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An Inter-Laboratory Comparison for the Urinary Acrolein Biomarker 3-Hydroxypropyl-Mercapturic Acid (3-HPMA)*

by

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SUMMARY

An inter-laboratory comparison study on the acrolein biomarker of exposure 3-hydroxypropyl- mercapturic acid (3-HPMA) with 12 laboratories from 7 globally distributed countries was performed. The laboratories received coded triplicates of 4 spiked and lyophilized urine samples (LU, 12 samples) as well as 5 authentic urine pool samples (PU, 15 samples) covering the 3-HPMA concentration range from background (non-smoking) to heavy smoking levels for analysis by using their own (in-house) analytical method. All laboratories applied liquid chromatography with tandem mass spectrometry (LC-MS/MS), with most of them (10 of 12) using solid phase extraction (SPE) as sample work-up procedure. The intra-laboratory variation (indicating repeatability) was determined by calculating the standard deviation (s_r) and the coefficient of variation (CV_r) of the triplicates, whereas the inter-laboratory variation (indicating reproducibility) was determined by calculating the standard deviation between laboratories (s_R) and the corresponding coefficient of variation (CV_R). After removal of outlier samples or laboratories, the mean CV_r values for LU and PU test samples ranged from 2.1–3.6% (mean: 2.8%) and 2.4–3.7% (mean: 3.3%), respectively, indicating good repeatability for the determination of 3-HPMA in both sample types. CV_R for LU and PU test samples ranged from 9.1–31.9% (mean: 18.8%) and 13.9–27.0% (mean: 18.5%), respectively, indicating limited reproducibility in 3-HPMA

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analysis for both sample types. Re-calculation of the PU results by applying an embedded calibration (EC), derived from the reported peak areas for the LU test samples, somewhat improved the CV_R values (range: 9.6–28.8%, mean: 16.7%).
It is concluded that the intra-laboratory variation (repeatability) in the determination of 3-HPMA in urine is in general acceptable in the participating laboratories, while the inter-laboratory variability requires further improvement. The relatively small reduction in the inter-laboratory variability (s_R and CV_R) by applying an EC suggests that other methodological factors than the standard reference material for 3-HPMA have to be addressed to achieve further improvement in reproducibility. [Beitr. Tabakforsch. Int. 27 (2017) 65–76]

KEYWORDS

Inter-laboratory comparison; 3-hydroxypropyl-mercapturic acid (3-HPMA); intra-laboratory variation; inter-laboratory variation; repeatability; reproducibility

ZUSAMMENFASSUNG

Es wurde eine Vergleichsstudie zwischen Laboren bezüglich des Acrolein-Expositionsbiomarkers 3-Hydroxypropyl-Mercaptursäure (3-HPMA) mit 12 Laboren aus 7 Ländern der Welt durchgeführt. Die Labore erhielten codierte Triplikate von 4 präparierten und lyophilisierten Urinproben (LU, 12 Proben) sowie 5 authentische Urin-Poolproben (PU, 15 Proben) für den Konzentrationsbereich von 3-HPMA von Hintergrund- (Nichtraucher-) bis Starkraucherkonzentrationen zur entsprechenden Analyse mit der eigenen (internen) Analysemethode. Alle Labore wandten Flüssigchromatographie mit Tandem-Massenspektrometrie (LC-MS/MS) an, wobei die meisten von ihnen (10 von 12) die Festphasenextraktion (SPE) zur Probenaufbereitung einsetzten. Die laborinterne Variation (Indikator für die Wiederholgenauigkeit) wurde durch Berechnung der Standardabweichung (s_r) und des Variationskoeffizienten (CV_r) der Triplikate bestimmt, während die Variation zwischen den Laboren (Indikator für die Reproduzierbarkeit) durch Berechnung der Standardabweichung zwischen Laboren (s_R) und des entsprechenden Variationskoeffizienten (CV_R) ermittelt wurde. Nach Aussortieren der Ausreißerproben bzw. -Labore lagen die mittleren CV_r-Werte für LU- und PU-Test-Proben bei 2,1–3,6% (Mittelwert: 2,8%) bzw. 2,4–3,7% (Mittelwert: 3,3%) und deuteten auf eine gute Wiederholgenauigkeit für die Bestimmung von 3-HPMA bei beiden Probentypen hin. Die CVR für LU- und PU-Test-Proben lagen bei 9,1–31,9% (Mittelwert: 18,8%) bzw. 13,9–27,0% (Mittelwert: 18,5%) und wiesen auf eine begrenzte Reproduzierbarkeit in der Analyse von 3-HPMA bei beiden Probentypen hin. Die Neuberechnung der PU-Ergebnisse unter Anwendung einer eingebettenen Kalibrierung, die aus den berichteten Peak-Bereichen für die LU-Testproben abgeleitet war, führte zu einer gewissen Verbesserung der CV_R-Werte (Bereich: 9,6–28,8%, Mittelwert: 16,7%).
Es wird geschlussfolgert, dass die laborinterne Variation (Wiederholgenauigkeit) bei der Bestimmung von 3-HPMA in Urin in den teilnehmenden Laboren im Allgemeinen akzeptabel ist, die Variabilität zwischen den Laboren jedoch noch verbessert werden muss. Die relativ geringe Reduktion der Variabilität zwischen den Laboren (s_R und CV_R) durch Anwendung einer eingebetteten Kalibrierung deutet darauf hin, dass andere methodische Faktoren als das Standardreferenzmaterial für 3-HPMA betrachtet werden müssen, um eine weitere Verbesserung der Reproduzierbarkeit zu erreichen. [Beitr. Tabakforsch. Int. 27 (2017) 65–76]

