Requirements for weighing instruments for calibration of pipettes.

Performance of gravimetric calibration procedure of a pipette requires use of a weighing instrument with metrological parameters as described in norm ISO 8655-6.

ISO 8655-6 determines the requirements for weighing instruments:

Tested volume *V*	Reading unit *d* mg	Repeatability and linearity mg	Standard deviation of a measurement mg
1ul *V* < 10ul	0,001	0,002	0,002
10 ul < *V* < 100 ul	0,01	0,02	0,02
100 ul < *V* < 1 000 ul	0,1	0,2	0,2
1 ml < *V* < 10 ml	0,1	0,2	0,2
10 ml< *V* < 200 ml	1	2	2

Minimal requirements for weighing instruments (according to ISO 8655-6)

In case, where standard deviation of liquid measurement is specified, than it is possible to use it as acceptance criterion instead of repeatability and linearity parameters of a weighing instrument. However, it needs to be assumed, that standard uncertainty is not bigger than twofold or threefold of reading unit *d*.

In order to maintain measurement retraceability, a weighing instrument that is utilized for pipette calibration, should have valid calibration certificate.

When analyzing requirements of the norm with regard to equipment of a workstation and ambient conditions which influence the result, there have been certain tests performed, which confirm the provisions from the norm. The results of such tests, and conclusions drawn from them are helpful in analyzing measurement process of pipette checking and calibration.

Metrological requirements for weighing instruments designed for pipettes calibration

As has been mentioned above, one of the basic measuring instruments for checking pipettes is a weighing instrument, i.e. a balance. The first series of tests concerned checking the characteristics of selected balances manufactured by MRC and used for gravimetric calibration of pipettes for confirmation of their compatibility with norm ISO 8655-6. The norm lists metrological requirements for balances used for calibration of pipettes, and for their two parameters: repeatability and linearity.

Weighing instrument repeatability

Norm ISO 8655-6 lists the metrological requirements for balances used in process of pipettes calibration with regard to two parameters characterizing the instrument: repeatability and linearity.

When analyzing the second chapter of ISO 8655-6 norm, which focuses on bounding documents, and chapter three, which discusses terms and definitions, it is only possible to unequivocally define term of “repeatability”. According to international dictionary of basic and general metrological terms, there are two kinds of repeatability.

In case of “linearity” if searching through norm ISO 8655-6 and referring documents (norms, metrological dictionary, OIML documents), and terminology in use, it is not possible to determine unique definition describing “linearity”.

Repeatability of a measuring instrument is characterized as a feature of this instrument according to which it indicates measurements similar to one another in case of multiple measurement of the same measured quantity in the same measurement conditions. These conditions refer to:

- reduction to minimum of changes caused by an observer
- the same measuring procedure
- the same observer
- the same measuring instrument, used in the same conditions
- the same place
- repetition within short period of time

Repeatability of measurement results is defined as a compatibility ratio between following measurements of the same measured quantity, performed in the same measurement conditions. The conditions, as characterized above, are called repeatability conditions.

Repeatability can be expressed in quantities, by means of characterizing dispersion of indications, most often presented as standard deviation.

Repeatability of a measuring instrument is, in most cases, specified by the manufacturer of balances on their technical folders. According to norm EN 45501 and document OIML R76-1, repeatability should be determined at least for two characteristic loads: for 50 % of maximal capacity and for maximal capacity (~100% Max). However, the specificity of small mass increment as in case of pipettes calibration, requires modification of the approach in determining repeatability of a balance.

In this test procedure, standard deviation has been determined with use of below methods:

- series of 10 repetitions in repetitive conditions for loads: 10mg, 500mg, 1g, 2g,

5g, 10g, 20g, 50,g (with respect to balance model),

- series of 10 repetitions in repetitive conditions for 100mg load with specific loads on TARE:

500mg, 1 g, 2g, 5g, 10g, 20g.

