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Development of a second–order standard addition fluorescence method for the direct determination of riboflavin in human urine samples without previous clean up and separation steps

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Abstract

A new strategy for the determination of riboflavin (RF, or vitamin B_2) in human urine samples has been developed, combining excitation–emission fluorescence matrix (EEFM) data in standard addition mode and second–order chemometric analysis. The method is simple, fast and eco–friendly because it complies with the green analytical chemistry principles, avoiding the need of previous clean up and separation steps that consume high amounts of organic solvents. Successful results were obtained by different chemometric algorithms, namely parallel factor analysis (PARAFAC), unfolded partial least–squares/residual bilinearization (U–PLS/RBL) and multidimensional partial least–squares/residual bilinearization (N–PLS/RBL), all in the modified standard addition. These algorithms allowed us to achieve selectivity in a system, which requires standard addition and shows a significant background spectral overlapping with the studied vitamin. The quality of the proposed strategy was evidenced on the basis of the analytical recoveries from urine samples spiked with RF. The detection limits achieved in urine samples are encouraging compared to those obtained using chromatographic approaches. The relative prediction errors were lower than 5.6 %.

Keywords: Second–order standard addition; Green analytical chemistry; Fluorescence; Chemometrics; Riboflavin; Human urine samples.

1. Introduction

Riboflavin (RF) or vitamin B_2 is a water–soluble vitamin long established as a vital nutrient. It is an essential component of the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) which are involved in the metabolism of many substances, e.g., glucose, fatty acids, amino acids, drugs, vitamins K and D [1,2]. It is found in a wide variety of foods: milk and cheese, meat and fish, eggs, wine and tea, these products provide about one–third of dietary requirements. Its deficiency may be due to insufficient intake, hormonal problems, drugs, alcohol or chelators reducing its bioavailability. Symptoms of RF deficiency include fatigue; digestive problems; cracks and sores around the corners of the mouth; swollen magenta tongue; soreness of the lips, mouth and tongue; red eyes and conjunctivitis [3,4]. Low intakes of RF can lead to poor growth and various functional abnormalities, including impaired iron handling and elevated plasma homocysteine concentration [5]. There is evidence that RF deficiency may increase both the risk of developing certain types of cancers [6] and the damage to proteins and DNA liver cells [7].

The amount in excess of RF in the body is rapidly excreted through urine because the vitamin is water–soluble and there is no capacity for storage. Free RF is the only flavin excreted by the body in significant amounts reflecting the short–term supply. When RF intake is low, excretion is proportional to the intake [3]. The most widely used analytical methods for the quantification of RF in human urine samples are liquid chromatography (LC)–fluorescence detection (FD) [8], LC–tandem mass spectrometry (MS/MS) [9,10], fluorescence with flow–injection analysis [11], capillary electrophoresis (CE) [12,13], voltammetry [14] and chemiluminescence [15]. These methods require rigorous extraction steps, the use of significant amounts of organic solvents and long analysis time.

Nowadays greener methodologies are very welcome [16], since they fulfill the principles of green analytical chemistry (GAC) [17] i.e. the absence of clean up and separation steps, and the minimization of the use of organic solvents and generation of toxic waste.

It is well known that the complexity of the biological matrices such as urine makes the determination of the target analyte difficult. The effect of a background on the analyte response leads to a change in the slope of the univariate signal–concentration relationship caused by inner filter effects or analyte–background interactions such as complex formation or protein binding. Nevertheless, this effect can be overcome by using the standard addition method [18]. Apart from that, the matrix constituents are potentially able to produce interference signal which does also affect the analyte response in a sample. In this case, the system requires both standard addition and second–order multivariate calibration achieving the second–order advantage for successful analyte quantification [19]. Second–order advantage refers the capacity of certain second–order algorithms to predict concentrations of sample components in the presence of any number of unsuspected constituents [20]. The algorithms that achieved the second–order advantage from standard addition data are parallel factor analysis (PARAFAC) [21], partial least–squares/residual bilinearization (U–PLS/RBL) [22,23] and multidimensional partial least–squares/residual bilinearization (N–PLS/RBL) [24,25].

In the present work, we have developed a GAC method to quantify RF in human urine samples. Second–order data were obtained by measuring excitation–emission fluorescence matrices (EEFMs) and processed by PARAFAC, U–PLS/RBL and N–PLS/RBL. The prediction capabilities of the employed algorithms were discussed.