RESUME

Consacrée au biomarqueur acide 3-hydroxypropyl mercapturique (3-HPMA) de l'exposition à l'acroléine, une étude comparative mit en regard 12 laboratoires implantés dans 7 pays répartis sur la planète. En vue d'une analyse par leur propre méthode analytique (en interne), les laboratoires reçurent des triplicats codés de 4 échantillons d'urine lyophilisés et enrichis (LU, 12 échantillons) ainsi que 5 échantillons authentiques de mélange d'urine (PU, 15 échantillons) couvrant les plages de concentration du 3-HPMA allant de l'échantillon de contrôle (non-fumeur) à des niveaux de tabagisme lourd. Tous les laboratoires recoururent à la chromatographie en phase liquide couplée à une spectrométrie de masse en tandem (LC-SM/SM) et la plupart (dix sur douze) employèrent une extraction liquide-solide (ELS) en guise de préparation des échantillons. La variation intralaboratoire (indicateur de répétabilité) fut déterminée par le calcul de l'écart-type (s_r) et du coefficient de variation (CV_r) des triplicats tandis que la variation interlaboratoire (indicateur de reproductibilité) fut déterminée par le calcul de l'écart-type entre les laboratoires (s_R) et leur coefficient correspondant de variation (CV_R). Après l'élimination des échantillons ou laboratoires aberrants, les valeurs CVr moyennes des échantillons d'épreuves LU et PU oscillèrent respectivement entre 2,1 et 3,6% (médiane : 2,8%) et 2,4 et 3,7% (médiane : 3,3%), indiquant une bonne répétabilité de la détermination du 3-HPMA pour les deux types d'échantillons. Les valeurs CV_R pour les échantillons d'épreuve LU et PU oscillèrent respectivement entre 9,1 et 31,9% (médiane : 18,8%) et 13,9 et 27,0% (médiane : 18,5%), indiquant une reproductibilité limitée pour l'analyse du 3-HPMA sur les deux types d'échantillons. Un nouveau calcul des résultats pour les échantillons PU par application d'un étalonnage intégré dérivé des aires de pic identifiées pour les échantillons d'épreuve LU permit d'améliorer quelque peu les valeurs CV_R (plage : 9,6–28,8%, médiane : 16,7%).
En conclusion, la variation intralaboratoire (répétabilité) dans la détermination du 3-HPMA dans l'urine est jugée, en règle générale, acceptable dans les laboratoires participants alors que la variabilité interlaboratoire devrait continuer à être améliorée. Le recul relativement léger de la variabilité interlaboratoire (s_R et CV_R) après l'application d'un étalonnage intégré laisse à penser que des facteurs méthodologiques autres que la substance de référence standard pour le 3-HPMA doivent être envisagés dans le but d'améliorer encore à l'avenir la reproductibilité. [Beitr. Tabakforsch. Int. 27 (2017) 65–76]

ABBREVIATIONS

3-HPMA	3-hydroxypropyl- mercapturic acid
CV_r	Coefficient of variation of intra-laboratory variation (repeatability)
CV_R	Coefficient of variation of inter-laboratory variation (reproducibility)
EC	Embedded calibration
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
LU	Lyophilized urine samples
PU	Urine pool samples
s_r	Standard deviation of intra-laboratory variation (repeatability)
s_R	Standard deviation of inter-laboratory variation (reproducibility)

INTRODUCTION

Given the health implication of tobacco smoking, tobacco harm reduction is an important public health activity (1). According to the reports of the INSTITUTE OF MEDICINE of 2001 and 2011 (2, 3), tobacco harm reduction is feasible and would require toxicological and clinical testing of newly developed modified risk tobacco products (MRTPs). The measurement of suitable biomarkers of exposure and effect is central to the evaluation of these products in humans. Comparability of quantitative biomarker data generated by different laboratories in biological samples from clinical and epidemiological studies is of fundamental importance for solid risk evaluation. Ring-trials are a suitable approach to prove comparability between laboratories and, if repeated on a regular basis, to ensure their quality of work on a continuous basis. In the past, inter-laboratory comparisons on the tobacco- and smoking-related biomarkers have been conducted and published for nicotine and cotinine (4, 5) and 3-hydroxypropyl-mercapturic acid (3-HPMA) (6). The international program of the German External Quality Assessment Scheme (G-EQUAS) provides proficiency testing for many biomarkers, including smoking-related ones such as nicotine, cotinine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), 3-HPMA (and a series of other mercapturic acids) as well as 1-/2-naphthol and 1-hydroxypyrene on a regular (twice yearly) basis (7).

One of the mandates of the Subgroup 'Biomarkers' of CORESTA (Cooperation Centre for Scientific Research Relative to Tobacco) is to undertake ring-trials, proficiency tests or inter-laboratory comparisons for selected biomarkers (for further information, see https://www.coresta.org/groups/biomarkers). The CORESTA Taskforce 'Nicotine uptake', a predecessor of the Subgroup 'Biomarkers' conducted 5 proficiency tests on nicotine and its 5 major metabolites in urine as well as 3 proficiency tests on salivary cotinine (8). In 2010, the CORESTA Subgroup 'Biomarkers' initiated an inter-laboratory comparison on 3-HPMA, which was actually conducted one year later.

3-HPMA in urine is an established biomarker of exposure to acrolein, a major toxicant in tobacco smoke (9), but is also formed during other combustion processes, fat pyrolysis as well as endogenously through lipid peroxidation (10). The mercapturic acid is formed by reaction of acrolein with glutathione (either spontaneously or enzymatically catalyzed by a glutathione transferase) and subsequent degradation of the tripeptide, followed by *N*-acetylation of the cysteine residue and reduction of the aldehyde group (Figure 1). Liquid chromatography with tandem mass spectrometry (LC-MS/MS) is the most frequently used methodology for 3-HPMA determination in urine (11–17).

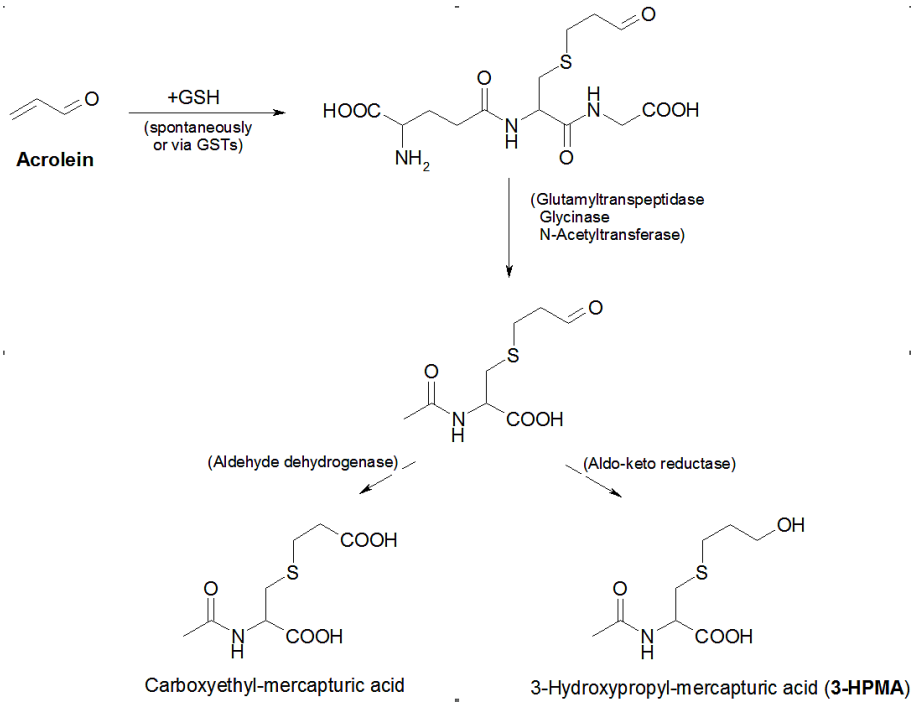

Figure 1. Simplified scheme for the metabolism of acrolein. GSH: glutathione, GSTs: glutathione-S-transferases (according to (10), modified).

