	–	34.6	–
P	8.4E−9	2.0E−6	0.00442	0.01438	0.50169
40	40.4 ± 2.3	61.6 ± 4.2	96.0 ± 9.2	140 ± 7.9	416 ± 27
RSD	5.7	6.8	9.6	5.6	6.5
Recovery (%)	100.8	102.5	86.5	90	*50*
LLOQ (μM)	–	–	**40**	–	–
rLLOQ (%)	N.A.	–	65	–	–
$LLOQ_{Rec}$ (μM)	N.A.	–	46.2	–	–
$rLLOQ_{Rec}$ (%)	N.A.	–	75.1	–	–
P	2E−10	2.7E−7	4.1E−4	0.00196	0.27552
50	51.0 ± 2.4	71.0 ± 4.2	115 ± 12	148 ± 5.7	434 ± 10
RSD	4.7	5.9	10.4	3.9	2.3
Recovery (%)	101.8	100.8	107.2	88	*76*
P	5.1E−11	5.2E−8	4.9E−5	4.0E−4	0.07051
60	60.6 ± 2.4	81.4 ± 4.2	128 ± 10	165 ± 7.9	446 ± 23
RSD	4.0	5.2	7.8	4.8	5.2
Recovery (%)	100.9	101.3	111.0	101.7	83.3
LLOQ (μM)	–	–	–	–	**60**
rLLOQ (%)	N.A.	–	–	–	15
$LLOQ_{Rec}$ (μM)	N.A.	–	–	–	72.0
$rLLOQ_{Rec}$ (%)	N.A.	–	–	–	18
P	1.1E−11	1.2E−8	5.9E−6	6.0E−5	0.0136
70	71.2 ± 2.4	91.0 ± 3.7	130 ± 10	173 ± 4.7	457 ± 22
RSD	3.4	4.1	7.7	2.7	4.8
Recovery (%)	101.6	100.6	98	98.6	87.1
P	2.9E−12	2.4E−9	4.1E−6	1.7E−5	0.00431
80	80.4 ± 1.5	104 ± 5.4	146 ± 7.4	179 ± 7.3	466 ± 12
RSD	1.9	5.2	5.1	4.1	2.6
Recovery (%)	100.4	104.3	105.8	93.8	87.5
P	2.9E−14	3.4E−9	3.0E−7	1.2E−5	7.3E−4
90	90.4 ± 2.3	111 ± 4.2	153 ± 5.7	193 ± 10	481 ± 17
RSD	2.5	3.8	3.7	5.2	3.5
Recovery (%)	100.4	100.4	101.8	98.9	94.4
P	3.2E−13	5.1E−10	8.4E−8	6.1E−6	3.3E−4
100	99.6 ± 3.9	122 ± 5.6	163 ± 5.7	206 ± 9.6	510 ± 24
RSD	3.9	4.6	3.5	4.7	4.7
Recovery (%)	99.5	101.4	101.6	102	114
P	1.0E−11	8.1E−10	3.7E−8	1.8E−6	1.0E−4

N.A., not applicable.

[a] Recovery was calculated using Formula (F2).

[b] Numbers in italics indicate unacceptable values for recovery and precision.

[c] *P*-value from *t*-test comparison with the respective basal level of *Drug* or *Endo*.

[d] Numbers in bold indicate acceptable values for recovery and precision.

concentration, i.e., C_{LLOQ}, to the biological sample that can be measured with acceptable accuracy (e.g., recovery of 100 ± 20%) and precision (e.g., RSD ≤ 20%) and that can be discriminated statistically significantly from the basal concentration $C_{0,Ln}$ of the analyte in relevant biological media. Alternatively, the total error concept with recovery + precision values of ≤30% may also be applied [4,5].

As mentioned above endogenous analytes occur at varying $C_{0,Ln}$ levels in biological samples, e.g., from $C_{0,L1}$ to $C_{0,L4}$ with $C_{0,L1} < C_{0,L2} < C_{0,L3} < C_{0,L4}$ (see Fig. 1), and therefore the actual LLOQ value of the method for such analytes may depend upon the extent of $C_{0,Ln}$ (Fig. 2), analogous to the recovery of the method (see above). This issue is exemplified in Table 1 and in Figs. 1 and 2 for a hypothetical pharmaceutical *Drug* and for a hypothetical physiological compound *Endo* that is assumed to occur at four different basal concentrations (i.e., $C_{0,Ln}$, $n = 1, 2, 3, 4$) in a biological system, for instance in plasma samples used in validation experiments.

