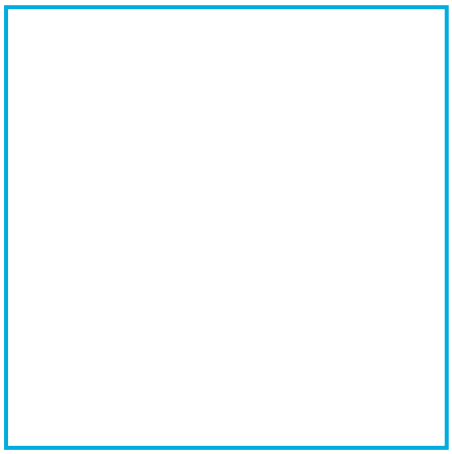
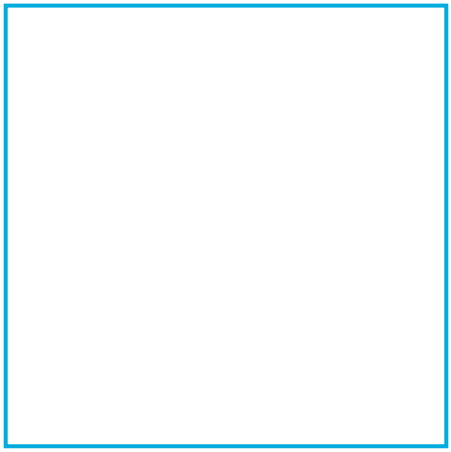
PTB bericht ThEx-16en
März 2021