In a series of studies, urinary 3-HPMA levels were analyzed in smokers and non-smokers, showing, on average, about 5-fold higher levels in smokers (12, 13, 16, 18–24). In most of these studies, a strong association between the smoking dose and urinary 3-HPMA excretion was found. Smoking cessation was observed to decrease the levels to non-smoker concentrations within a few days (25–29). The Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area (working under the auspices of the German Research Community, DFG) evaluated for 3-HPMA a biological reference value (BAR) for non-smokers of 600 µg/g creatinine, which corresponds to 2.1 nmol/mL approximately (30). The BAR was derived from the 95th percentile of the 3-HPMA distribution in the German non-smoking general population of working age. In a recent report of the INSTITUTE OF MEDICINE (3), 3-HPMA mean excretion rates of 5,869–11,190 and 1,131–1,847 nmol/24 h for smokers and non-smokers, respectively, were reported. This corresponds to urinary concentrations of about 4–7.5 and 0.8–1.2 nmol/mL for smokers and non-smokers, respectively. These urinary concentration ranges are covered by the inter-laboratory comparison study reported here.

The primary goal of this inter-laboratory comparison on urinary 3-HPMA was to determine the intra- and inter-laboratory variation between the participating laboratories, which had different levels of experience in 3-HPMA analysis and all used their own analytical method. Furthermore, the test samples provided included 3-HPMA fortified samples, which were used for an embedded calibration (EC), so that all samples could be evaluated with the laboratories' own calibration and with the EC allowing differentiation between laboratory variation and differences in the used standard reference materials. Finally, the inclusion of spiked and lyophilized urine samples (LU) and of authentic urine pool (PU) test samples derived from smokers and non-smokers provided the opportunity to test the suitability of LU samples as standard reference material for 3-HPMA in terms of storage stability and shipment conditions.

EXPERIMENTAL

Test samples

Two types of samples were prepared and shipped for analysis to the participating laboratories:

(i) 3-HPMA-spiked and lyophilized non-smoker urine pool samples (LU) including 4 different levels identical with those used in an earlier inter-laboratory comparison (6): unspiked (0.14–0.23) and spiked with 400, 1200 and 3600 ng/mL (1.81, 5.42 and 16.27 nmol/mL);

(ii) 5 different pool urine (PU) samples from a non-smoker (PU1) and from a range of slight to heavy smokers (PU2 to PU5).

All urine samples used for the generation of the PU samples were derived from ethically approved clinical studies. All samples were provided to the participants in blinded triplicates, thus each laboratory received 12 LU test samples (randomly coded LU1 to LU12) and 15 PU test samples (randomly coded PU1 to PU15). PU sample preparation, aliquotting, coding and shipments were performed by ABF (reference laboratory). Each laboratory received volumes of test samples sufficient for at least a duplicate analysis.

Participating laboratories and shipments

In total, 12 laboratories participated in the 3-HPMA inter-laboratory comparison (Table 1), located in 7 countries: China (4 labs), Germany (3), France (1), Canada (1), USA (1), Japan (1) and South Korea (1). Test samples were shipped by ABF from Munich, Germany to the various countries on dry ice. Shipments reached the addressee within 4 days, at the latest. All samples stayed frozen during shipping.

Table 1. Participating laboratories (in alphabetic order).

Institute / Company	Country
ABF Analytisch-Biologisches Forschungslabor GmbH	Germany
Celerion	USA
China National Tobacco Quality Supervision and Test Centre	China
China Tobacco Zhejiang Industrial Company Ltd.	China
Japan Tobacco Inc.	Japan
Institute of Occupational and Social Medicine, RWTH Aachen	Germany
Institute and Outpatient Clinic of Occupational, Social and Environmental Medicine (IPASUM), University of Erlangen-Nuremberg	Germany
KT&G Research Institute	South Korea
Labstat International UTC	Canada
SEITA-Imperial Tobacco Group Ltd.	France
Shanghai Tobacco Group Co. Ltd.	China
Zhengzhou Research Institute of CNTC	China

Data reporting

Each laboratory received an Excel template for providing the following information to the statistical evaluation group:

i. Calibration equation ('own' calibration) generated and used by the lab for calculation of 3-HPMA results
ii. Entry of 3-HPMA results from all study samples (12 LU + 15 PU = 27 samples)
iii. Peak area of 3-HPMA for all study samples
iv. Peak area of the internal standard (IS) for all study samples
v. Limit of quantification (LOQ) for 3-HPMA
vi. Principle of the analytical method used

Statistical evaluation

The statistical evaluation was performed by statisticians, who also were the only persons having access to the assignment of the laboratory codes (A to L). The intra- and inter-laboratory variation, which were the main objectives of this inter-laboratory comparison, were determined by calculating the intra-laboratory standard deviations (s_r) based on each triplicate of all test samples reported by each laboratory as well as the inter-laboratory standard deviation

(s_R) based on the means of the triplicates for each test sample. s_r and s_R are used as indicators for the repeatability (r) and reproducibility (R), respectively, as defined in the spirit of the ISO 5725 guidelines for inter-laboratory comparisons (31). The relative intra- and inter-laboratory variability is expressed by calculating the coefficient of variations (CV_r and CV_R, respectively). Outliers were identified and removed prior to calculating any inter-laboratory parameters using the Grubbs' and Cochran's C tests as appropriate (31). Embedded calibration (EC) functions were calculated for each laboratory based on the reported peak areas for 3-HPMA and the internal standard (IS) of the spiked lyophilized urine samples (LU). For this purpose, the linear regression between the peak area ratio 3-HPMA/IS and the outlier-cleared means for the 3-HPMA concentration in the 4 LU samples was calculated. The regression line was forced through the origin.