2. The proposal

2.1. The relative lower limit of quantification—the rLLOQ

The ratio of the C_{LLOQ} value – for instance of the lowest added analyte concentration (i.e., $C_{LLOQ,LOW+}$) – to the respective basal concentration $C_{0,Ln}$, i.e., C_{LLOQ}:$C_{0,Ln}$, may represent an additional useful analytical parameter to characterize the analytical performance of an analytical method and to compare more reliably and objectively analytical methods on the basis of the LLOQ. This work proposes the use of the *relative lower limit of quantification*, i.e., *rLLOQ*. The value of rLLOQ is calculated by Formula (F4), i.e., by dividing the value of the lower limit of quantification C_{LLOQ} by the experimentally measured value of $C_{0,Ln}$ and by multiplying the observed molar ratio by 100:

$$\text{rLLOQ} = (C_{LLOQ} : C_{0,Ln}) \times 100 \quad \text{(F4)}$$

By definition, the rLLOQ expresses the percentage fraction of the analyte which, upon addition to the biological sample that contains this analyte in the basal concentration $C_{0,Ln}$, can be measured therein with acceptable accuracy (e.g., recovery of 100 ± 20%) and imprecision (e.g., RSD ≤ 20%) or with an acceptable total error (recovery + precision) of ≤30%, and can be discriminated from $C_{0,Ln}$. From this definition it results that the smaller the rLLOQ value the greater the discriminatory power of the analytical method for the analyte.

In a further step it may be proposed that LLOQ and rLLOQ be corrected, i.e., divided, for the recovery values with which the LLOQ values (i.e., C_{LLOQ}) have been determined experimentally. Formulas (F5) and (F6) describe these issues for the *recovery-corrected LLOQ*, i.e., $LLOQ_{Rec}$:

$$\text{LLOQ}_{Rec} = C_{LLOQ} : Rec@C_{LLOQ} \quad \text{(F5)}$$

$$\text{rLLOQ}_{Rec} = [(C_{LLOQ} : C_{0,Ln}) : Rec@C_{LLOQ}] \times 100 \quad \text{(F6a)}$$

$$\text{rLLOQ}_{Rec} = \text{rLLOQ} : Rec@C_{LLOQ} \quad \text{(F6b)}$$

whereas $Rec@C_{LLOQ}$ is the value of the recovery with which the C_{LLOQ} is determined. Instead of the recovery, the total error could also be used in these formulas.

2.2. Definition and calculation of methods accuracy

In the context of this proposal the author would like to suggest adoption of the definition of the analytical factor *accuracy* in the quantification of endogenous substances. Thus, the term Accuracy could be defined as follows:

Under precisely prescribed analytical conditions, the *Accuracy* for endogenous compounds, the endobiotica, which occur in biological samples at varying basal concentrations $C_{0,Ln}$, describes the degree of closeness of the concentration difference C_M–$C_{0,Ln}$ (i.e., the difference of endobiotic concentration measured C_M in the spiked biological sample and the endobiotic basal concentration $C_{0,Ln}$ measured in the unspiked biological sample) to the known concentration C_+ of the reference synthetic endobiotic, which was added to the (native) biological sample within a physiologically and pathologically relevant concentration range of the endogenous analyte.

The numerical value of *Accuracy* is termed *Recovery* (*Rec*), is determined by using Formula (F2) and is expressed in units of percent (%). The deviation of the experimentally observed recovery value *Rec* from the ideal value of 100% is termed *relative Bias (rBias)* and is expressed in units of percent (%): $rBias = Rec - 100$. Thus, numerical values for *rBias* can be positive or negative.