Henning Wolf

Final report on the project Volume Determination in the Microlitre Range





ISSN 1434-2391
ISBN 978-3-95606-582-8

Physikalisch-Technische Bundesanstalt

Thermodynamik und Explosionsschutz
PTB-ThEx-16en
Braunschweig, März 2021

Henning Wolf

Final report on the project
Volume Determination in the Microlitre Range

English version of the original report written in German
from 2001-02-15

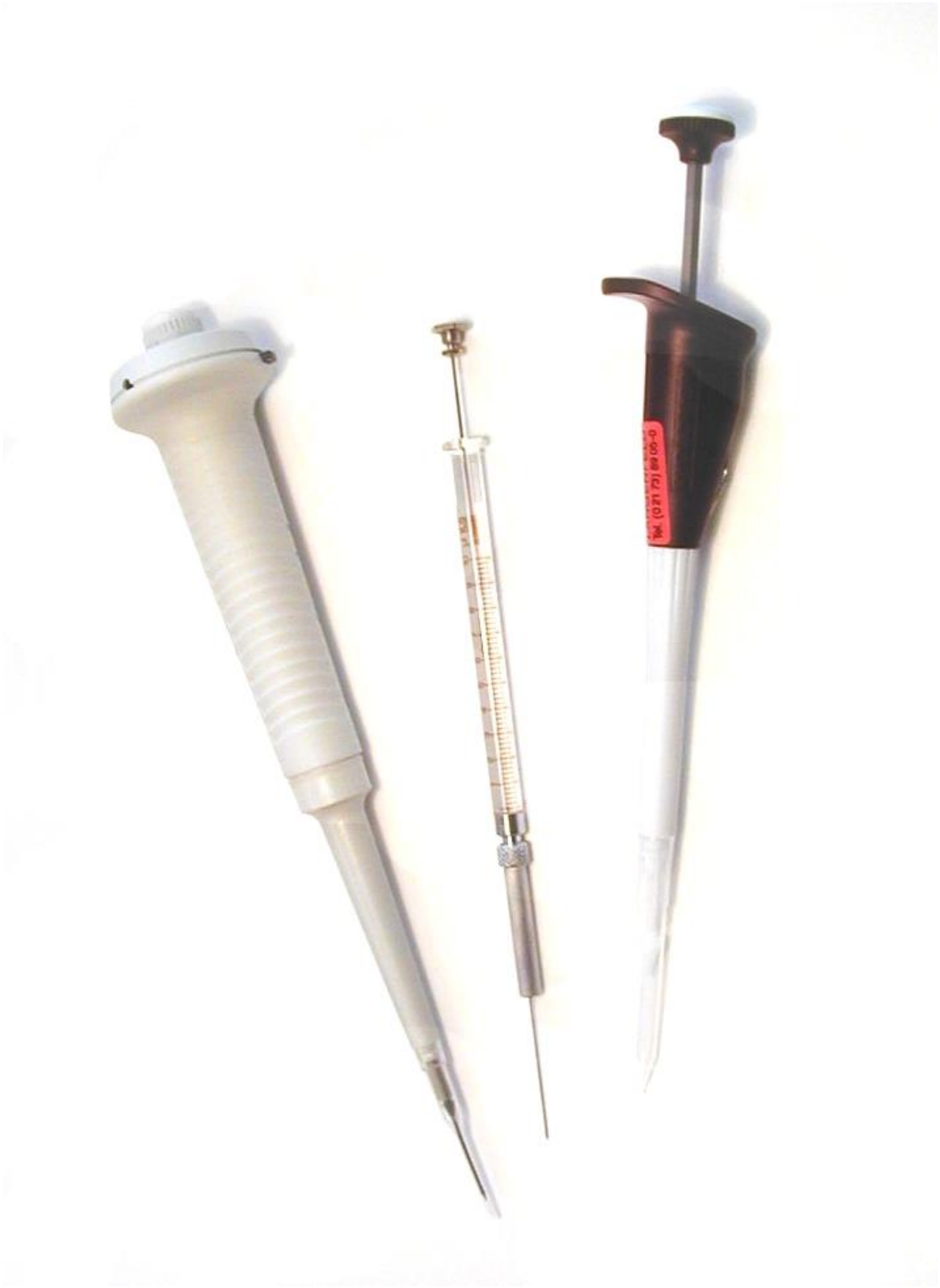
ISSN 1434-2391
ISBN 978-3-95606-582-8

Herausgeber:
Physikalisch-Technische Bundesanstalt
ISNI: 0000 0001 2186 1887

Presse und Öffentlichkeitsarbeit

Bundesallee 100
38116 Braunschweig

Telefon: (05 31) 592-93 21
Telefax: (05 31) 592-92 92
www.ptb.de



Volumetric apparatus for the dispensing of liquids in the microlitre range

Acknowledgements

Dr. G. Schumann, Institut für Klinische Chemie der Medizinischen Hochschule Hannover (MHH),
Prof. Dr. L. Siekmann, Institut für Klinische Biochemie of the University of Bonn,
A. Kessler, Institut für Klinische Biochemie of the University of Bonn, and
R. Klauke, Institut für Klinische Chemie der Medizinischen Hochschule Hannover (MHH), supported the Volume Determination in the Microlitre Range project through discussions and invaluable input, and by providing human serum and control material. I am particularly appreciative of Mr Klauke's commitment to preparing and analysing the major part of the serum samples.

At this point, I would like to thank all persons involved most cordially.

The volumetric apparatuses used within the scope of the investigations were provided by:

Abimed, Langenberg,Brand GmbH + Co, Wertheim,
Hirschmann Laborgeräte GmbH &Co. KG, Eberstadt,
Hamilton Deutschland GmbH, Darmstadt,
Eppendorf-Netheler-Hinz GmbH, Hamburg, and
ILS Innovative Labor Systeme, Stützerbach.

I would therefore also like to thank these companies for their precious support.

Table of contents

List of the symbols used

Symbol	Quantity
A	Absorbance of a contrast agent solution
c	Concentration of a contrast agent solution
d	Thickness of the liquid layer
I_o	Intensity of the outgoing radiation
I_i	Intensity of the incident radiation
k	Extinction coefficient of a contrast agent
k	Coverage factor in uncertainty statements
k_B	Boltzmann constant
k_c	Molar extinction coefficient
K_P	Protein concentration
K_S	Sodium chloride concentration
m	Mass
M_i	Mass of a type-i particle
N	Number of particles (gas molecules)
N_i	Number of type-i particles
p	Pressure
p_i	Partial pressure of the type-i gas
p_A	Ambient pressure
T	Absolute temperature
t	Temperature in °C
t_A	Air temperature in °C
U	Expanded measurement uncertainty
u	Measurement uncertainty contribution
V	Volume
W	Weighing value
$\Delta\rho_{protein}$	Density fraction in the serum density
$\Delta\rho_{NaCl}$	Density fraction in the serum density
$\Delta\rho_{res}$	Density fraction in the serum density
φ	Relative humidity
ρ	Density
$\rho_{components}$	Density fraction in the serum density
ρ_W	Density of the weights
ρ_A	Air density
ρ_{serum}	Serum density
ρ_{water}	Water density

1 Introduction

One of the key skills a chemist needs to master is the dispensing of liquids, in particular the ability to measure liquids accurately, i.e. to determine the quantity, volume or mass.

Working in chemical analysis (e.g. in chromatographic investigations) today, you will be working mainly with volumes of liquids in the microlitre range. Such small volumes can be dispensed routinely in discrete procedures with piston-operated pipettes and microlitre syringes. Since their introduction in 1958, piston-operated pipettes in particular have increasingly established themselves due to their easy handling for dispensing liquids, in particular in the volumetric range from 1 µl to 1000 µl.

In all piston-operated volumetric apparatus, the liquid volume to be measured is first determined by the geometric volume of the piston travel. Due to the interaction of the liquid with the device and its environment, however, corrections have to be applied to this purely geometric consideration. The smaller the volume measured, the more significant the corrections. Physical material parameters that influence those interactions are thermal expansion, vapour pressure, density, viscosity, interface tension and surface tension. If different liquids are dispensed, their different parameters may lead to differences in the dispensed volume and also in the associated uncertainty. Calibrating the volumetric apparatus with a certain liquid is therefore not necessarily applicable to the dispensing of other liquids.