RESULTS AND DISCUSSION

Analytical methods used

All 12 participating laboratories used LC-MS/MS methodology for 3-HPMA analysis in the test samples, adding stable-isotope-labeled 3-HPMA as internal standard (IS, [^{13}C3-^{15}N]-3-HPMA, AptoChem, Montreal, Canada) prior to sample work-up. Ten laboratories applied solid phase extraction as sample work-up procedure, whereas 2 laboratories (F, J) reported centrifugation/filtration as sample preparation procedure. All laboratories calibrated their method by linear regression, with 5 labs (D, G, I, J, K) applying 1/x or $1/x^2$ weighting. Reported limits of detection (LOD) and limits of quantification (LOQ) were in the range of 0.002–0.02 and 0.005–0.113 nmol/mL, respectively. Two participants (E, K) reported to achieve LOD and LOQ levels at least 2 orders of magnitude lower (which, however appears to be irrelevant for authentic human urine samples).

Lyophilized and spiked urine samples (LU)

Table 2 shows the individual results for all LU test samples as reported by the laboratories A–L. Intra-laboratory variation, as indicated by the absolute (s_r) and relative standard deviations (CV_r) are in a good to acceptable range. CV_r values are mostly in the range of 1–10%, which demonstrates a high repeatability for measuring 3-HPMA in the 4 LU test samples, in general. Laboratories E and L were outliers according to the Grubbs' test for sample LU2 and, therefore, not considered in the calculation of the means for these test samples. For illustration purposes, a graphical presentation of the individual results for test sample LU2 is shown in Figure 2.

The inter-laboratory variations (indicated by the outlier-adjusted means of the standard deviations s_R) almost linearly increased with the 3-HPMA concentrations in the LU test samples and is 4- to 9-fold higher than the intra-laboratory variation (s_r). As expected, the relative standard deviation (CV_R) is highest (31.9%) in the test sample with the lowest 3-HPMA concentration (LU1). The lowest CV_R value (9.1%) is unexpectedly observed in LU2, which had the second lowest 3-HPMA concentration. This can be explained by the fact that two laboratories (E and L) have to be excluded from the s_R calculation for this test sample. The data in Table 2 show that Laboratories E and L significantly contribute to the s_R values of LU3 and LU4. The mean CV_R for all reported and the outlier-adjusted results amounted to 22.1 and 18.8%, respectively.

Figure 3 shows intra- and inter-laboratory variation in relation to the increasing 3-HPMA concentrations of test samples LU1–LU4. The large difference between intra- and inter-laboratory variations (5- to 8-fold) is evident.

Authentic urine pools (PU)

Table 3 shows the individual results for all PU test samples. Intra-laboratory variation, as indicated by the absolute standard deviations (s_r) and the relative standard deviations (CV_r) are again in a good to acceptable range. CV_r values

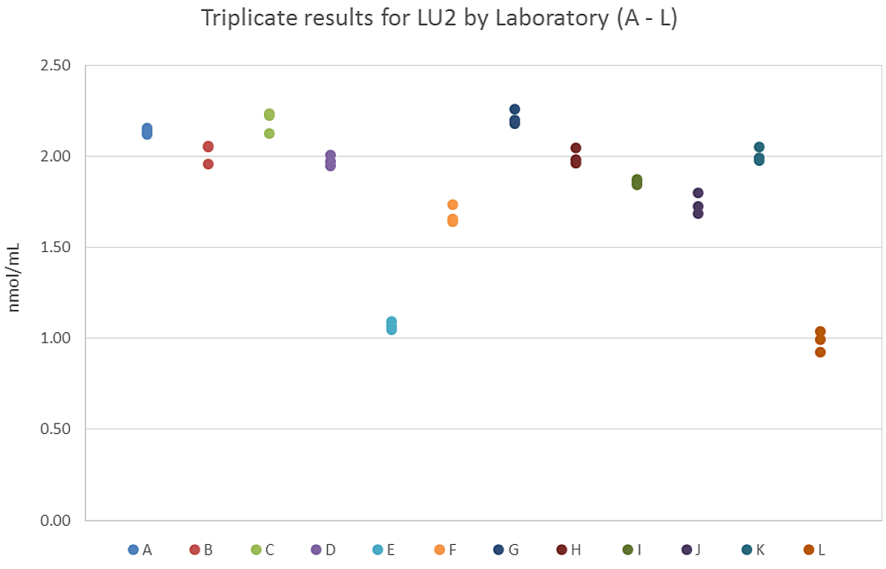

Figure 2. Results of all laboratories (A–L) for triplicate analysis of the LU2 test sample.

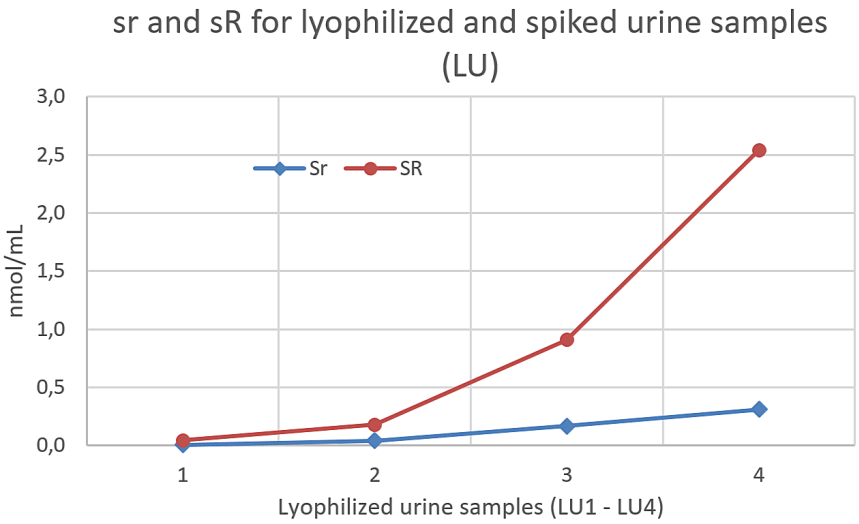

Figure 3. Intra- (s_r) and inter-laboratory variation (s_R) in LU test samples.