Analogous to analytical methods for drugs (e.g., Ref. [7]), analytical methods for the quantification of endobiotica in their specific biological samples should be characterized as accurate when recovery values are 100 ± 20%, i.e., $rBias \leq \pm 20\%$, in relevant concentration ranges for added analyte concentrations C_+. Because the basal concentration $C_{0,Ln}$ of endobiotica may vary greatly in particular biological samples, notably in urine, the concentration range for added analyte concentration C_+ should be directed to the basal concentration $C_{0,Ln}$; this basal concentration should be measured precisely in the biological sample prior to start with methods validation or quality control. The upper range for C_+ should not be arbitrary, but it should be chosen on the basis of the highest analyte concentration expected to occur in certain pathological conditions. In the personal opinion of this author, experiments on methods validation for endobiotica should involve 5–7 concentration points including the basal concentration $C_{0,Ln}$, both in a wide and in a narrow concentration range, with the highest added concentration, i.e., C_{HIGH+}, regularly not exceeding the value of 4× $C_{0,Ln}$ in the narrow-range validation experiment (see below). Issues on validation have been addressed in this journal [1–3,5] and elsewhere [4,6].

2.3. Definition and calculation of methods lower limit of quantification

For endogenous substances the term lower limit of quantification could be defined as follows:

Under precisely prescribed analytical conditions, the *Lower Limit of Quantification* for endogenous compounds, the endobiotica, is the lowest concentration C_{LOW+} of the synthetic reference analytes which, upon addition to the biological sample that contains the endogenous substances at the measured basal concentration $C_{0,Ln}$, can be experimentally measured in the spiked sample with suitable precision and accuracy (as defined above), i.e., it can be distinguished from the basal analytes concentration $C_{0,Ln}$. The numerical value of the LLOQ, i.e., C_{LLOQ}, is expressed in units of concentration with respect to the biological sample.

Commonly, the LLOQ values of analytical methods for endogenous and exogenous analytes are determined by analyzing matrix-free dilutions of the reference synthetic analytes, for instance dilutions in organic solvents, water or aqueous organic solvents. However, this approach is incorrect as it disregards the well-known and well-recognized severe matrix-effects. Actually, this approach provides the LLOD value of the method.

A suitable experimental procedure to gain reliable values for the LLOQ (i.e., C_{LLOQ}) of endogenous substances is that described above for accuracy, namely by working in the narrow range for

added analytes concentrations C_+. Due to the varying basal concentration $C_{0,Ln}$, the C_{LLOQ} value may vary to a considerable degree in dependence upon $C_{0,Ln}$. As shown in this work, this shortcoming can be overcome by using the relative lower limit of quantification, *rLLOQ*, i.e., by correcting the C_{LLOQ} value for the basal analyte concentration $C_{0,Ln}$. The lowest added analyte concentration C_{LOW+} may not fulfill generally accepted stringent criteria for the LLOQ, e.g., recovery of 100 ± 20% and RSD ≤ 20%. However, correction of such an experimentally obtained value by the respective recovery value with which the C_{LOW+} is determined, i.e., the use of $rLLOQ_{Rec}$, or alternatively with the total error, may be useful in estimating the potential of analytical methods to discriminate between the lowest added analyte concentration C_{LOW+} and the basal analyte concentration $C_{0,Ln}$.

3. Examples supporting the proposal

3.1. A theoretical example

Table 1 shows the calculated data for rLLOQ and $rLLOQ_{Rec}$ for the hypothetical endogenous compound *Endo* which is assumed to be present at four different basal levels (L1, L2, L3 and L4), i.e., at four different basal concentrations ($C_{0,Ln}$, n = 1–4, i.e., $C_{0,L1}$, $C_{0,L2}$, $C_{0,L3}$ and $C_{0,L4}$) in the same biological matrix (e.g., plasma) which was used in validation experiments. It can be demonstrated that very different values for recovery can be obtained when the wrong Formula (F3) is used instead of the correct Formula (F2), notably for $C_{0,Ln} > C_{LOW+}$ (data not shown). The data of Table 1 suggest that calculation of recovery values by Formula (F2) allows for evaluation of the discriminatory power of the analytical method for added concentrations being a small fraction of the basal analyte concentration $C_{0,Ln}$.