When it comes to the accreditation of reference and calibration laboratories, traceable calibration of the measuring instruments used is one of the most important requirements. The question as to whether calibrations carried out with a given liquid can be transferred to the dispensing of other liquids prompted PTB's Advisory Board for Medical Metrology (Beirat für Medizinische Messtechnik an der PTB) to recommend that PTB set up a project to investigate the traceability of volume determinations in the microlitre range [1]. In this recommendation, particular emphasis was placed on the investigation of human serum dispensing. Human serum is the most important liquid that is analysed in medical analysis. It is therefore probably one of the liquids that is the most frequently dispensed in analytical chemistry.

The present paper therefore deals with the question as to whether it is possible to transfer the dispensing results obtained with water to other liquids. Hereby, particular emphasis is placed on the dispensing of human serum.

2 Current state of research and standardization

2.1 Published investigations

Although piston-operated pipettes have been increasingly used for dispensing liquids (especially in the volumetric range from 1 µl to 1000 µl) for more than 40 years, they have been very little investigated by independent institutes. Apart from the issue dealt with in the publication by Lochner et al. (see below) regarding the influence of different liquids on the air interface of displacement pipettes with air interface, all other published investigations referred to water as the liquid to be dispensed. Systematic investigations into the dispensing behaviour of piston-operated pipettes or microlitre syringes when dispensing different liquids have not been published to date.

In 1978, R. Belac, W. Gögge, H. Groß and W. Ludwig [2] published a paper which provided important advice with regard to the correct handling of a piston-operated pipette. The test liquid used was water, and commercially available piston-operated pipettes were used for dispensing. The DIN 12650-2 and DIN 12650-6 standards were based on the results of this work.

In 1983, S. Mieke [3] published a proposal for computer-aided gravimetric pipette calibration. This procedure is particularly well-suited to arithmetically detect the quantity of liquid that evaporates during the weighing procedure, which can be problematic for the gravimetric calibration of small dispensing volumes. This new procedure allowed volumes in the range from 1 µl to 10 µl to be calibrated with a small measurement uncertainty. Mieke tested this procedure using commercially available piston-operated pipettes with water as the test liquid.

The gravimetric procedure is also the object of an investigation conducted by F. Michel, K. Sommer and F. Spieweck [4]. This paper was published in 1995. It describes a measuring device for measuring small volumes of liquids with a very low measurement uncertainty. In this measuring device, the evaporation of the liquid is reduced by means of an "evaporation trap" used to increase the humidity inside the weighing chamber. The performance of this device is demonstrated by measurements regarding the influence of the piston striking and of the device's temperature when dispensing volumes smaller than 50 µl. The objects of the investigation were air displacement and positive displacement piston-operated pipettes. Water

was used as a test liquid. The gravimetric measuring device used within the scope of the present work to measure small volumes of different liquids was set up based on the device described in [4].

In 1996, K.H. Lochner, T. Ballweg and H.-H. Fahrenkrog published an investigation in which water and liquids other than water were considered[5]. In this paper, the measurement accuracy of piston-operated pipettes with an air interface was investigated. It focused on the interaction of the dispensed liquid with the air interface of the pipette. A model pipette was constructed especially for the investigations. It was used to investigate the influence of numerous parameters such as the inclination angle, the way of pre-humidifying the air interface, the air pressure and the temperature experimentally and to compare the results with theoretical considerations. The examination also contained a quantitative statement on the influences experienced by the air interface and the resulting deviations of the dispensed volumes obtained when using different liquids due to the differences in density and vapour pressure of the liquids. Values for methanol (high vapour pressure) and sulphuric acid (high density) were given as examples.

2.2 Current state of standardization

Piston-operated pipettes are standardized in the ISO 8655 (Part 2) [6] series of standards (this standard replaces Part 2 of the expiring German DIN 12650 standard [7]). There is currently no standard available for microlitre syringes. The series of standards 8655 prescribes a calibration of the volumetric apparatus by means of the gravimetric test procedure (Part 6 of ISO 8655 [8] standard) or by means of the photometric test procedure (Part 7 of the ISO 8655 [9] standard). The corresponding, expiring German standards are DIN 12650, Part 6 [10] and DIN 12650, Part 7 [11]. The series of standards ISO 8655 was adopted in 2002 and transposed into the German DIN EN ISO 8655 standard.

The test liquid prescribed for the gravimetric test procedure is ultrapure water as described in Part 6 of the ISO 8655 standard. This standard does not deal with the dispensing of other liquids.

3 Objective of the investigation

The main question of the present investigation is to find out ***whether a calibration using water as a calibration liquid can be transferred to the dispensing of other liquids***.