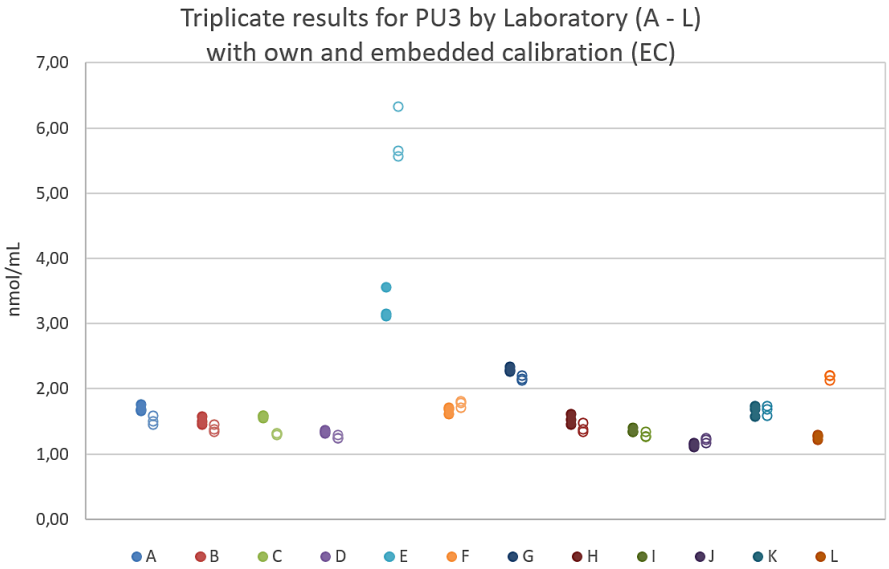

Figure 4. Results of all laboratories (A–L) for triplicate analysis of PU3 test sample. Closed circles: own calibration, open circles: embedded calibration (EC).

are mostly in the range of 1–10%, with one exception: Laboratory L showed a relative standard deviation for PU2 of 14%. Overall, the laboratories' precision (or repeatability) for measuring 3-HPMA in the 5 PU test samples is, in general, good. Laboratory E was an outlier according to the Grubbs' test for all 5 PU test samples and excluded from the calculation of the mean levels for these samples. In contrast to the results for the LU test samples of Laboratory E, results for the PU samples were higher by a factor of 2 compared to the other laboratories. For reasons explained in the next section, Laboratory L was also not considered for calculation of the mean values of all laboratories. For illustration purposes, a graphical presentation of the individual results for test sample PU3 is shown in Figure 4.

The inter-laboratory variation (indicated by the outlier-adjusted means of the standard deviations s_R) almost linearly increased with the 3-HPMA concentrations in the PU test samples and is 6- to 10-fold higher than the intra-laboratory variation (s_r). The ratio s_R/s_r decreased to 5- to 7-fold after removing Laboratory E and L (Figure 5). The coefficient of variation (CV_R) ranged from 17.3–41.3% (mean: 29.7%) for the unadjusted PU results and decreased to 16.1–27.0% (mean: 18.5%) after removal of the outlier laboratories (E, L). The difference between intra- and inter-laboratory variations were still found to be substantial.

Embedded calibration (EC)

With the reported peak areas for the analyte (3-HPMA) and the internal standard (IS) and the outlier-adjusted means (unit: nmol/mL) of the LU test samples (Table 2), embed-

Table 2. 3-HPMA concentrations (nmol/mL) in spiked and lyophilized triplicates (a – c) of test samples (LU1 to LU4) reported by the participating laboratories (A – L).

		A	B	C	D	E	F	G	H	I	J	K	L	Means*				
														3-HPMA	s_R	CV_R (%)	s_r	CV_r (%)
LU1	a	0.16	0.14	0.17	0.11	0.16	0.19	0.16	0.03	0.11	0.11	0.16	< LOQ					
	b	0.16	0.13	0.16	0.12	0.16	0.19	0.16	0.04	0.11	0.11	0.17	< LOQ					
	c	0.16	0.14	0.16	0.12	0.19	0.19	0.16	0.03	0.11	0.10	0.16	< LOQ					
Mean		*0.162*	*0.134*	*0.164*	*0.114*	*0.166*	*0.188*	*0.163*	*0.032*	*0.114*	*0.108*	*0.162*		*0.137*	*0.044*	*31.9*		
s_r		*0.003*	*0.006*	*0.002*	*0.005*	*0.017*	*0.003*	*0.002*	*0.004*	*0.002*	*0.004*	*0.007*					*0.005*	
CV_r (%)		*1.7*	*4.5*	*1.5*	*4.5*	*10.1*	*1.6*	*0.9*	*13.2*	*2.1*	*3.4*	*4.2*						*3.6*
LU2	a	2.12	2.05	2.22	2.01	1.07	1.64	2.18	1.96	1.86	1.80	1.99	0.92					
	b	2.14	1.96	2.12	1.97	1.10	1.73	2.20	2.04	1.87	1.69	2.05	1.00					
	c	2.16	2.06	2.24	1.95	1.05	1.66	2.26	1.98	1.84	1.73	1.98	1.04					
Mean		*2.138*	*2.022*	*2.194*	*1.975*	*1.071**	*1.677*	*2.213*	*1.997*	*1.860*	*1.738*	*2.008*	*0.986**	*1.982**	*0.180**	*9.1**		
s_r		*0.016*	*0.056*	*0.061*	*0.030*	*0.023**	*0.049*	*0.042*	*0.043*	*0.015*	*0.057*	*0.040*	*0.059**				*0.041**	
CV_r (%)		*0.8*	*2.8*	*2.8*	*1.5*	*2.1**	*2.9*	*1.9*	*2.2*	*0.8*	*3.3*	*2.0*	*6.0**					*2.1**
LU3	a	6.03	5.57	6.07	5.40	3.12	4.94	5.38	5.83	5.41	4.75	5.48	3.64					
	b	5.93	5.54	6.15	5.63	3.02	4.70	5.65	5.83	5.34	4.75	5.44	4.52					
	c	6.05	6.24	6.02	5.49	2.88	4.77	5.87	5.80	5.44	5.18	5.62	3.87					
Mean		*6.006*	*5.782*	*6.082*	*5.509*	*3.006*	*4.802*	*5.633*	*5.821*	*5.397*	*4.891*	*5.513*	*4.012*	*5.204*	*0.907*	*17.4*		
s_r		*0.064*	*0.397*	*0.064*	*0.114*	*0.118*	*0.125*	*0.245*	*0.016*	*0.050*	*0.248*	*0.094*	*0.457*				*0.166*	
CV_r (%)		*1.1*	*6.9*	*1.0*	*2.1*	*3.9*	*2.6*	*4.4*	*0.3*	*0.9*	*5.1*	*1.7*	*11.4*					*3.2*
LU4	a	16.71	16.0	18.36	15.84	8.19	14.19	16.40	16.87	15.80	14.27	15.12	12.03					
	b	17.07	16.5	18.28	16.03	8.37	14.38	15.70	16.05	15.87	14.86	14.62	13.75					
	c	16.62	16.1	18.40	16.25	8.55	14.23	16.10	16.13	16.12	13.74	14.76	12.39					
Mean		*16.800*	*16.205*	*18.346*	*16.042*	*8.371*	*14.270*	*16.067*	*16.351*	*15.931*	*14.289*	*14.831*	*12.726*	*15.019*	*2.540*	*16.9*		
s_r		*0.235*	*0.264*	*0.061*	*0.205*	*0.181*	*0.099*	*0.351*	*0.448*	*0.167*	*0.560*	*0.257*	*0.906*				*0.311*	
CV_r (%)		*1.4*	*1.6*	*0.3*	*1.3*	*2.2*	*0.7*	*2.2*	*2.7*	*1.0*	*3.9*	*1.7*	*7.1*					*2.1*