The results of standard deviation calculation as a measure of repeatability are presented for series of 10 measurements and for series of 6 measurements, as specified in requirements of a norm

PN-EN 45501 on non-automatic weighing instruments. Results of all measurements are presented in figures no. 3 and no. 4 below:

Balance model			Load on weighing pan of a balance [g]							
			0,1	0,5	1	2	5	10	20	50
Max=21g d=0,001 mg	S for n=10	[mg]	0,0021	0,0022	0,0023	0,0024	0,0023	0,0024	--	--
	S for n= 6	[mg]	0,0021	0,0021	0,0022	0,0023	0,0023	0,0024	--	--
	S=ISO8655-6	[mg]	0,002							
Max=21g d=0,01 mg	S for n=10	[mg]	0,012	0,007	0,007	0,007	0,012	0,008	0,0014	0,012
	S for n=6	[mg]	0,013	0,008	0,006	0,008	0,012	0,008	0,014	0,008
	S=ISO8655-6	[mg]	0,02							
Max=60g d=0,01 mg	S for n=10	[mg]	0,03	0,04	0,03	0,03	0,02	0,03	0,04	0,04
	S for n=6	[mg]	0,03	0,03	0,02	0,03	0,02	0,02	0,03	0,04
	S=ISO8655-6	[mg]	0,02							

Fig. 3. Standard deviation of tested balances in different loads

Balance model			Load TARE [g]							
			0	0,5	1	2	5	10	20	50
Max=21g d=0,001 mg	S for n=10	[mg]	0,0023	0,0022	0,0023	0,0022	0,0023	0,0023	--	--
	S for n= 6	[mg]	0,0016	0,0020	0,0018	0,0018	0,0015	0,0018	--	--
	S=ISO8655-6	[mg]	0,002							
Max=21g d=0,01 mg	S for n=10	[mg]	0,002	0,002	0,000	0,000	0,002	0,001	0,002	--
	S for n=6	[mg]	0,002	0,002	0,000	0,000	0,000	0,000	0,002	--
	S=ISO8655-6	[mg]	0,02							
	S for n=10	[mg]	0,02	0,02	0,01	0,02	0,02	0,02	0,02	--
	S for n=6	[mg]	0,02	0,02	0,01	0,02	0,02	0,02	0,02	--
	S=ISO8655-6	[mg]	0,02							

Fig. 4 Standard deviation of tested balances for 100 mg load with respective TARE loads

When analyzing results presented in Fig. 3 and 4, it can be noticed that, tested electronic balances have different standard deviation if repeatability criteria are applied. The procedure of pipette checking requires dosing of tested volumes to a vessel located on balance weighing pan, and tarring it as weighing result is saved. Testing of repeatability was also performed with small load with specified TARE loads.

During analysis of measurement results for balance with reading unit *d* = 0,001mg, and the test for repeatability by means of two methods, it occurs that both methods of calculation are compatible with norm ISO 8655-6. It should be stressed here, that average value of standard deviation determined for small capacities is approximately 1/3 lower than standard deviation determined for each load separately (in particular for *Max*). If assumed, that this is a common situation (i.e. that determination of standard deviation for small loads with various TARE loads), than it would be sufficient to meet the requirements specified in Norm EN 45501. However, such assumption can not be accepted without tests for its confirmation, thus the procedures for determination of repeatability, for balances used for gravimetric pipettes calibration, should focus on actual character of balance operation.

The obtained results of tests performed on MRC balances, confirm that all balances meet the requirements determined for balances that are used in process of pipettes calibration; standard deviation if calculated from series of 6 measurements (according to norm EN 45501) give better numerical value, but for 6 and for 10 series of measurements, the result is positive.

Linearity

In case of linearity, as has been mentioned above, the ISO 8655-6 norm and reference documents (norms, metrological dictionary, OIML documents) and terminology in use do not provide unique definition of “linearity”. It is possible to accept a colloquial definition, which in metrology of mass is described as the ability of a weighing instrument to maintain certain tolerance value in its full weighing range. There is also a problem of determining this parameter. If one takes into consideration the upper mentioned fact of determining very small increments of mass in short period of time, than determining this parameter should be different from the classic procedure, as in case of weighing instruments for general use.

The test has been performed on the same balances, that were used for determining parameter of repeatability. The test was to establish error indications of balances in two ways of proceeding:

- determining error of indications of balances for 6 specified points according to method described in point 8.2.2 of EN 45501 norm with application of E_1 standard masses with valid calibration certificate
- determining error of indications of balances with use of 100mg load and accruing (from 100mg to 1g) for five TARE values and application of standard masses class E_1 100mg and set of ten standard masses class F_2 100 mg each with valid calibration certificate.