It is relevant to highlight that it was the first time that the selectivity offered by the chemometric analysis was evaluated for the determination of RF using EEFMs and second–order standard addition calibration in a complex matrix. The feasibility of determining the

target vitamin in human urine samples using sustainable resources was demonstrated. The new method represents other example of the power of coupling non–sophisticated analytical equipment with second–order data for the resolution of interfering samples.

2. Experimental

2.1. Reagents and solutions

RF and ofloxacin (OFL) were purchased from Sigma (St. Louis, MO, USA). The stock solution of RF (104 mg L^{-1}) was prepared in ultrapure Milli−Q water and OFL (204 mg L^{-1}), tested as a potential interferent, was prepared in 5×10^{-2} mol L^{-1} acetic acid. From these solutions, more diluted aqueous working solutions were daily obtained. Ultrapure Milli–Q water was used throughout the work.

2.2. Instrumentation

Fluorescence measurements were performed on an Aminco Bowman (Rochester, NY, USA) Series 2 luminescence spectrophotometer, equipped with a 150 W xenon lamp. EEFMs were measured from 320 to 460 nm (each 4 nm, excitation) and from 470 to 600 nm (each 1 nm, emission). In this way, the matrices were of size 36×131. Both the excitation and emission slit widths were of 8 nm using 1.00 cm quartz cells. The photomultiplier tube (PMT) sensitivity was fixed at 425 V and the temperature of the cell compartment was regulated at 21.0 ± 0.1 °C using a thermostatic bath (Cole–Parmer, IL, USA). The EEFMs were saved in ASCII format, and transferred to a PC for subsequent chemometric analysis.

2.3. Validation and test samples

RF is a light sensitive vitamin, so a pool of urine samples was placed in a clear glass bottle and exposed to white light of a lamp for 120 hours [8,10]. This sample was analyzed to confirm that the concentration of RF was not detected by the proposed method. After this process, the pool sample was considered "blank urine" and used to prepare validation and test samples.

A validation set of 10 samples, by duplicate, was prepared. An aliquot of 40.00 µL diluted blank urine (1:125) and concentrations of RF at random numbers in the range 0.02–0.10 mg L^{-1} were placed in a 5.00 mL volumetric flask which was completed to the mark with ultrapure water. Afterwards, new solutions were prepared starting from the blank urine samples in order to carry out three successive additions of RF. Concentrations were increased by 0.02, 0.05 and 0.10 mg L^{-1} for RF on different aliquots of the original samples. We estimated the uncertainties in all these analyte concentrations to be of the order of ± 0.01 mg L^{-1}. Finally, the four solutions were cooled to 21.0 ºC and their EEFMs were recorded in the conditions described in Section 2.2.

OFL is an antibiotic widely used and its excess is excreted by urine. As it will be demonstrated below, OFL has fluorescence signal that significantly overlaps with that of the studied compound. With the purpose of evaluating the method in the presence of this interferent drug, 8 test samples, by duplicate, were prepared containing 40.00 µL diluted blank urine (1:125), random RF concentrations in the range 0.02–0.10 mg L^{-1} and OFL in the concentration within the therapeutic range in human urine, in the order of 0.05–1.02 mg L^{-1} [26]. In a similar way than in validation samples, new solutions were prepared with three successive additions of RF to carry out the standard addition method.

2.4. Urine samples

The urine samples were collected from fasting healthy adult volunteers (female and male from 20 to 60 years). Fresh urine samples were immediately stored in a dark glass bottle at 4 ºC after a preliminary centrifugation step at 10000 rpm for 15 minutes. The urine samples were diluted 1:25, an aliquot of 200.0 µL was placed in a 5.00 mL volumetric flask and completed to the mark with ultrapure water. The subsequent procedure was the same as described above for validation and test samples. A recovery study was carried out by spiking each urine sample with RF, by duplicate, at a final concentration level in the range 0.5–2.5 mg L^{-1}.

2.5. Chemometric algorithms and software

The theory of the second–order multivariate calibration algorithms applied in the present work is well established and can be found in the relevant references: PARAFAC [21], U–PLS [27], N–PLS [28] and PLS/RBL [22, 23,29].

All routines of employed chemometrics algorithms were written in MATLAB 7.10 [30], and implemented using the graphical interface MVC2 [31], available on the Internet [32].