To be able to answer this question, the following goals were formulated for the investigation:

- Setting up a gravimetric standard measurement apparatus for the calibration of piston-operated pipettes and microlitre syringes in the microlitre range
- Determining and quantitatively describing effects which influence the gravimetric volume determination of different liquids
- Measuring the differences in the dispensed volume when dispensing different liquids with an emphasis on the comparison between the dispensing of water and of human serum, and determining the physical effects causing these differences
- Developing methods that can be applied to the calibration of volumetric apparatus used to dose human serum

3.1 Practical realization

- **Volume range to be investigated: 0.2 µl to 50 µl**
  The smallest volumes dispensed with manual piston-operated pipettes and microlitre syringes are 0.2 µl and, in some cases also 0.1 µl. 0.2 µl is therefore the lower limit of the volume range to be investigated. It must be expected that physical interactions between the liquid and the apparatus will become more visible with decreasing volumes; the volume variation from 50 µl down to smaller volumes is therefore meaningful.
- **Volumetric apparatus to be investigated: commercially available piston-operated pipettes and microlitre syringes of various types**

  The investigation is designed to cover a wide spectrum of different devices to allow statements on whether the type or the material of the apparatus has an influence on

the dispensing. To limit the effort in terms of measurements, only one specimen of each type of apparatus will be investigated.

- **Operating the volumetric apparatus: manual operation close to daily practice**
  All devices investigated are devices which can be operated manually. In the investigations, they were therefore operated manually to remain as close as possible to actual practice. The disadvantage residing in the fact that very small differences in the dispensing behaviour may, under certain circumstances, be concealed by the insufficient reproducibility when operating the apparatus manually is condoned.

- **Setting up a gravimetric measurement apparatus to determine small volumes of liquid**
  The gravimetric method is selected as the primary method. It is a "conventional" method used to determine small volumes of liquid. This method consists in determining the mass of the liquid volume gravimetrically and converting it into a volume by means of the density. The quantity of *volume* is thus traced to the quantity of *mass*. The apparatus must be conceived in such a way that it can be used as a standard volumetric apparatus for volumes in the microlitre range.

- **Testing the photometric method as to its suitability for volume measurement**
  Another method that is often used to determine volume is based on the photometric measurement of light absorption in a contrast agent solution. A contrast agent solution with known contrast agent concentration is dispensed into a basic liquid of known volume and known absorption. From the absorption measured after the dispensing, it is possible to determine the contrast agent concentration in the mixture and, from that, the volume of the contrast agent solution added. This method is to be compared with the gravimetric method.

- **Fundamental investigations on the characterization of the gravimetric method**
  These are in particular investigations into the influence of the evaporation of liquid during the measurement, into the reproducibility of the measurement results and into drawing up a measurement uncertainty budget for the gravimetric measurement.

- **Investigations into the dispensing of different liquids**

  Measurements should first be carried out with simple liquids whose physical properties are well known. These measurements are to show whether differences in the dispensed volume occur. Section 3.2 explains in detail why certain liquids should be selected.

- **Investigations into the dispensing of human serum**

  After performing investigations with "simple" liquids, human serum shall be investigated. Human serum, as a biological fluid, is very complex; a simple physical description stating its density, viscosity and surface tension is therefore not sufficient to characterize it as to its dispensing behaviour. Human serum is a liquid that consists of more than 90 % water in which a large number of different components are dissolved. Its composition may vary in each human being. In the sick, the deviations from "normal values" may be very large. Correspondingly, the physical material properties of different samples of human serum may differ. Moreover, biochemical reactions may result in changes in the liquid during its storage.

- **Replacement liquid to model the dispensing behaviour of human serum**

  The lack of stability of human serum makes it difficult to collect larger quantities of this liquid and to transport it to a different laboratory for traceable density determination. This makes it difficult to use human serum itself as a calibration liquid.

  If the calibration results obtained with water are not transferable to human serum, another liquid must be found. This liquid should allow good modelling of the dispensing behaviour of human serum, and it should be possible to use this liquid to calibrate volumetric apparatus.

  The most important additional criteria are that large quantities of this liquid must be available, and the data concerning its density must be traceable.

3.2 Selecting the liquids

Physical material parameters of the liquids that may influence its dispensing are its viscosity, surface and interface tensions (which are interrelated with capillary forces), density and vapour pressure.

The present investigation aims to clarify whether an influence of these material parameters on the dispensed volume can be detected quantitatively when using positive displacement piston-operated pipettes and microlitre syringes. The liquids selected were water, ethanol and nonane. Hereby, water is used as a reference liquid. In addition, it represents liquids with high surface tension. Ethanol and nonane both have a very low surface tension, and their density is lower than that of water. Ethanol and nonane differ from each other in particular with regard to their vapour pressure. Ethanol's vapour pressure is approximately ten times higher than that of nonane. All of these three liquids stretch across a parameter field that includes the parameters of human serum. Solely the density of human serum is slightly higher than that of water. The most relevant physical material parameters are compiled in Table 1.