The results are presented in Fig. 5:

Balance model								
Max=21g d=0,001 mg	Load	[g]	0,1	0,5	1	2	5	10
	Error = EN 45501	[mg]	0,006	-0,004	0,004	-0,030	-0,042	-0,060
	Error with TARE load	[mg]	+/-0,002	--	--	--	+/-0,002	+/-0,000
	Linearity = ISO 8655-6	[mg]	0,002					
Max=21g d=0,01 mg	Load	[g]	0,1	1	5	10	20	50
	Error = EN 45501	[mg]	+/-0,00	+0,01	-0,02	-0,06	-0,06	-0,07
	Error with TARE load	[mg]	+/-0,00	--	+/-0,00	+/-0,00	+/-0,00	--
	Linearity = ISO 8655-6	[mg]	0,002					
Max=60g d=0,01 mg	Load	[g]	0,1	1	5	10	20	50
	Error = EN 45501	[mg]	+0,02	-0,02	-0,04	-0,05	-0,07	-0,15
	Error with TARE load	[mg]	+/-0,02	+/-0,02	+/-0,02	+/-0,02	+/-0,02	+/-0,02
	Linearity = ISO 8655-6	[mg]	0,002					

Fig.5 Comparison between balances test – indication error (linearity) with TARE load

When analyzing obtained test results, it can be stated, that as in case of repeatability, the way linearity of a balance is determined makes a difference for balance in gravimetric checking of pipettes. Indication errors if determined in accordance with EN 45501 norm in the full measuring range of a balance, differ in their numeric value from indication errors determined with small loads for specified TARE load.

Stabilization time

An important factor in measuring procedure is time of measurement stabilization, i.e. a time that elapses from the moment of placing a load on weighing pan of a balance till obtaining stable measurement result, and in most cases marked with graphic pictorial. Stabilization time depends on many ambient factors, like: breeze, vibrations, drifts caused by temperature change, and other important aspects while using pipettes: liquid evaporation during weighing process.

The test was to determine the stabilization time for selected balances and specified loads: for balance model MXA 21/P (chart a) and XA60/220/X (chart b) and loads 1g, 2g, 5g, 10g, 20g, 30g, 40g, 50g.

The results are presented in Fig. 6 and graphically in Figure 6.

Balance model	Load	Stabilization time
d = 0,001 mg	10 g	1-5g – 8sec; 10g – 9sec; 20-30g – 10sec
d = 0,01 mg	50 g	1g – 9sec; 2-5g – 10sec; 10-30g – 11sec; 40-50g – 13 sec

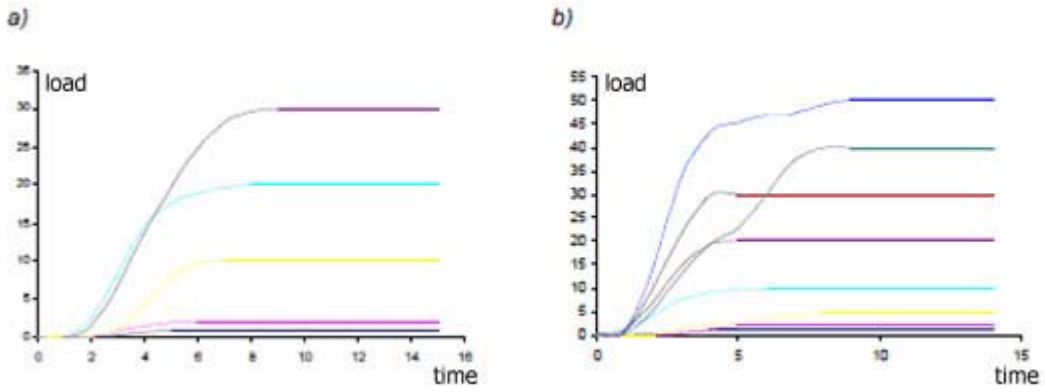

Fig. 6 Graphic interpretation of stabilization time of balances

The above presented table and figure refers to stabilization time for each load separately. As observed, the lower the load, the shorter stabilization time is. It is quite obvious in case of an electronic balances operating well, i.e. its filters are set properly. In case of pipettes checking, as mentioned above, a balance is loaded with very small masses for specified TARE load. An instance of balance stabilization time in case of checking pipette volume 100ul is presented on below figure:

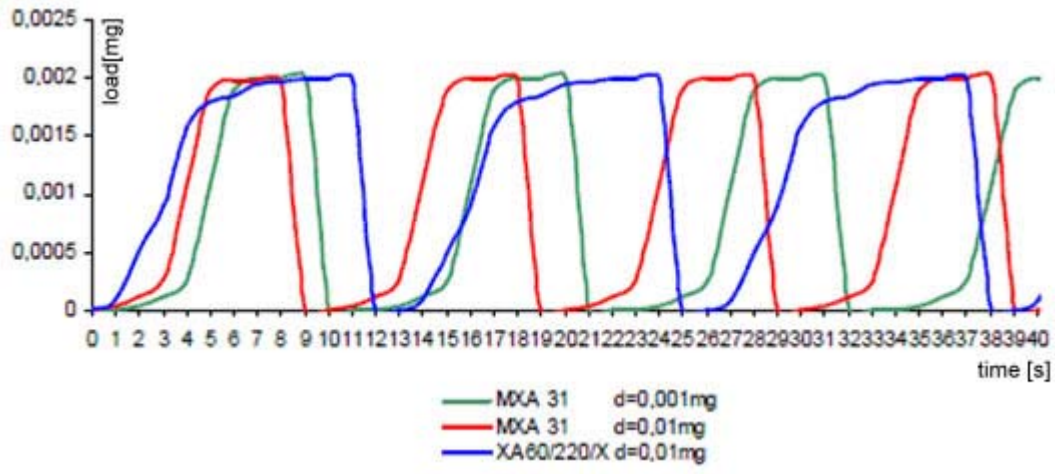

Fig. 7 stabilization time during pipette checking

Stabilization time for each balance is marked with separate colour:

- green – balance d = 0,001mg – stabilization time is approximately 9 seconds
- red – balance d = 0,01mg – stabilization time is approximately 8 seconds
- blue – balance d = 0,01mg – stabilization time is approximately 15 seconds

In case of balance with resolution d = 0,01mg the extended stabilization time is caused by ambient conditions, which influence the test procedure.

Stabilization time for one selected volume of a pipette, with series of 10 repetitions, and application of various balances is presented on below table:

Pipette volume [ul]	Balance model		
	MXA 21/P		XA 60/220/X
	d = 0,001 mg	d = 0,01 mg	d = 0,01 mg
2	85 seconds	--	--
10	85 seconds	75 seconds	100 seconds
100	--	85 seconds	110 seconds
1000	--	--	120 seconds

Fig 7 Stabilization time during pipette checking for selected balances – for one series of 10 repetitions for single volume

2.3 Humidity stabilization time in a weighing chamber

The test was to check the stabilization time in a weighing chamber. In case of a balance, an important factor which decides on start of measuring procedure, is humidity stabilization in a weighing chamber with “evaporation trap” container filled with liquid. Humidity influences evaporation process of a liquid during pipette calibration process, which influences the accuracy and repeatability of measuring results. One of solutions for this problem is, as mentioned before, use of small liquid volumes (below 50 ul) and low volume vessels with covers. The measurement were performed with application of analytical balance series MXA 21/P equipped with “evaporation trap” vessel, thermohygrometer and a clock.

Hygrometer probe was located in weighing chamber, as presented on neighbouring picture. The test of humidity contest was initiated, and measurements were recorded in 2 minutes intervals. As the humidity in weighing chamber has stabilized, distilled water was put into the “evaporation trap” vessel, and observation of humidity content was continued until stabilization.

As result of performed test of humidity in a weighing chamber, the time of humidity stabilization has been scheduled i.e. period of time and “evaporation trap” vessel filled with liquid, after which it is possible to initiate the test procedure.