Table 1 suggests that the lowest added concentration C_{LOW+}, i.e., 10 μM, is not the LLOQ value of the method for *Endo* when its basal concentration of level 1 (L1), i.e., $C_{0,L1}$, is 20.6 μM, because the added concentration of 10 μM is measured with the unacceptable recovery value of 124% [1]. On the other hand, this recovery value, which is not far from the upper acceptable value of 120% [6], suggests that the LLOQ value of the method is rather of the order of 10 μM. Consideration of the recovery value by which this added concentration was measured, provides the recovery-corrected LLOQ value, i.e., $LLOQ_{Rec}$, which is calculated to be 8.1 μM and corresponds to a $rLLOQ_{Rec}$ value of 39.1%. Formally, the added concentration of 20 μM for *Endo* with the lowest basal level L1 is the LLOQ value of the method and corresponds to an rLLOQ value of 103%. Consideration of the recovery value with which this added concentration was measured, yields $LLOQ_{Rec}$ and $rLLOQ_{Rec}$ values of 18 μM and 88.3%, respectively. These data suggest that $LLOQ_{Rec}$ and $rLLOQ_{Rec}$ may be better useful than LLOQ and rLLOQ for estimating the discriminatory power of the method. Similar results are also obtained for *Endo* at the higher basal levels L2, L3 and L4 (Table 1).

3.2. Quantitative determination of ADMA in human plasma by GC–MS/MS

ADMA (i.e., asymmetric dimethylarginine) is an endogenous compound that occurs in human plasma at basal concentrations in the range 400–500 nM [8–10]. We have developed, validated and used a GC–MS/MS method for the quantification of ADMA in human plasma and urine [11,12]. Parts of the data generated by this method [12] were used in the present study to test the usefulness of the proposal for rLLOQ. Table 2 shows the results from the GC–MS/MS quantitative determination of ADMA in a pooled human plasma sample before and after spiking with 100 nM ($C_{+,L1}$) or 200 nM ($C_{+,L2}$) of synthetic ADMA on five different days. The measured basal ADMA concentration ($C_{0,Ln}$) ranged between 317 and 363 nM (Table 2). For unspiked and spiked samples the imprecision values were acceptable throughout (i.e., RSD < 20%). By contrast, the LLOQ values, defined as the lowest added ADMA concentration that was measured with a recovery of 100 ± 20% and precision < 20%, were 100 nM on days 1, 4 and 5, but 200 nM on days 2 and 3. The data of Table 2 suggest that consideration of the recovery value (or total error value) with which the lowest added concentration C_{LOW+} of the analyte is determined, not being the actual LLOQ value ($C_{LOQ,A}$) of the method, may provide LLOQ values very close to the actual LLOQ values.

3.3. Measuring of stable-isotope labeled analogs of endogenous compounds

An example that resembles the difference between xenobiotica and endobiotica regarding the LLOQ may represent the

Table 2
Calculation of rLLOQ and $rLLOQ_{Acc}$ values from original experimentally generated validation data for ADMA in human plasma [12][a].

Day	C_+ (nM)	C_M (nM)[b]	Recovery (%)[c]	Precision (RSD, %)	Total error (%)[d]	P-value (t-test)[e]	LLOQ (nM)	rLLOQ (%)	$LLOQ_{Rec}$ (nM)	$rLLOQ_{Rec}$ (%)
Day 1	0	362 ± 2	N.A.	0.6	N.A.	N.A.	–	–	–	–
	100	479 ± 5	117.0	1.0	18.0	3.97E−6	100	27.6	85.5	23.6
	200	570 ± 4	104.0	0.7	4.7	1.92E−5	–	–	–	–
Day 2	0	363 ± 9	N.A.	2.5	N.A.	N.A.	–	–	–	–
	100	437 ± 11	74.0	2.5	28.5	8.95E−5	–	–	135	37.1
	200	536 ± 26	86.5	4.9	18.4	0.0037	200	55.1	–	–
Day 3	0	317 ± 12	N.A.	3.8	N.A.	N.A.	–	–	–	–
	100	452 ± 10	135.0	2.2	37.2	9.93E−5	–	–	74.1	23.4
	200	534 ± 32	108.5	6.0	14.5	0.013	200	63.1	–	–
Day 4	0	339 ± 28	N.A.	8.3	N.A.	N.A.	–	–	–	–
	100	421 ± 3	82.0	0.7	18.7	0.0295	100	29.5	122	35.6
	200	544 ± 20	102.5	3.7	6.2	0.0129	–	–	–	–
Day 5	0	340 ± 13	N.A.	3.8	N.A.	N.A.	–	–	–	–
	100	449 ± 36	109.0	8.0	17.0	0.0568	100	29.4	91.7	27.0
	200	559 ± 7	109.5	1.3	10.8	0.0511	–	–	–	–