3. Results and discussion

3.1. Preliminary studies

In a first stage, the fluorescence properties of the studied analyte were evaluated. As previously reported [11,12], RF in aqueous medium presents two excitation maxima at

approximately 368 and 440 nm, and a fluorescence emission maximum at 524 nm. The pH changes in the range 4–8 did not produce any significant modifications in the fluorescence signal [33]. Because the pH of the analyzed urine samples were between 5 and 7, it was not adjusted. The influence of the temperature of the cell was studied in the range 18.0–28.0 °C, recording the fluorescence intensity at 368 and 524 nm as excitation and emission wavelengths, respectively. It was found out that the optimum value obtained was at 21.0 ºC.

3.2. Quantitative analysis

The need of standard addition method was corroborated through the different slopes of the univariate calibration curves for RF in water solution and in the presence of the urine matrix. The results for RF in water with five different concentrations in the range 0.0–0.25 mg L^{-1} were slope = 43.0(4), intercept = 0.16(8), r^2 = 0.998 (standard deviation in the last significant figures in parenthesis), while in a typical urine background, slope = 37.9(3), intercept = 4.74(4) and r^2 = 0.998. The results suggest a significant change in slope (a decrease of 5.1 units in slope, ca. 15 times larger than the average standard deviation (0.35)). These results can be attributed, in principle, to analyte–background interactions requiring standard addition for successful analyte quantitation.

Fig. 1 shows the normalized fluorescence spectra for RF, diluted urine (1:25) and the potential interferent OFL under the employed working conditions. As it can be observed, the overlapping is significant and occurs in both the excitation and emission spectra between the investigated compound and a typical urine sample. This overlapping hinders the direct spectrofluorimetric determination of the analyte through a zeroth–order calibration. The situation regarding selectivity becomes more serious if other fluorescent compounds, as OFL, are also present. With the objective of overcoming this problem and avoiding separation

steps, second–order calibration applying algorithms is necessary. Therefore, in this particular system, standard addition method should be complemented with the measurement of second–order EEFM data, so as to be able to achieve the second–order advantage [19]. As already indicated, this second–order advantage implies analyte quantitation in the presence of unsuspected constituents in samples [20]. The algorithm of choice to obtain the second–order advantage from standard addition data in the classical mode (mode 1) is PARAFAC [21]. Initially, U–PLS/RBL [22,23] and N–PLS/RBL [24] could not be employed with standard addition data because the model requires the nominal analyte concentration of the calibration samples [34]. However, PLS/RBL algorithms can be applied when the urine sample matrix data is subtracted digitally from the three standard addition matrices (modified standard addition or mode 2), so three new virtual samples are created. These virtual samples contain the analyte at three known concentrations, i.e. the three added concentrations, and now the quantification is processed by a classical external calibration procedure [25].

3.2.1. Validation samples

As can be seen in Fig. 2, the urine matrix changed after it was irradiated with white light of a lamp with the purpose of eliminating the endogenous RF to use it later as blank urine. This behavior might be due to the degradation and/or formation of new fluorescent compounds, but blank urine remained an interfering matrix in the analysis of RF.

EEFMs under optimal working conditions were recorded for validation samples (Fig. 3A) where only the studied analyte and the blank urine were present. A set of EEFMs could be arranged as a three–way array, which in general complies with the trilinearity conditions [35] and, therefore, the algorithm of choice for data processing should be PARAFAC [34]. When PARAFAC analysis of the different experimental data sets was carried out, the first step was

the estimation of the number of responsive components. This can in principle be assessed using either the diagnostic tool known as the core consistency test [36] or the consideration of the residual fit of the PARAFAC model [37], as the number of components is increased. When PARAFAC standard addition mode 1 was employed for validation samples, the core consistency values were 100, 99.9, 2.3 and 0 for 1–4 components, respectively, while the residuals fit decreased as follows: 0.65, 0.051, 0.034 and 0.03 arbitrary fluorescence units. The progression of core consistency and the residual fit values suggest two components, which are reasonable (RF as analyte and one interference as urine). The prediction results showed that the root mean square error of prediction (RMSEP) was 0.656 units, corresponding to a relative error of prediction (REP) of 8.0%. However, inspection of Fig. 4 (algorithm 1) reveals a bias in the results for the complete set of validation samples when PARAFAC was applied in standard addition mode 1.

In the modified standard addition mode 2 the core consistency values were 100, 100, 37 and 39.2 for 1–4 components, respectively, while the residuals of the PARAFAC fit decreased as follows: 0.28, 0.041, 0.035 and 0.032 arbitrary fluorescence units. Once again, the tests suggest two components. The satisfactory prediction results for the 10 validation samples are shown in Fig. 4 (algorithm 2), the analyte prediction results in this second mode were considerably better than those for the first mode of analysis. As can be seen in Table 1, the RMSEP decreased to 0.332 concentration units and the REP to 4.8%. The reason of those poorest results is unclear, but may be related to the strong correlations when mode 1 is used [25].