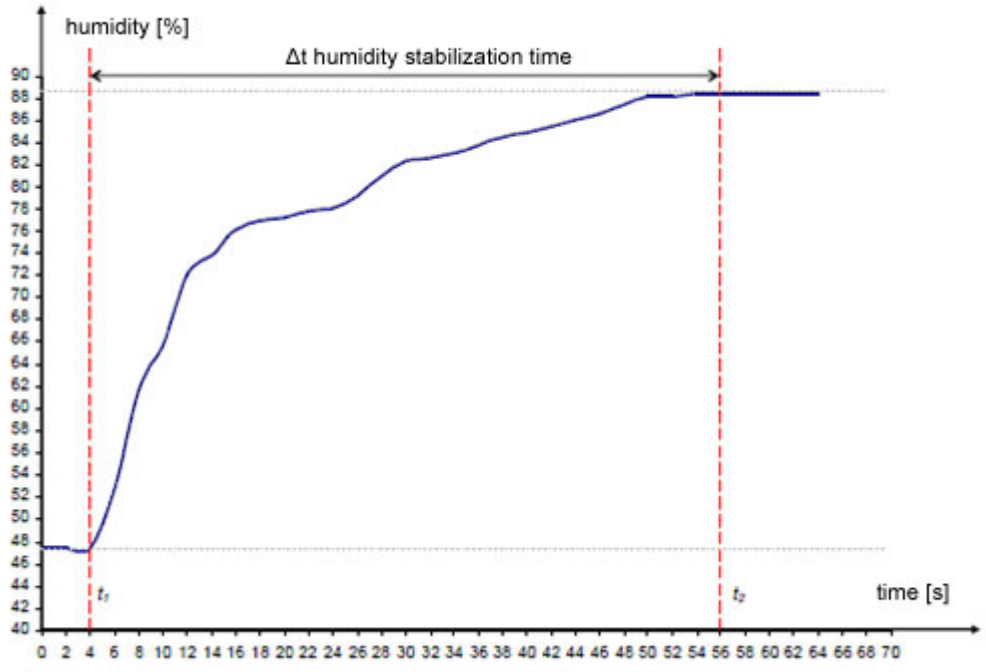

Fig. 8 Chart of humidity changes in a weighing chamber in time for balance model MXA 21/P

Figure 8 presents a graph of changes in humidity and in time in a weighing chamber. For the purpose of graph analysis below data was introduced:

t_1 – time [min], in which the "evaporation trap" was filled with liquid

t_2 – time [min], in which humidity stabilization was observed

Δt_1 – time of humidity stabilization in a weighing chamber

$$\Delta t_1 = t_2 - t_1 \ [min] \quad (15)$$

The measurement was done by locating a probe in the weighing chamber. The weighing chamber was closed tight. In time t_1 (after approximately 4 minutes) water was poured into "evaporation trap". Then, the indication on hygrometer was observed, until the measurement has stabilized.

Stabilization was observed in time t_2.

The test made it possible to establish stabilization time Δt_1 with a formula (15). Stabilization time is approximately 50 minutes.

2.4 Influence of „evaporation trap" on liquid evaporation speed in time and in relation to evaporation surface

At this stage, the measurements of mass were taken in 10 second time intervals.

Before the start of measuring process, the „evaporation trap" - B vessel was inserted, which increased the direct humidity in a weighing chamber. The test was to place various vessels filled with distilled water – A in weighing chamber. The mass was read out from balance indicator in 10 seconds time intervals. The measuring time took approximately 600 seconds (10 minutes) for each vessel diameter. Test results are presented charts, table 8 below.

	Vessel diameter		Vessel diameter	
	Ø 14,5 mm		Ø 22,5 mm	
	With evaporation trap	Without evaporation trap	With evaporation trap	Without evaporation trap
Δm/t	0,003 mg / 10 min	0,015 mg / 10 min	0,54 mg / 10 min	2,87 mg / 10 min

Fig. 8 mass change in time as result of liquid evaporation with application of „evaporation trap" vessel.

As result of performed tests, it can be stated, that a "evaporation trap" vessel eliminates or maximally delays the process of liquid evaporation during weighing, which is very important for the purpose of gravimetric pipette calibration procedure.