* Laboratories E and L were Grubbs' outliers for sample LU2 and, therefore, not considered when calculating the means

Table 3. 3-HPMA concentrations (nmol/mL) in triplicates (a–c) of authentic urine pool test samples (PU1 to PU5) reported by the participating laboratories (A–L).

		A	B	C	D	E*	F	G	H	I	J	K	L*	Means*				
														3-HPMA	s_R	CV_R(%)	s_r	CV_r (%)
PU1	a	0.25	0.20	0.24	0.18	0.52	0.22	0.266	0.26	0.20	0.17	0.20	<LOQ					
	b	0.25	0.20	0.22	0.18	0.49	0.20	0.273	0.25	0.19	0.19	0.19	<LOQ					
	c	0.26	0.21	0.24	0.17	0.48	0.23	0.281	0.28	0.19	0.17	0.20	<LOQ					
Mean		*0.252*	*0.203*	*0.234*	*0.180*	*0.498*	*0.217*	*0.273*	*0.262*	*0.195*	*0.174*	*0.195*		*0.219*	*0.035*	*16.1*		
s_r		*0.005*	*0.005*	*0.009*	*0.006*	*0.016*	*0.014*	*0.008*	*0.013*	*0.004*	*0.011*	*0.001*					*0.007*	
CV_r(%)		*1.8*	*2.2*	*3.8*	*3.3*	*3.3*	*6.4*	*2.7*	*4.8*	*2.1*	*6.2*	*0.5*						*3.4*
PU2	a	0.77	0.66	0.69	0.59	1.81	0.93	1.17	0.55	0.59	0.49	0.82	0.94					
	b	0.77	0.71	0.71	0.60	1.67	0.88	1.16	0.60	0.62	0.57	0.82	0.83					
	c	0.79	0.62	0.72	0.57	1.78	0.99	1.13	0.53	0.60	0.48	0.83	1.10					
Mean		*0.778*	*0.665*	*0.706*	*0.587*	*1.753*	*0.933*	*1.153*	*0.559*	*0.602*	*0.512*	*0.825*	*0.957*	*0.732*	*0.197*	*27.0*		
s_r		*0.009*	*0.046*	*0.018*	*0.014*	*0.074*	*0.054*	*0.021*	*0.036*	*0.019*	*0.050*	*0.007*	*0.134*				*0.027*	
CV_r(%)		*1.2*	*6.9*	*2.5*	*2.4*	*4.2*	*5.8*	*1.8*	*6.4*	*3.2*	*9.8*	*0.8*	*14.0*					*3.7*
PU3	a	1.69	1.44	1.59	1.33	3.16	1.61	2.29	1.61	1.33	1.16	1.74	1.26					
	b	1.67	1.50	1.59	1.32	3.55	1.71	2.28	1.46	1.33	1.18	1.69	1.28					
	c	1.75	1.57	1.55	1.36	3.11	1.69	2.35	1.52	1.40	1.11	1.57	1.23					
Mean		*1.705*	*1.506*	*1.578*	*1.335*	*3.271*	*1.672*	*2.307*	*1.529*	*1.354*	*1.148*	*1.665*	*1.256*	*1.580*	*0.310*	*19.6*		
s_r		*0.037*	*0.062*	*0.022*	*0.020*	*0.240*	*0.052*	*0.038*	*0.073*	*0.040*	*0.035*	*0.089*	*0.028*				*0.047*	
CV_r(%)		*2.2*	*4.1*	*1.4*	*1.5*	*7.4*	*3.1*	*1.6*	*4.8*	*3.0*	*3.1*	*5.4*	*2.2*					*3.0*
PU4	a	3.29	3.32	3.39	2.99	4.14	2.58	4.15	3.58	2.88	2.70	3.16	2.59					
	b	3.29	4.11	3.27	2.94	4.66	2.42	4.05	3.53	2.93	2.58	3.14	2.76					
	c	3.30	3.66	3.32	2.98	4.39	2.41	4.2	3.50	2.85	2.56	3.08	2.55					
Mean		*3.291*	*3.694*	*3.326*	*2.971*	*4.397*	*2.470*	*4.133*	*3.534*	*2.887*	*2.613*	*3.126*	*2.633*	*3.204*	*0.504*	*15.7*		
s_r		*0.007*	*0.397*	*0.059*	*0.029*	*0.260*	*0.093*	*0.076*	*0.041*	*0.040*	*0.078*	*0.045*	*0.115*				*0.087*	
CV_r(%)		*0.2*	*10.7*	*1.8*	*1.0*	*5.9*	*3.8*	*1.8*	*1.2*	*1.4*	*3.0*	*1.4*	*4.4*					*2.7*
PU5	a	10.41	10.5	11.67	10.08	13.44	8.24	12.2	10.74	8.76	8.18	9.92	8.01					
	b	10.58	10.7	12.21	10.08	13.62	8.43	12.2	11.61	8.75	7.76	10.32	7.83					
	c	10.59	10.5	11.55	9.99	13.48	8.66	12.3	10.11	8.26	8.13	9.92	7.78					
Mean		*10.528*	*10.593*	*11.812*	*10.049*	*13.514*	*8.444*	*12.233*	*10.819*	*8.591*	*8.026*	*10.051*	*7.871*	*10.115*	*1.404*	*13.9*		
s_r		*0.099*	*0.122*	*0.353*	*0.052*	*0.094*	*0.209*	*0.058*	*0.749*	*0.289*	*0.229*	*0.235*	*0.120*				*0.240*	
CV_r(%)		*0.9*	*1.2*	*3.0*	*0.5*	*0.7*	*2.5*	*0.5*	*6.9*	*3.4*	*2.9*	*2.3*	*1.5*					*2.4*

* Laboratory E was an outlier according to the Grubbs' test for all PU samples. For reasons discussed in the text, Laboratory L was also not considered when calculating the means

ded calibration functions were calculated for each participating laboratory. Linear regression with line enforcement through the origin was applied. Slopes and R^2 values for the regressions of all laboratories are shown in Table 4. The R^2 values ranged from 0.9791 to 0.9996, indicating that acceptable to excellent calibration functions were obtained for all laboratories.