N.A., not applicable.
[a] Data were taken from a previously reported study of our group describing validation of a GC–MS/MS method for ADMA in human plasma using d_7-ADMA as internal standard [12].
[b] d_7-ADMA was added to plasma samples at a final concentration of 800 nM.
[c] Recovery was calculated using Formula (F2).
[d] Total error is defined as relative bias (%) + precision (RSD,%).
[e] Comparison between neighbours. Values are given as mean ± SD, n = 3.

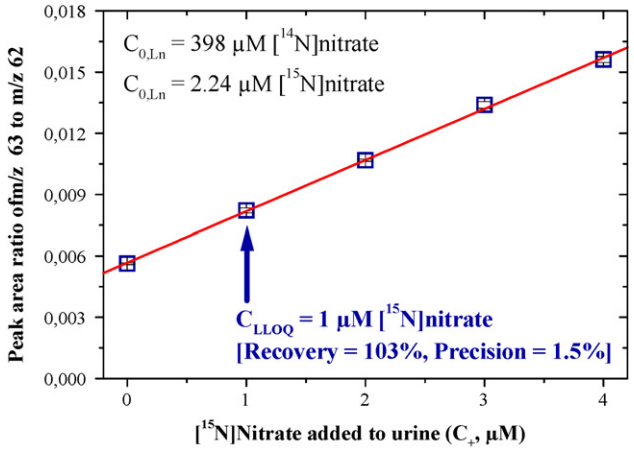

Fig. 3. Lower range of the standard curve generated by quantifying [^{15}N]nitrate (0–80 μM) added to a human urine sample that contained total nitrate at a basal concentration ($C_{0,Ln}$) of about 400 μM. Nitrate was analyzed by GC–MS as its pentafluorobenzyl ester derivative [14]. The ions at m/z 63 for [^{15}N]nitrate and at m/z 62 for [^{14}N]nitrate were detected in the selected-ion monitoring (SIM) mode as described elsewhere [14]. The lowest added [^{15}N]nitrate concentration of 1 μM (C_{LOW+}) is defined as the LLOQ value of the method for [^{15}N]nitrate in this urine sample. For more details see Table 3 and the text. This figure was constructed with data published by our group elsewhere [15].

measuring of stable-isotope labeled analogs of endogenously produced compounds such as [^{15}N]nitrate. The natural abundance of ^{15}N is 0.36%. This means that in a urine sample that contains nitrate (NO_3^-) at a basal concentration of 1000 μM about 996 μM are due to [^{14}N]nitrate and only about 4 μM are due to [^{15}N]nitrate. With respect to the total nitrate concentration (i.e., [^{14}N]nitrate + [^{15}N]nitrate), the concentration of [^{15}N]nitrate would be negligible as compared to [^{14}N]nitrate, i.e., $C_{0,Ln} \approx 0$ μM. In other words, [^{15}N]nitrate behaves as a drug in relation to [^{14}N]nitrate.

By means of mass spectrometry-based methods such as GC–MS [13], [^{15}N]nitrate and [^{14}N]nitrate can be discriminated each other because of their different m/z values of the anions $^{15}NO_3^-$ (m/z 63) and $^{14}NO_3^-$ (m/z 62). For the majority of other possible analytical methods including HPLC, discrimination between [^{15}N]nitrate and [^{14}N]nitrate is not possible. It results from this that the LLOQ value of a GC–MS method for [^{15}N]nitrate would be completely different, i.e., much lower as compared to a HPLC method. The results from the GC–MS analysis of [^{15}N]nitrate added to a human urine that contained basally about 398 μM of nitrate are summarized in Table 3 for the whole concentration range investigated (i.e., 0–80 μM of [^{15}N]nitrate) and are shown in Fig. 3 for the lower [^{15}N]nitrate range (i.e., 0–4 μM of [^{15}N]nitrate) [14,15]. These data suggest that the LLOQ value for [^{15}N]nitrate in the urine sample is C_{LLOQ} = 1 μM. In accordance with Formula (F4), the rLLOQ for [^{15}N]nitrate would be 123% with respect to the basal [^{15}N]nitrate concentration, but only 0.25% with respect to the basal [^{14}N]nitrate or to the total nitrate concentration. The very low rLLOQ value of 0.25% for [^{15}N]nitrate indicates the great discriminatory power of the GC–MS method for [^{15}N]nitrate and the superior advantage of using stable-isotope labeled analogues of endobiotica.