Below figures present influence of "evaporation trap" to liquid evaporation with relation to dimension of weighing vessels.

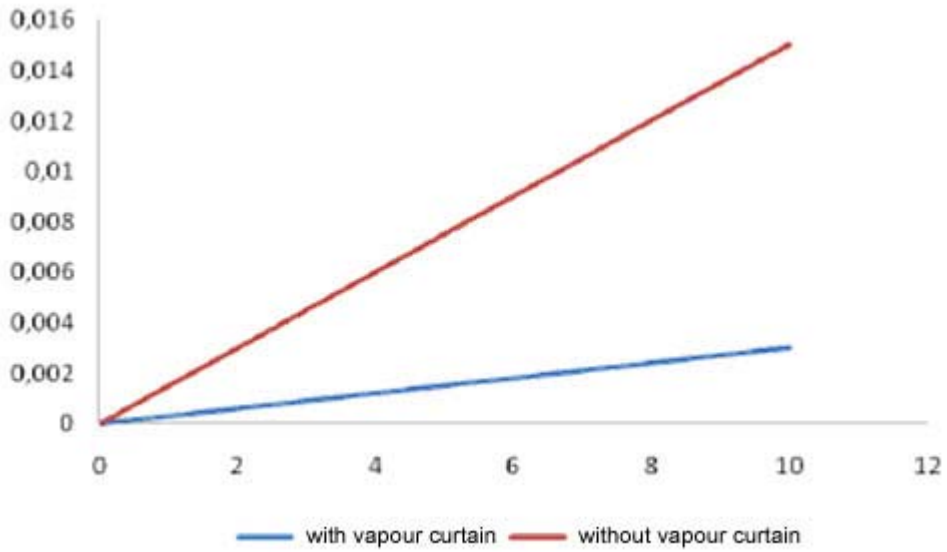

Fig. 9a influence of „evaporation trap" on evaporation process – balance model MXA 21/P (blue – with evaporation trap; red w/o evaporation trap)

The chart includes results of performed test. It is visible that in case of balance with Max 21 g application of a vessel with diameter 14,5mm has eliminated the effect of evaporation . Long measurements, performed in a long period of time would demonstrate presence of such effect, but for the purpose of pipettes calibration, the time intervals are short.

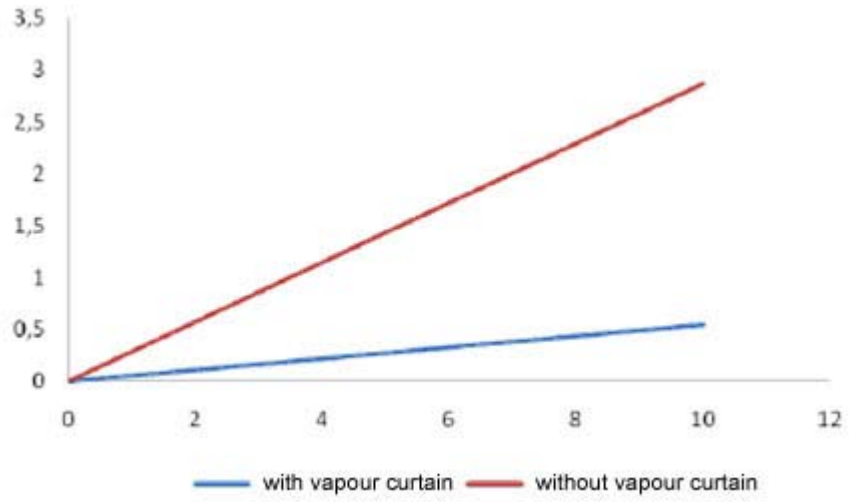

Fig. 9b influence of „evaporation trap" onto evaporation – balance model XA60/220/X (blue – with evaporation trap; red – w/o evaporation trap)

A graph prepared according to performed tests, presents, that in case of a balance with Max 220g, application of a vessel with diameter 22,5mm has slowed down evaporation process.
When analyzing evaporation speed for each of the balances in µg/mm2s, the results are as presented below:

Blance model		Evaporation speed in unit [mg/Pp 600s]	Evaporation speed in unit [ug/mm2s]
Max 21 g	With evaporation trap	0,003	0,00003
	Without evaporation trap	0,015	0,00015
Max 220 g	With evaporation trap	0,54	0,00226
	Without evaporation trap	2,87	0,01204

Fig. 9 Evaporation speed for selected balances.