U–PLS/RBL and N–PLS/RBL algorithms were applied using the only possible standard addition strategy: mode 2. The optimum number of latent variables was estimated according to the cross–validation method [38]. Calibration was performed using a single latent variable and one component corresponding to the unexpected constituent presents in the urine sample

was included in the RBL phase. The prediction results for the validation samples applying both algorithms in mode 2 show RMSEP and REP, which are similar to PARAFAC in mode 2 (Table 1). As clearly displayed in Fig. 4 (algorithms 3 and 4), the results are comparable to the best PARAFAC results. According to these results, PARAFAC, U–PLS/RBL and N–PLS/RBL, all in the modified standard addition or mode 2, were the algorithms selected for the analysis of test and urine samples.

3.2.2. Test samples

Many compounds, as pharmaceuticals, are potentially able to produce interference. The potential interferent OFL displays signal which strongly overlaps that for the studied analyte (Fig. 1). Therefore, with the purpose of simulating a genuine situation, test samples containing RF, blank urine and the above compound, which could be concomitantly present in real samples, were analyzed. Fig. 3B shows the three–dimensional plots and the corresponding contour plots of the EEFM of a typical test sample with interference.

When PARAFAC in mode 2 was applied to the test samples, both the progression of core consistency and the residual fit values suggested two components. This could be because the algorithm PARAFAC considers the profiles of the two interferences (OFL and urine) as one mathematical components, and it is able to distinguish these combined signals from the analyte signal.

Fig. 5A illustrates the satisfactory predictions corresponding to the application of PARAFAC in mode 2 to the test samples. With the purpose of assessing the accuracy of the predicted concentrations, the elliptical joint confidence region (EJCR) test was performed [39]. From the EJCR test (Fig. 5D), we conclude that the ellipse includes the theoretically expected point (1,0), suggesting that PARAFAC in mode 2 is appropriate for resolving the

system under investigation. The analytical performance of this algorithm applied to test samples is appreciated from the statistical results shown in Table 1.

When U– and N–PLS/RBL in mode 2 algorithms were applied to test samples, the optimum number of factors for the calibration set was one, and they required the introduction of the RBL procedure with one additional number of components corresponding to the unexpected sample constituents (OFL and urine). Adding more unexpected components did not improve the RBL fit, showing a similar behavior to that of PARAFAC in mode 2. Fig. 5B and 5C show the prediction results corresponding to the application of U–PLS/RBL and N–PLS/RBL in mode 2 to the test samples containing OFL as interferent, respectively. The EJCR test (Fig. 5D) corroborated that both ellipses had a similar size and included the theoretically expected values of (1,0), demonstrating the accuracy of the used methodologies. The statistical results are comparable to those for PARAFAC in mode 2 (Table 1). The REPs indicate acceptable precision taking into account that a very simple and rapid methodology is applied to a complex matrix. Considering that EEFMs measurements are performed in about 15 minutes, a throughput of about four urine samples per hour is achieved.

3.2.3. Urine samples

A set of twenty–four urine samples obtained in early morning from different healthy adult volunteers was analyzed. The concentration of urinary RF vary greatly depending on dietary intake, nutritional supplement use, health and physical condition. The ranges of RF urinary previous reported varied from 0.10 to 7.80 mg L^{-1} [8,9]. As it can be observed in Table 2 the prediction results obtained for the three algorithms in the studied urine samples are in agreement with the values previously mentioned.

A recovery study was carried out by spiking the urine samples with the analyte, by duplicate, at one concentration level. The signal of the analyte highly overlapped with the fluorescent matrix constituents (Fig. 3C). However, the physical removal of these interferences is not necessary when using an appropriate second–order calibration methodology, highlighting the value of the chemometric approach.

When algorithms are applied to real samples, PARAFAC in mode 2 requires two components while U– and N–PLS/RBL in mode 2 require one latent variable and one additional component included in the RBL procedure. Table 2 and Fig. 6 display the satisfactory prediction results obtained for these spiked urine samples, suggesting that the proposed methodology can overcome the problem of interactions with the background and the presence of the unexpected compounds. The good analytical performance for the selected algorithms in mode 2 of standard addition can be appreciated from the statistical results shown in Table 1. These results indicate that both the limit of detection (LOD) and the limit of quantification (LOQ) obtained are appropriate for determining low concentrations of RF in urine samples from individuals with deficiency due to insufficient intake of the vitamin. It is important to indicate that the statistical results have been calculated according to the recommendation of the International Union of Pure and Applied Chemistry (IUPAC) [40]. Besides, the LODs are achieved in real samples and reflect the benefits of the proposed methodology in comparison with other reported methods, most of them applying chromatographic approaches, whose LODs varied from 0.010 to 0.090 mg L^{-1} [8–12].