3.4. Examples from the literature [16–26]

Table 4 summarizes the calculated rLLOQ and $rLLOQ_{Rec}$ values from published work by our group and other groups on the quantification of endobiotica from the L-arginine/nitric oxide (NO) and oxidative/nitrative stress areas of research. In the studies considered here, validation data had been published in such a way that they could be used to test the proposal presented in this work. In

Table 3
Calculation[a] of recovery values from the GC–MS analysis of [^{15}N]nitrate in human urine.

[^{15}N]Nitrate added (μM)	$R_{63/62}$ (×1000)[b] measured (μM)	Precision (RSD, %)	P (t-test)[c]	[^{14}N]Nitrate (basal) (μM)	[^{15}N]Nitrate (basal) (μM)	[^{15}N]Nitrate (measured) (μM)	[^{15}N]Nitrate (measured – basal) (μM)	Recovery [^{15}N]nitrate (%)
0	5.63 ± 0.040	0.71	N.A.	398	1.43	2.24	0.81	N.A.
1	8.22 ± 0.125	1.52	4.4E−6	398	1.43	3.27	1.84	103.0
2	10.7 ± 0.055	0.51	6.3E−6	398	1.43	4.26	2.83	101.0
3	13.4 ± 0.165	1.23	1.1E−5	398	1.43	5.33	3.90	103.0
4	15.6 ± 0.148	0.95	6.4E−5	398	1.43	6.21	4.78	99.3
5	19.2 ± 0.190	0.99	1.3E−5	398	1.43	7.64	6.21	108.0
7.5	24.5 ± 0.325	1.33	1.7E−5	398	1.43	9.75	8.32	100.1
10	30.8 ± 0.127	0.41	6.4E−5	398	1.43	12.26	10.83	100.2
20	54.6 ± 1.050	1.92	2.6E−6	398	1.43	21.73	20.3	97.5
30	80.6 ± 1.590	1.97	1.9E−5	398	1.43	32.08	30.65	99.5
40	105 ± 0.496	0.47	1.4E−5	398	1.43	41.79	40.36	98.9
80	205 ± 2.070	1.01	1.4E−7	398	1.43	81.59	80.16	99.2

N.A., not applicable
[a] Original data were taken from a study previously reported by our group [15]. See also Fig. 3.
[b] $R_{63/62}$ is the peak area ratio of m/z 63 (for [^{15}N]nitrate) and m/z 62 (for [^{14}N]nitrate) from triplicate analyses.
[c] Comparison between next concentrations. rLLOQ for ^{15}N versus ^{15}N: rLLOQ = (1 μM/0.81 μM) × 100 = 123%; rLLOQ for ^{15}N versus ^{14}N: rLLOQ = (1 μM/398 μM) × 100 = 0.25%.

Table 4
Summary of calculated rLLOQ values from some reported analytical methods on endogenous compounds in human plasma samples.