Table 4. Slopes and R^2 values for the embedded calibration (EC) functions of all participating laboratories.

Laboratory	Slope (mL/nmol)	R^2
A	1.344	0.9995
B	3.955	0.9987
C	0.762	0.9994
D	0.107	0.9996
E	0.182	0.9986
F	0.368	0.9993
G [a]	0.294	0.9791
H	0.136	0.9987
I	0.336	0.9994
J	0.786	0.9976
K	0.546	0.9987
L [b]	0.158	0.9928

[a] Results for test sample LU2 (a–c) were eliminated (outlier)
[b] Result of LU1 c eliminated (outlier)

Table 5 shows the individual results for all PU test samples as calculated with the EC functions for the laboratories A–L. Intra-laboratory variation, as indicated by the absolute (s_r) and relative standard deviations (CV_r) are only slightly different from those obtained with the own calibration (Table 5, Figure 5). Except for two laboratories (E, L), the EC-calculated concentrations for 3-HPMA are only slightly different from the reported values and shifted (either decreased or increased) to the direction of the overall mean levels. This appears to be different for Laboratories E and L, which were shifted by a larger extent compared to the reported level and, beyond that, in the 'wrong direction'. This can be also seen in Figure 4, which shows the triplicate results obtained with the own and embedded calibration for the test sample PU 3. Obviously, analytical issues other than calibration problems are responsible for the observed deviation with Laboratories E and L. Therefore, these two laboratories were not considered in the EC-based calculations of the overall means in Table 5. In order to allow comparison of the inter-laboratory variations with the reported results (own calibration, Table 3) with those obtained after re-calculation with EC functions (Table 5), Laboratories E and L were not considered in the calculations of the means. The same applies to Figure 5, which shows the intra- and inter-laboratory standard deviations when evaluating the reported results (laboratories' own calibration) and the EC-based results for the PU test samples. A significant decrease in s_R is observed for the two test samples with the highest 3-HPMA concentrations (PU4 and PU5). Improvements (i.e., decreases of s_R) for the lower level PU test samples (PU1, PU2, PU3) are small or non-existent. Relative inter-laboratory variability as expressed by CV_R for the EC-calculated results range from 9.6 to 28.8% (mean: 16.7%) compared to 13.9–27.0% (mean: 18.5%) for the result obtained with the laboratories' own calibration (labs E and L excluded in both cases). These results show that re-calculation with EC leads to some improvement (decrease) in inter-laboratory variability. Nevertheless, the effect is minor, and EC may not be crucial for the comparability of 3-HPMA analysis between well experienced laboratories. For two laboratories (E and L), an impairment (i.e., increase in deviation from the overall mean) was observed, suggesting that other analytical issues could have caused the increase in inter-laboratory variation.

CONCLUSIONS

The inter-laboratory comparison for the determination of urinary 3-HPMA concentrations presented here is an extension of a ring-trial on the same analyte reported earlier

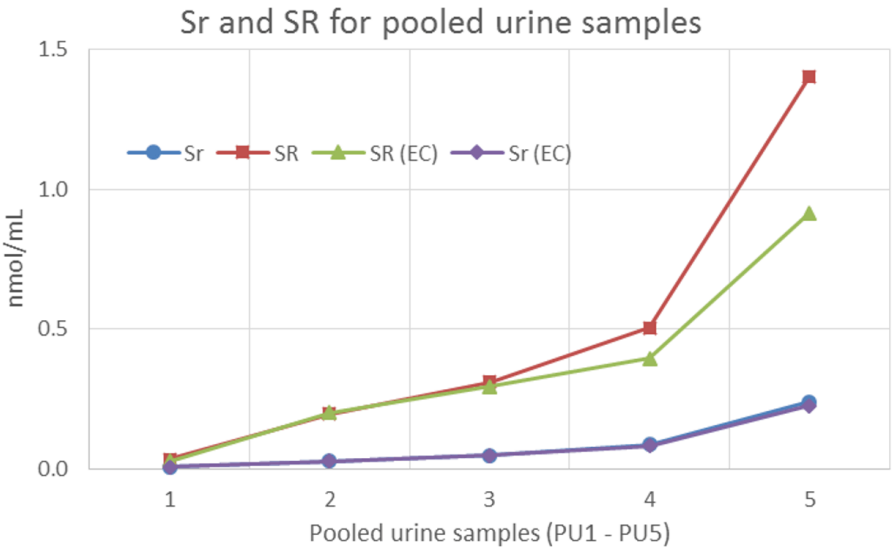

Figure 5. Intra- (s_r) and inter-laboratory variations (s_R) in PU test samples. Some improvement in s_R is observed for PU4 and PU5 when applying EC for re-calculation. Laboratories E and L have not been considered for the calculation of s_r and s_R.

Table 5. 3-HPMA concentrations (nmol/mL) in triplicates (a–c) of authentic urine pool test samples (PU1 to PU5) calculated with the embedded calibration (EC) functions of all participating laboratories (A–L).