Results of the tests made it possible to determine the influence of a "evaporation trap" on stopping evaporation process. Previous test, gave a result which confirmed increase of humidity in a weighing chamber if a "evaporation trap" is applied. Common physic law says, that speed of evaporation process depends on humidity of ambient conditions in which it takes place. The performed calculations helped to determine how much the process was stopped. Also, these results help is setting most appropriate time for test cycle of a pipette with specific volume.

2.5 Influence of “evaporation trap” application on test results of pipette checking

The test was to determine the direct influence of a „evaporation trap” onto the process of pipette calibration. The errors of a pipette were specified with and without application of a “evaporation trap”. The measurements were made with analytical balance model Max 21g, and a “evaporation trap” vessel. Workstation is presented on a picture.

A tested object was a pipette with variable volume 20-200ul, and initial setting to 20ul. The measurements were made with empty and full “evaporation trap” vessel. All the measurements and calculations were performed in accordance to procedure valid in MRC Measuring Laboratory, and according to ISO 8655-6, as discussed in Chapter 1 of this article. The results from tests are presented in table no. 10.

The test result made it clear, that „evaporation trap” is very important, and it should be applied in tests. As has been mentioned above, evaporation speed depends on humidity content. The application of a “evaporation trap” as described above, has demonstrated increase of humidity in weighing chamber to approximately 90 %. Thus, now it is checked and tested, that application of a „evaporation trap” does increase humidity in weighing chamber. Previous tests have also checked the decrement of liquid mass in time.

The next test was to determine the influence of evaporation process onto Maximal Permissible Error (MPE) of a pipette as specified for particular volumes in norm ISO 8655-2.

The test was performed on volumes: 2ul, 20ul, 100ul and 1000ul. Each of volumes had 10 average volume measurements out of 10 series of measurements.

No.	Tested volume – 2 ul				Tested volume – 20 ul			
	Volume		MPE		Volume		MPE	
	Without evaporation trap	With evaporation trap	ISO 8655-6	Manufacturer	Without evaporation trap	With evaporation trap	ISO 8655-6	Manufacturer
	[ul]	[ul]	[ul]	[ul]	[ul]	[ul]	[ul]	[ul]
1	1,95	1,96	0,04	0,04	19,73	19,86	0,02	0,06
2	1,97	1,98			19,76	19,85		
3	1,97	1,97			19,76	19,84		
4	1,95	1,99			19,73	19,85		
5	1,97	1,98			19,76	19,86		
6	1,96	1,97			19,76	19,85		
7	1,98	1,99			19,79	19,85		
8	1,96	1,98			19,73	19,86		
9	1,97	1,97			19,79	19,85		
10	1,95	1,98			19,74	19,87		
Average	**1,96**	**1,98**			**19,78**	**19,85**		

No.	Tested volume – 100 ul				Tested volume – 1000 ul			
	Volume		MPE		Volume		MPE	
	Without evaporation trap	With evaporation trap	ISO 8655-6	Manufacturer	Without evaporation trap	With evaporation trap	ISO 8655-6	Manufacturer
	[ul]	[ul]	[ul]	[ul]	[ul]	[ul]	[ul]	[ul]
1	98,9	99,2	0,03	0,15	999,3	999,9	3,0	1,5
2	99,4	98,7			999,3	999,8		
3	99,5	99,3			999,1	999,8		
4	99,3	98,7			999,3	1000,1		
5	99,3	99,5			999,2	1000,0		
6	99,5	99,8			999,2	1000,1		
7	99,4	99,6			999,1	999,8		
8	99,5	99,6			999,4	999,8		
9	99,3	99,2			999,2	999,9		
10	99,3	99,3			999,3	999,9		
Average	**99,29**	**99,34**			**999,24**	**999,91**		

Fig. 10 Measurement results for volume with and without application of "evaporation trap".

Measurement results of maximal permissible values according to ISO 8655-2 and permissible errors as defined by the manufacturer are presented on the table 10 above.