4. Conclusions

A second–order standard addition method was developed to quantify riboflavin (RF, vitamin B_2) in human urine samples using excitation–emission fluorescence matrices

(EEFMs). The satisfactory results indicate that the overlapping between RF and urine background in both spectral dimensions is overcome by applying algorithms which have achieved the second–order advantage, namely parallel factor analysis (PARAFAC), unfolded partial least–squares/residual bilinearization (U–PLS/RBL) and multidimensional partial least–squares/residual bilinearization (N–PLS/RBL), all in the modified standard addition.

The proposed strategy is significantly simple and green because the determination of RF in complex matrices is carried out in aqueous solutions without previous clean up or separation steps and using a non–sophisticated equipment. In addition, the method is fast, allowing a sample throughput of about four urine samples per hour.

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Figure Captions

Fig. 1. Normalized excitation and emission fluorescence spectra of human urine diluted (1:25) (blue line), RF (green line) and OFL (red line). C_{RF} = 0.05 mg L^{-1}, C_{OFL} = 0.25 mg L^{-1}.

Fig. 2. Three–dimensional plots and the corresponding contour plots of excitation–emission fluorescence matrices for (A) a diluted (1:25) human urine and (B) diluted (1:125) blank urine.

Fig. 3. Three–dimensional plots and the corresponding contour plots of excitation–emission fluorescence matrices for (A) a validation sample containing 0.10 mg L^{-1} RF, (B) a test sample containing 0.08 mg L^{-1} RF and 0.98 mg L^{-1} OFL, and (C) a spiked human urine diluted (1:25) with 0.10 mg L^{-1} RF.

Fig. 4. Box and whisker plot of prediction results corresponding to the validation samples for RF. Algorithms are numbered in the horizontal axis as follows: (1) PARAFAC standard addition in mode 1, (2) PARAFAC standard addition in mode 2, (3) U–PLS/RBL standard addition in mode 2, and (4) N–PLS/RBL standard addition in mode 2. For each algorithm, the gray boxes are bounded by the 25% and 75% quartiles with the median inside, whereas the extreme levels correspond to 5% and 95% quartiles.

Fig. 5. Plots for RF predicted concentrations in test samples with interference as a function of the nominal values using (A) PARAFAC in mode 2, (B) U–PLS/RBL in mode 2, (C) N–PLS/RBL in mode 2 and (D) Elliptical joint regions (at 95% confidence level) for slope and intercept of the regression of PARAFAC in mode 2 (blue line), U–PLS/RBL in mode 2 (red line), and N–PLS/RBL in mode 2 (green line). The cross marks the theoretical (intercept = 0, slope = 1) point.

Fig. 6. Plots for RF predicted concentrations in spiked human urine samples as a function of the nominal values using PARAFAC in mode 2 (blue triangle up), U–PLS/RBL in mode 2 (red square) and N–PLS/RBL in mode 2 (green circle). The inset shows the corresponding elliptical joint regions (at 95% confidence level) for slope and intercept of the regression of PARAFAC in mode 2 (blue line), U–PLS/RBL in mode 2 (red line), and N–PLS/RBL in mode 2 (green line). The cross marks the theoretical (intercept = 0, slope = 1) point.

Table 1

Statistical results for RF in validation, test and human urine samples using PARAFAC, U–PLS/RBL and N–PLS/RBL standard addition in mode 2.

	PARAFAC mode 2	U–PLS/RBL mode 2	N–PLS/RBL mode 2
Validation samples[a]			
LOD (mg L^{-1})	0.008	0.004	0.004
LOQ (mg L^{-1})	0.024	0.012	0.011
RMSEP (mg L^{-1})	0.332	0.332	0.316
REP (%)	4.8	4.8	3.2
Test samples[b]			
LOD (mg L^{-1})	0.008	0.006	0.004
LOQ (mg L^{-1})	0.025	0.019	0.013
RMSEP (mg L^{-1})	0.906	0.812	0.906
REP (%)	11.2	9.6	11.2
Urine samples[c]			
LOD (mg L^{-1})	0.010	0.006	0.007
LOQ (mg L^{-1})	0.029	0.016	0.020
RMSEP (mg L^{-1})	0.071	0.071	0.063
REP (%)	5.6	5.6	5.3

[a] Ten validation samples.