Table 1: Material parameters of the investigated liquids

Liquid	**Temperature**	**Density**	**Thermal volumetric expansion coefficient**	**Vapour pressure**	**Dynamic viscosity**	**Kinematic viscosity**	**Surface tension**
	°C	**kg/m³**	**1/K**	**hPa**	**Pa s**	**m²/s**	**mN/m**
Water	20	998.20	$2.12 \cdot 10^{-4}$	23	$1.00 \cdot 10^{-3}$	$1.00 \cdot 10^{-6}$	72.60
Ethanol	20	789.23	$1.10 \cdot 10^{-3}$	51	$1.20 \cdot 10^{-3}$	$1.52 \cdot 10^{-6}$	22.40
Nonane	20	718.10	$1.08 \cdot 10^{-3}$	4.8	$7.10 \cdot 10^{-4}$	$9.90 \cdot 10^{-7}$	22.85
Human serum	20	1025.7	?	?	?	?	56.2
Human serum	37	?	?	?	$1.12 \cdot 10^{-3}$	$1.15 \cdot 10^{-6}$	?

As previously mentioned, however, it must be assumed that human serum will not be easily characterized by means of the physical properties listed above. Chemical biological reactions with the surfaces of the volumetric apparatus may lead to a behaviour deviating from that of a "simple" liquid. The most notable of these properties resides in human serum's tendency to "adhere". This is caused by reactions of certain components of the serum with the inner surfaces of the apparatus.

4 Gravimetric measurement apparatus

4.1 Theoretical fundamentals

A gravimetric method is used as primary method to determine small volumes of liquid. Here, the mass of the liquid is determined by means of a weighing instrument. If the density of the liquid is known, then its volume can be determined directly via the relation

(4-1) $$V = \frac{m}{\rho}$$

where V describes the volume, m the mass, and ρ the density of the liquid. Volume determination is thus replaced by mass determination. Traceability of the quantity of volume is established to the SI base unit of mass.

A weighing instrument measures a weighing value W which must be converted into the mass m with an air buoyancy correction

(4-2) $$m = W \cdot \frac{1-\frac{\rho_A}{\rho_W}}{1-\frac{\rho_A}{\rho}}$$

where ρ_L is the density of the ambient air, and ρ_G is the density of the weights used with the weighing instrument. (Even in modern electromagnetic weighing instruments, this reference of the balance reading to applied weights is maintained.)

Thus, gravimetric volume determination requires – in addition to determining the weighing value of the liquid mass – knowledge of the density of the liquid, of the density of the ambient air, and of the density of the weights. The air density can be expressed by means of the equation below.

(4-3) $$\rho_A = \frac{k_1 p_A + \varphi(k_2 t_A + k_3)}{t_A + t_{A0}}$$

Hereby, the air pressure in hPa is to be inserted for p_A, the relative humidity in % for φ, and the air temperature in °C for t_A. For the constants k_1, k_2, k_3 and t_{A0}, the values $k_1 = 0.34844$ (kg/m^3) · °C/hPa, $k_2 = -0.00252$ kg/m^3, $k_3 = 0.020582$ (kg/m^3) · °C and $t_{A0} = 273.15$ °C are to be inserted. Equations (4-1) to (4-3) are described in detail in [12].

Figure 1 is a representation of the connections between the quantities in the form of a flow chart.

The influence of the relative humidity on the air density – and thus on the air buoyancy correction – is so small that it is not necessary to determine its exact value for volume