Analyte	Matrix	Method	LLOQ[a] (C_{LOW+})	$C_{0,Ln}$	rLLOQ (%)	*Rec* (%)	$LLOQ_{Rec}$	$rLLOQ_{Rec}$ (%)	Ref.
ADMA	Urine	GC–MS/MS	3 μM	20 μM	15	90	3.3 μM	17	[11]
ADMA	Plasma	GC–MS/MS	100 nM	455 nM	22	95	105 nM	23	[11]
ADMA	Plasma	LC–MS/MS	50 nM	660 nM	7.6	106	47 nM	7.1	[16]
ADMA	Plasma	GC–MS	250 nM	880 nM	28	112	223 nM	25	[23]
ADMA	Serum	ELISA	250 nM	590 nM	42	101	248 nM	42	[24]
SDMA	Plasma	LC–MS/MS	50 nM	610 nM	8.2	84	60 nM	9.8	[16]
L-Arginine	Plasma	GC–MS	2 μM	75.4 μM	2.7	102	1.96 μM	2.6	[11]
L-Arginine	Plasma	LC–MS/MS	0.5 μM	43.8 μM	1.1	113	0.44 μM	1.0	[16]
Dimethylamine	Urine	GC–MS	100 μM	446 μM	22	95	105 μM	24	[26]
Nitrate	Plasma	GC–MS	10 μM	70.8 μM	14	105	9.5 μM	13	[14]
Nitrate	Urine	HPLC-UV	360 μM	1093 μM	33	103	350 μM	32	[19]
3-Nitrotyrosine	Plasma	GC–MS/MS	1.0 nM	4.18 nM	24	80	1.25 nM	30	[17]
3-Nitrotyrosine	Plasma	GC–MS/MS	0.5 nM	0.83 nM	60	99	0.51 nM	61	[18]
dn-dh-8-*iso*-$PGF_{2\alpha}$[b]	Urine	GC–MS/MS	307 pM	721 pM	43	92	334 pM	46	[20]
15(*S*)-8-*iso*-$PGF_{2\alpha}$	Urine	GC–MS/MS	113 pM	279 pM	41	108	105 pM	37	[21]
Oleic acid oxide	Plasma	GC–MS/MS	10 nM	58.6 nM	17	89	11 nM	19	[22]
Creatinine	Plasma	HPLC-UV	10 μM	60.7 μM	16	93	10.8 μM	18	[25]
Creatinine	Urine	HPLC-UV	100 μM	925 μM	11	112	89 μM	9.6	[25]

[a] In most cases the reported lowest added concentration was used for calculations.
[b] dn-dh-8-*iso*-$PGF_{2\alpha}$, 2,3-dinor-5,6-dihydro-8-*iso*-$PGF_{2\alpha}$.

most cases the reported lowest added concentration of the analyte (C_{LOW+}) was used to calculate the values for rLLOQ and $rLLOQ_{Rec}$. Table 4 shows that the calculated rLLOQ values range from 1.1% to 2.7% for L-arginine measured in human plasma by LC–MS/MS [16] and GC–MS [11], respectively, to 61% for 3-nitrotyrosine measured in human plasma by GC–MS/MS [17].

Plotting of the (absolute) LLOQ values summarized in Table 4 against $C_{0,Ln}$ revealed a clear dependence of the LLOQ value upon the basal analyte concentration $C_{0,Ln}$ ($R = 0.82$, $P < 0.0001$). By contrast, plotting of the rLLOQ values versus $C_{0,Ln}$ shows no dependence at all ($R = 0.0005$, $P = 0.99$). This finding suggests that use of rLLOQ instead of LLOQ should be a more reliable analytical factor in methods comparison.

4. Discussion, conclusions and outlook

Reliable quantification of endogenous substances in biological samples, notably of those present therein at very low basal concentrations, is by far a more challenging analytical endeavor than quantification of drugs. Assessment of "true" concentrations of numerous endobiotica in their specific biological media requires use of highly sensitive analytical methods. However, high sensitivity in terms of small LLOD values is always a prerequisite for the quantification of large a number of physiological substances in biological material such as human plasma and urine. Accuracy, precision or more correctly imprecision, and lower limit of quantification are key analytical parameters by means of which the performance of methods of quantitative analysis can be characterized, and analytical methods can be compared each other on a quantitative basis. Thus, only high accuracy paired with high precision enables accurate measurement and analytical distinction of small changes in their basal concentrations as a result of disease, life style and/or pharmacological intervention. Consequently, analytical methods intended for use for the quantification of endogenous substances in their typical biological samples, notably in the frame of clinical and animal research studies, deserve special attention, even more attention than that paid to drugs, thus far.