		A	B	C	D	E*	F	G	H	I	J	K	L*	Means*				
														3-HPMA	s_R	CV_R (%)	s_r	CV_r (%)
PU1	a	0.22	0.19	0.20	0.18	0.94	0.24	0.25	0.23	0.20	0.18	0.20	0.36					
	b	0.23	0.18	0.18	0.18	0.90	0.21	0.26	0.23	0.19	0.20	0.19	0.32					
	c	0.24	0.19	0.19	0.17	0.89	0.24	0.26	0.25	0.19	0.18	0.20	0.31					
Mean		*0.229*	*0.188*	*0.193*	*0.180*	*0.908*	*0.229*	*0.254*	*0.234*	*0.191*	*0.183*	*0.196*	*0.328*	*0.208*	*0.026*	*12.6*		
s_r		*0.006*	*0.004*	*0.007*	*0.006*	*0.028*	*0.015*	*0.007*	*0.013*	*0.004*	*0.011*	*0.001*	*0.025*				*0.007*	
CV_r (%)		*2.8*	*2.2*	*3.8*	*3.1*	*3.1*	*6.4*	*2.8*	*5.5*	*2.0*	*6.0*	*0.4*	*7.7*					*3.5*
PU2	a	0.72	0.61	0.56	0.57	3.25	0.99	1.10	0.50	0.56	0.52	0.82	1.82					
	b	0.72	0.66	0.59	0.57	3.00	0.93	1.08	0.54	0.60	0.60	0.82	1.71					
	c	0.68	0.57	0.59	0.55	3.19	1.04	1.06	0.48	0.57	0.50	0.83	2.00					
Mean		*0.704*	*0.615*	*0.581*	*0.562*	*3.148*	*0.986*	*1.081*	*0.506*	*0.577*	*0.538*	*0.826*	*1.844*	*0.698*	*0.201*	*28.8*		
s_r		*0.023*	*0.043*	*0.015*	*0.012*	*0.131*	*0.057*	*0.019*	*0.032*	*0.018*	*0.052*	*0.005*	*0.145*				*0.028*	
CV_r (%)		*3.3*	*6.9*	*2.5*	*2.1*	*4.2*	*5.8*	*1.7*	*6.4*	*3.2*	*9.7*	*0.7*	*7.9*					*4.0*
PU3	a	1.45	1.34	1.31	1.25	5.66	1.71	2.14	1.47	1.26	1.22	1.74	2.20					
	b	1.51	1.39	1.31	1.25	6.34	1.81	2.14	1.33	1.27	1.24	1.70	2.21					
	c	1.59	1.45	1.28	1.28	5.57	1.79	2.20	1.38	1.33	1.17	1.59	2.14					
Mean		*1.516*	*1.393*	*1.300*	*1.263*	*5.854*	*1.769*	*2.159*	*1.394*	*1.289*	*1.210*	*1.675*	*2.182*	*1.497*	*0.295*	*19.7*		
s_r		*0.071*	*0.057*	*0.018*	*0.020*	*0.423*	*0.055*	*0.033*	*0.069*	*0.038*	*0.037*	*0.081*	*0.036*				*0.048*	
CV_r (%)		*4.7*	*4.1*	*1.4*	*1.6*	*7.2*	*3.1*	*1.5*	*5.0*	*3.0*	*3.1*	*4.9*	*1.7*					*3.2*
PU4	a	2.95	3.07	2.79	2.81	7.40	2.73	3.88	3.28	2.73	2.85	3.18	3.67					
	b	2.97	3.80	2.69	2.77	8.36	2.56	3.80	3.23	2.78	2.71	3.16	3.86					
	c	2.94	3.38	2.73	2.81	7.82	2.55	3.94	3.21	2.71	2.70	3.08	3.63					
Mean		*2.951*	*3.416*	*2.739*	*2.795*	*7.860*	*2.612*	*3.875*	*3.237*	*2.739*	*2.754*	*3.139*	*3.720*	*3.026*	*0.394*	*13.0*		
s_r		*0.014*	*0.367*	*0.048*	*0.026*	*0.484*	*0.099*	*0.071*	*0.036*	*0.038*	*0.085*	*0.051*	*0.124*				*0.083*	
CV_r (%)		*0.5*	*10.8*	*1.8*	*0.9*	*6.2*	*3.8*	*1.8*	*1.1*	*1.4*	*3.1*	*1.6*	*3.3*					*2.8*
PU5	a	9.52	9.74	9.61	9.46	23.95	8.71	11.35	9.86	8.30	8.60	9.95	9.70					
	b	9.68	9.93	10.06	9.46	24.29	8.92	11.33	10.61	8.29	8.17	10.34	9.57					
	c	9.61	9.72	9.52	9.36	24.06	9.16	11.51	9.29	7.82	8.56	9.94	9.53					
Mean		*9.604*	*9.795*	*9.728*	*9.428*	*24.097*	*8.930*	*11.398*	*9.917*	*8.136*	*8.445*	*10.078*	*9.597*	*9.546*	*0.915*	*9.6*		
s_r		*0.082*	*0.113*	*0.290*	*0.057*	*0.176*	*0.222*	*0.097*	*0.660*	*0.273*	*0.238*	*0.223*	*0.089*				*0.225*	
CV_r (%)		*0.9*	*1.2*	*3.0*	*0.6*	*0.7*	*2.5*	*0.8*	*6.7*	*3.4*	*2.8*	*2.2*	*0.9*					*2.4*

* Laboratories E and L were not considered when calculating the means (see text).

(6). In contrast to the previous comparison, in which 4 experienced laboratories participated, the present trial was performed with 12 globally distributed laboratories, which had different degrees of experience with the analysis of 3-HPMA in urine. Both ring-trials included the same LU samples. The mean inter-laboratory variability was reported to be 7.0% in the earlier study (6), whereas it amounted to 22.1 or 18.8% in this comparison, when all or only the outlier-adjusted results, respectively, were included for the LU test samples. For the 5 authentic PU test samples (which were different in the two ring-trials), a mean inter-laboratory variability of 16.2% was reported (6), whereas the mean amounted to 29.7% for all 12 laboratories in the present ring-trial. These results reflect the different levels of laboratory experience in the 2 studies very well. After removal of the outlying Laboratories E and L, the mean inter-laboratory variability decreased to 18.5% and was further reduced to 16.7%, when re-calculating the results for the PU test samples by using the embedded calibration (EC) functions. Results from the outlier laboratories, however, showed similar relative differences between the various PU test samples as the other laboratories, suggesting that also Laboratories E and L would generate valid results in terms of the relative exposure to acrolein. An EC approach based on the LU test samples was also applied in the earlier study (6). However, only minor differences between own calibration- and EC-based results were found, showing that EC does not significantly reduce the inter-laboratory variability in more experienced laboratories.

The results of this inter-laboratory comparison show that further methodological improvements at least for some laboratories are necessary and reemphasize the requirement of continuous external quality control, e.g., within the frame of periodically organized proficiency tests or inter-laboratory comparisons. Finally, spiked, lyophilized urine samples (LU) proved to be a suitable standard reference material for 3-HPMA.

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