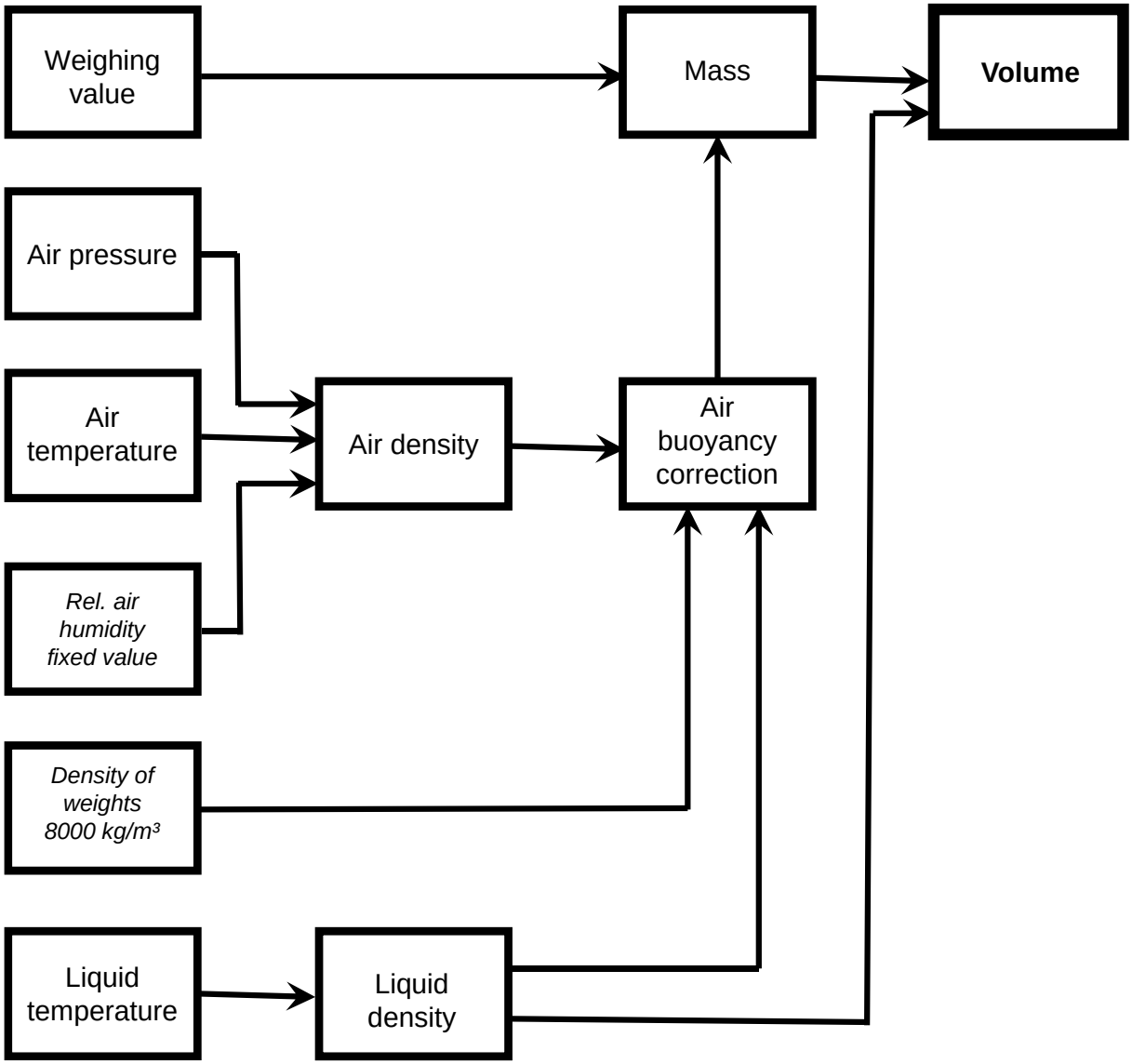


Fig. 1: Flow chart on the relations between the quantities required to determine volume gravimetrically. The boxes in italic font contain fixed values that are used instead of measured values.

determinations in the microlitre range. It is possible to work with mean values instead. (Cf. also the statements provided in the measurement uncertainty budget and the remarks in Section 4).

The density ρ as a function of the temperature t can, for some liquids (e.g. water, ethanol), be taken from tables or be calculated by means of a numerical equation (for this purpose, cf. [12] too). However, since these data are only available for few liquids, the density must, as a general rule, be measured as a function of the temperature for the temperature range in which the dispensing is to take place. Other influences (such as the change in density due to air pressure variations or due to air dissolving in the liquid) may be neglected. For example, the difference in density between water saturated with air and water that is free of air amounts to approx. $2 \cdot 10^{-3}$ kg/m^3 (i.e. a relative value of only $2 \cdot 10^{-6}$) at 20 °C.

4.2 Description of the gravimetric apparatus

Figure 2 shows the gravimetric apparatus. The apparatus is set up on a weighing table affixed to a massive wall. The weighing instrument itself is located on a granite plate, decoupled from the weighing table and mounted on vibration-absorbing material. The laboratory is air-conditioned; the room temperature is stabilized at ±0.5 °C.

The apparatus thus set up consists of the following components:

- A comparator balance for mass determination

  resolution: 100 ng, maximum capacity: 5 g.

  It is possible to humidify the air in the (closed) weighing chamber[1] by means of an "evaporation trap".

  During a measurement series, the weighing chamber is only accessible by means of an opening located in the lid of the weighing chamber (diameter: 10 mm). This opening is closed by means of a glass lid that is only removed when a pipette or a microlitre syringe has to be introduced to dispense a liquid into the weighing vessel.

1 In this part of the paper, the (ambiguous) term of 'weighing chamber' designates the closed part of the weighing instrument where the weighing platform and the product to be weighed are located. The part of the building where the entire apparatus is accommodated is called 'laboratory'.