[b] Eight test samples containing OFL as interferent.

[c] Twenty–four urine samples.

LOD, limit of detection; LOQ, limit of quantification; RMSEP, root–mean–square error of prediction and REP, relative error of prediction.

Table 2

Predicted concentrations for RF in human urine samples using PARAFAC, U–PLS/RBL and N–PLS/RBL standard addition in mode 2.

Urine		PARAFAC mode 2		U–PLS/RBL mode 2		N–PLS/RBL mode 2	
	Added ($mg\ L^{-1}$)	Found[a] ($mg\ L^{-1}$)	Rec (%)	Found[a] ($mg\ L^{-1}$)	Rec (%)	Found[a] ($mg\ L^{-1}$)	Rec (%)
1		0.90(6)		0.91(6)		0.92(8)	
	2.0	2.9(2)	100	2.9(2)	100	2.9(2)	99
2		0.96(1)		0.96(1)		0.98(1)	
	1.2	2.129(8)	97	2.129(8)	97	2.132(8)	96
3		0.26(1)		0.26(1)		0.28(1)	
	0.8	1.06(4)	100	1.06(4)	100	1.07(4)	99
4		1.69(1)		1.69(1)		1.70(1)	
	2.5	4.1(2)	96	4.1(1)	96	4.2(1)	100
5		1.29(4)		1.30(4)		1.35(2)	
	1.5	2.86(4)	105	2.86(4)	104	2.89(4)	103
6		1.79(4)		1.80(4)		1.81(3)	
	2.0	3.83(1)	102	3.83(1)	102	3.83(1)	101
7		1.482(7)		1.486(7)		1.490(7)	
	1.2	2.71(3)	102	2.71(3)	102	2.71(3)	102
8		0.190(2)		0.21(3)		0.21(2)	
	0.8	0.963(2)	97	0.98(2)	96	0.982(8)	97
9		0.25(2)		0.25(2)		0.27(2)	
	1.0	1.220(7)	97	1.222(7)	97	1.23(1)	96
10		0.298(6)		0.36(8)		0.37(7)	
	1.6	1.878(8)	99	1.90(4)	96	1.91(3)	96
11		0.37(3)		0.37(3)		0.40(2)	
	1.6	1.93(3)	98	1.93(3)	98	1.95(3)	97
12		0.40(7)		0.41(7)		0.43(7)	
	2.4	2.74(4)	98	2.75(4)	98	2.76(4)	97
13		0.37(4)		0.37(4)		0.38(3)	
	1.2	1.52(5)	96	1.53(5)	97	1.53(5)	96
14		0.28(1)		0.28(1)		0.31(1)	
	2.0	2.3(1)	101	2.3(1)	101	2.3(1)	100
15		0.25(6)		0.26(6)		0.26(3)	
	1.6	1.68(3)	89	1.68(3)	89	1.71(3)	91
16		0.74(8)		0.74(8)		0.77(8)	
	1.6	2.28(3)	96	2.28(3)	96	2.29(3)	95
17		0.35(4)		0.35(4)		0.37(5)	
	1.6	1.84(4)	93	1.84(4)	93	1.85(4)	93
18		0.41(2)		0.41(2)		0.44(3)	
	2.5	2.84(7)	97	2.84(7)	97	2.85(6)	96
19		0.55(3)		0.55(3)		0.59(4)	
	2.5	3.15(7)	104	3.15(7)	104	3.15(8)	102
20		0.59(2)		0.60(2)		0.61(2)	
	1.2	1.93(2)	112	1.92(2)	110	1.91(2)	108
21		0.8705(2)		0.878(4)		0.875(3)	
	1.2	2.16(8)	107	2.15(8)	106	2.14(8)	105
22		0.838(4)		0.85(1)		0.849(2)	
	0.6	1.44(6)	100	1.44(3)	98	1.44(4)	99
23		0.59(4)		0.59(4)		0.60(4)	
	2.0	2.7(2)	106	2.7(2)	106	2.7(2)	105
24		0.073(1)		0.078(3)		0.086(8)	
	0.5	0.59(4)	103	0.59(6)	102	0.59(2)	101

[a] The corresponding standard deviations in the last significant figure are given between parentheses.