Concluding from analysis of results and value of maximal permissible errors, it can be stated that:

– in case of volume 2ul the contribution of pipette error indication caused by distilled water evaporation is 0,02ul with maximal permissible value 0,04ul which constitutes 50% of error as specified by norm, and is according to manufacturer specification;

– in case of volume 20ul the contribution of pipette error indication caused by distilled water evaporation is 0,13ul with maximal permissible value 0,1ul which constitutes 35% of error as specified by norm and 117% according to manufacturer specification;

– in case of volume 100ul the contribution of pipette error indication caused by distilled water evaporation is 0,05ul with maximal permissible value 0,3ul which constitutes 17% of error as specified by norm and 33% according to manufacturer specification;

– in case of volume 1000ul the contribution of pipette error indication caused by distilled water evaporation is 0,67ul with maximal permissible value 3,0ul which constitutes 22% of error as specified by norm and 45% according to manufacturer specification.

According to norm ISO 8655 errors resulting from water evaporation should be considered as valid. Thus, for low volumes, i.e. below 50 ul, it is recommended to use weighing vessels with a cover, or an additional application to a balance, such as "evaporation trap". The correctness of above norm requirements is confirmed by above tests.

Apart from an appropriate vessel or "evaporation trap", time is another very important factor in measuring process it is important to make the complete cycle as short as possible. The recommendation says, one complete cycle, that is sampling and expelling of a liquid should be as regular as possible. Independently from above specified factors, an error may occur as result of experience of an operator who does the measurements.

2.6 Influence of an additional kit for pipettes checking on analytical balances

A kit consists of an additional chamber equipped with “evaporation trap”, which is located inside the draft shield (weighing chamber) of an analytical balance. It has been designed to minimize the process of liquid during weighing process.

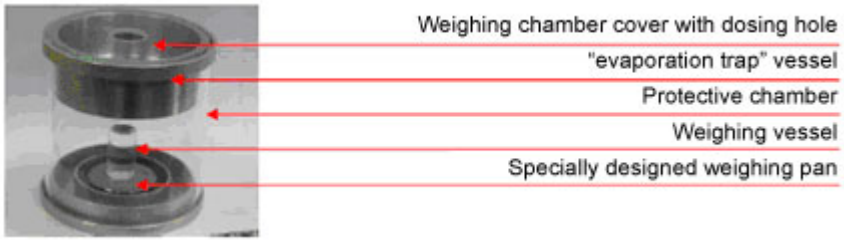

Fig. 10 Kit for checking pipettes on RADAG balances series AS and XA/X

Results of checking a pipette before and after application of kit for pipette checking

No.	Tested volume - 20 ul				Tested volume – 100 ul			
	Volume		MPE		Volume		MPE	
	Without „evaporation trap”	With „evaporation trap”	ISO8655-2	Manufacturer	Without „evaporation trap”	With „evaporation trap”	ISO8655-2	Manufacturer
	[ul]	[ul]	[ul]	[ul]	[ul]	[ul]	[ul]	[ul]
1	19,63	19,87	0,2	0,06	98,5	99,6	0,3	0,15
2	19,66	19,75			99,4	98,7		
3	19,66	19,82			99,3	99,6		
4	19,63	19,78			99,2	98,7		
5	19,66	19,76			99,1	99,5		
6	19,66	19,74			99,2	99,7		
7	19,69	19,81			99,2	99,5		
8	19,63	19,78			99,2	99,6		
9	19,69	19,73			99,3	99,5		
10	19,64	19,79			99,3	99,6		
Average	19,67	19,78			99,17	99,40		

Fig. 11 Results of volume measurements with application of „evaporation trap” for pipette checking

Analysis of above results and values of MPE, gives below conclusions:

– in case of volume 20ul the contribution of pipette error indication caused by distilled water evaporation is 0,13ul with maximal permissible value 0,1ul which constitutes 55% of error as specified by norm and 183% according to manufacturer specification;

– in case of volume 100ul the contribution of pipette error indication caused by distilled water evaporation is 0,05ul with maximal permissible value 0,3ul which constitutes 77% of error as specified by norm and 153% according to manufacturer specification;