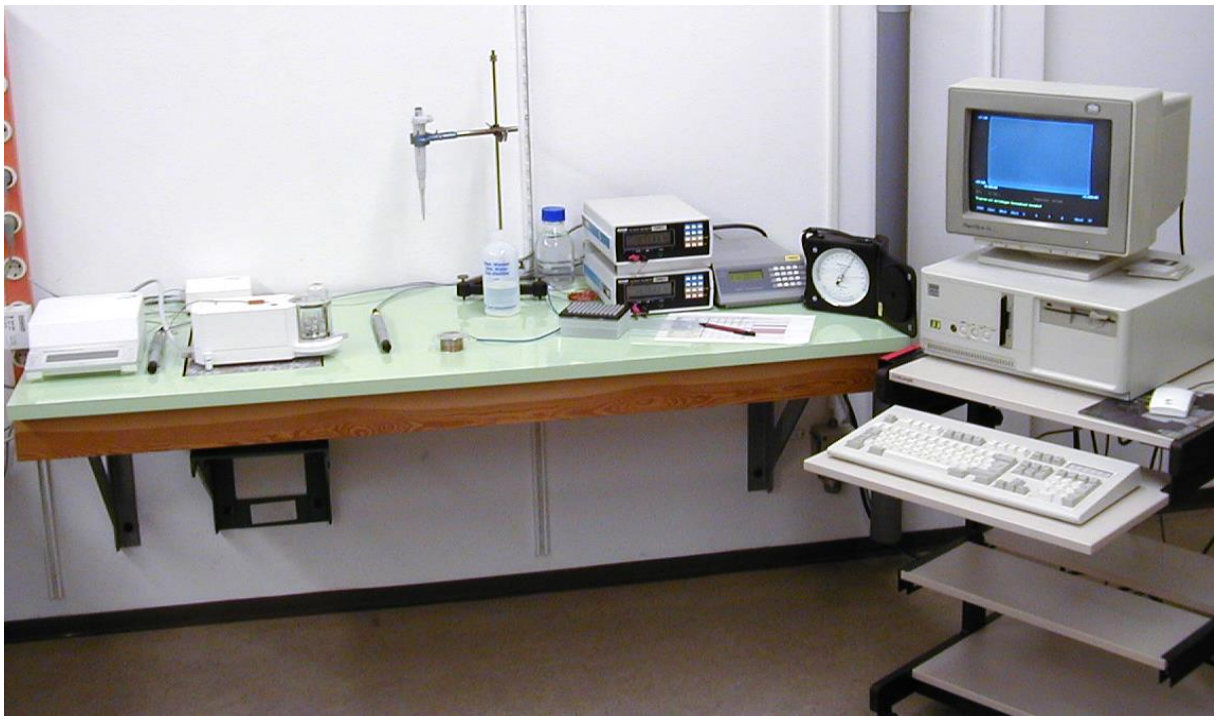


Fig. 2: Photo of the measurement apparatus
Left: the weighing instrument, consisting of the weighing module, the control module and the mains adapter. The reservoir for the liquid is located on the free surface in the centre of the table; the volumetric apparatus is located on the tripod above the reservoir. Right: the digital ohmmeter to measure the thermometer resistances, and measuring instruments for air pressure and humidity. A computer for data acquisition is located next to the measuring table.

- Barometer to determine air pressure

  Resolution: 1 mbar

- Measuring instrument to determine the temperature and relative humidity of the room

  Resolution: 0.1 °C and 0.1 % rel. humidity

- 2 thermistors to determine the temperature of the liquid and the temperature of the weighing chamber. The thermistor resistances are measured by means of digital ohmmeters with a resolution of 0.1 Ω; this corresponds to a temperature resolution smaller than 1 mK (depending on the temperature, since the characteristic of the thermistors is nonlinear).

- Computer to import, record and process the measurement data.

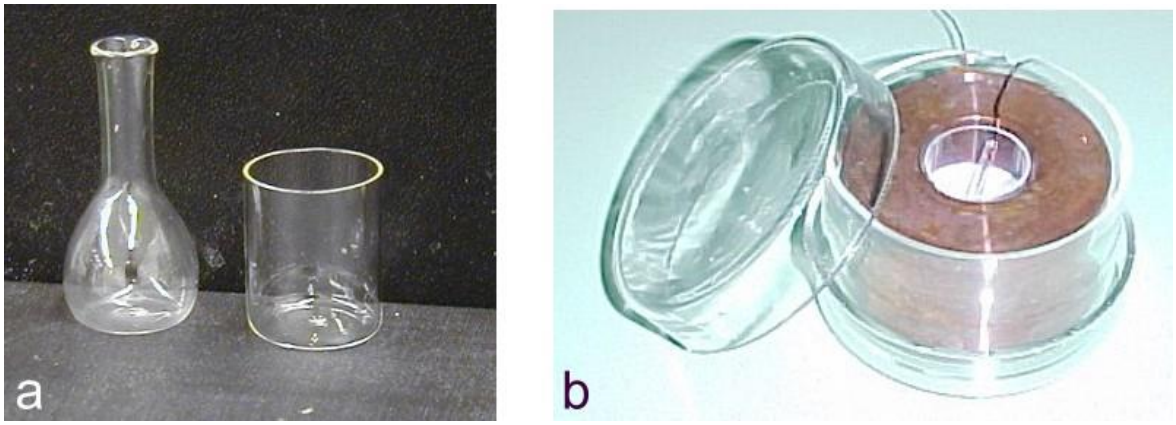


Fig. 3: a) Handmade glass vessels used as the reservoir and the weighing vessel for the liquid. b) Glass vessel with a copper ring for thermal stabilization of the liquid reservoir. Inside the glass vessel: the thermistor used to determine the temperature.