Definitions and rules originally drew up for the quantitative analysis of pharmaceuticals may also be useful for endogenous substances. However, the inherent analytical difference between xenobiotica and endobiotica makes mandatory a careful adaptation of definitions and rules to the class of the physiological substances. With regard to the quantification in biological samples of endogenous substances that occur at very low basal concentrations, the LLOQ value of the method may be by far the most relevant analytical factor, because LLOQ value implies that the method is accurate and precise at this analyte concentration. This work proposes the use of the relative lower limit of quantification rLLOQ and adaptations for the accuracy and the LLOQ in quantitative analyses of endogenous substances. Recently, the total error approach is increasingly applied in analytical methods validation and comparison [4,5]. The total error approach "is a quality parameter derived from two performance parameters (precision and accuracy) which contribute mutually to the quality of a test result" [5]. Adaptation of the total error approach in the proposal of the relative lower limit of quantification is possible and should be striven for.

Unfortunately, LLOQ values are determined incorrectly or LLOD values are either erroneously reported as the LLOQ values or they are used to calculate LLOQ values considering the respective signal-to-noise (S/N) ratio. In such cases methods comparison is actually not possible. A typical example for analytes, for which several different methods have been reported but reliable comparison of the methods used is actually not possible, represents nitrite [27]. By contrast, for ADMA, another member of the L-arginine/nitric oxide pathway, an endogenous substance of high clinical importance, analytical methods based on various principles are satisfactorily reported and a reliable comparison among these methods is possible on the basis of several key analytical parameters including LLOQ, accuracy and precision [5,28,29]. The rLLOQ proposed in the present work could be a suitable parameter to describe numerically the potential of analytical methods to discriminate among little differing concentrations of endobiotica. Also, the rLLOQ may allow for a more objective comparison of analytical methods for quantitative analysis of endobiotica.

Accurate measuring of basal concentrations of endogenous compounds is of outstanding value, because solid knowledge of basal concentrations in physiological conditions is of fundamental importance in defining reference values and intervals. This goal can be reached by clear definitions for relevant analytical parameters that describe quantitatively the quality of the performance of analytical methods and techniques intended for quantitative measurement of endogenous substances in biological systems. It is believed that the adaptations proposed here will be useful in the analytical chemistry of physiological compounds and will contribute to clarify uncertainties concerning LLOQ and accuracy of endogenous substances and will allow for a more objective method comparison.

Abbreviations and formulas

Rec	recovery (%), numerical value for accuracy
	$Rec = (C_M : C_+) \times 100$ (F1)
	$Rec = [(C_M - C_{0,Ln}) : C_+] \times 100$ (F2)
	$Rec = [C_M : (C_{0,Ln} + C_+)] \times 100$ (F3)
$Rec@C_{LLOQ}$	recovery value (%) with which the LLOQ concentration C_{LLOQ} is determined
$C_{0,Ln}$	basal concentration of the analyte of the level Ln (e.g., nM), $n = 1, 2, 3, \ldots$.
C_+	added analyte concentration (e.g., nM)
C_{LOW+}	lowest added analyte concentration (e.g., nM)
C_{HIGH+}	highest added analyte concentration (e.g., nM)
C_{LLOQ}	LLOQ concentration of the analyte (e.g., nM)
$C_{LLOQ,A}$	actual LLOQ concentration of the analyte (e.g., nM)
$C_{LLOQ,LOW+}$	lowest added LLOQ concentration of the analyte (e.g., nM)
C_M	measured analyte concentration (e.g., nM)
$LLOQ_{Rec}$	recovery-corrected LLOQ concentration of the analyte (e.g., nM)
	$LLOQ_{Rec} = C_{LLOQ} : Rec@C_{LLOQ}$ (F5)
rLLOQ	relative LLOQ (%)
	$rLLOQ = (C_{LLOQ} : C_{0,Ln}) \times 100$ (F4)
$rLLOQ_{Rec}$	relative recovery-corrected LLOQ (e.g., nM)
	$rLLOQ_{Rec} = [(C_{LLOQ} : C_{0,Ln}) : Rec@C_{LLOQ}] \times 100$ (F6a)
	$rLLOQ_{Rec} = rLLOQ : Rec@C_{LLOQ}$ (F6b)

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