- Vessels for the liquid

  Very small vessels were chosen to receive the liquid. In the case of the reservoir for the liquid to be dispensed, this was done in view of the investigations on human serum, and, in the case of the weighing vessel, due to the low maximum capacity of the weighing instrument. The glass vessels are handmade; they have an empty mass between 1.5 g and 2.5 g and a capacity from 3 ml to 5 ml for liquids. Different shapes of vessels have been tested. Two of these are shown in Fig. 3a.

  The small vessels react very quickly to changes in the ambient temperature, which are unavoidable due to the presence of the experimenter. To stabilize the reservoir with regard to such temperature variations, it was placed in a massive copper ring (mass: 283 g). This copper ring and the reservoir are, in turn, placed in a glass vessel with a glass lid for additional thermal insulation from the environment. This arrangement is shown in Fig. 3b. In addition, the glass lid reduces the evaporation of liquid from the reservoir. This prevents the liquid from cooling down due to evaporation. In the case of liquid mixtures (in the present investigation, human serum is considered as such), this arrangement prevents the volatile components from evaporating – and thus the concentration of the least volatile components from increasing.

  Figure 3b also shows the thermistor used to measure the temperature of the liquid. This sensor allows the temperature of the liquid to be checked continuously. The thermistor has a power of approx. 1 µW; warming up of the liquid due to the thermistor is negligible.

4.3 Description of the dispensing process

The liquid is dispensed on the basis of the procedure described in Part 6 of the ISO 8655 standard [8].

The reserve liquid is located in the reservoir inside the copper ring (Fig. 3b) described in Section 4.2. The liquid is retrieved from the reservoir by aspiration using the piston-operated pipette or the microlitre syringe. The tip of the device is then wiped off against the wall of the reservoir. The length of this wiping motion is approx. 10 mm; the tip is touched to the wall at an angle of approx. 30°. The filled piston-operated pipette or microlitre syringe is then introduced into the weighing vessel through the opening in the cover of the weighing chamber where it is applied to the wall of the vessel, directly above the surface of the liquid. The liquid is then expelled via the delivery mechanism, and the tip of the volumetric apparatus is then wiped off against the wall of the vessel over a length of approx. 10 mm, with its tip at an angle of approx. 30°.

Prior to the first measurement, the weighing vessel is filled with approx. 0.5 ml to 1 ml of liquid. Subsequent doses may only be added as long as the wall of the weighing vessel that is not covered by liquid is at least 10 mm to allow the tip of the volumetric apparatus to be wiped off. The opening in the cover of the weighing chamber must be closed by means of a lid after each dispensing operation. This lid may only be removed again immediately before the next measurement.

Contrary to the procedure described in Part 6 of the ISO 8655 standard [7], which prescribes 10 individual measurements, a measurement series here consists of 15 individual doses. The individual dispensing operations are carried out at intervals of 40 s each. The total measurement time amounts to 660 s. This value slightly exceeds 600 s (resulting from 15 times 40 s) as the measurement time prior to and following the actual dispensing operation must also be taken into account. During this total measuring time, the balance reading is recorded every 0.4 s. A measurement curve "weighing value as a function of time" is generated with measurement points at 0.4 s intervals. This measurement curve is staircase-shaped and has 15 steps. This is shown in Fig. 4 based on the example of a dispensing series in which wiping off the tip has been dispensed with for more clarity.

4.4 Assessing the measurement curves

After the measurement, the measurement curve "weighing value as a function of time" is assessed. This assessment aims to determine the height of the individual steps which is a measure of the weighing value of the respective liquid dispensed.

The steps are, however, not ideal square steps, since the weighing instrument needs a finite step response time before stabilizing to the new value after a new dose has been added. The step response time increases due to the fact that wiping off the tip against the wall of the weighing vessel at the end of the dispensing operation exerts a force onto the balance, hereby applying a strong weight for a short time. The balance signal therefore considerably fluctuates and even reaches the overload range during the dispensing operation. The stabilizing process thus takes longer due to the necessary recovery time. The mechanical strain applied to the weighing instrument due to the tip of the apparatus touching the wall of the weighing vessel is not well reproducible due to the fact that this operation is carried out manually, so that the step response time also varies. To better illustrate this effect, Fig. 4 shows a measurement curve in which the tip has **not** been wiped off during the dispensing operation – i.e. the dispensing has **not** taken place the usual way – whereas Fig. 5 shows a measurement curve in which the tip was wiped off during the dispensing operation. Whereas the measurement series without wiping off the tip exhibits only short stabilizing processes, which lead to a rounding of the steps, wiping off the tip causes the extreme balance deflections described above. The recovery time of the balance is therefore considerably longer.

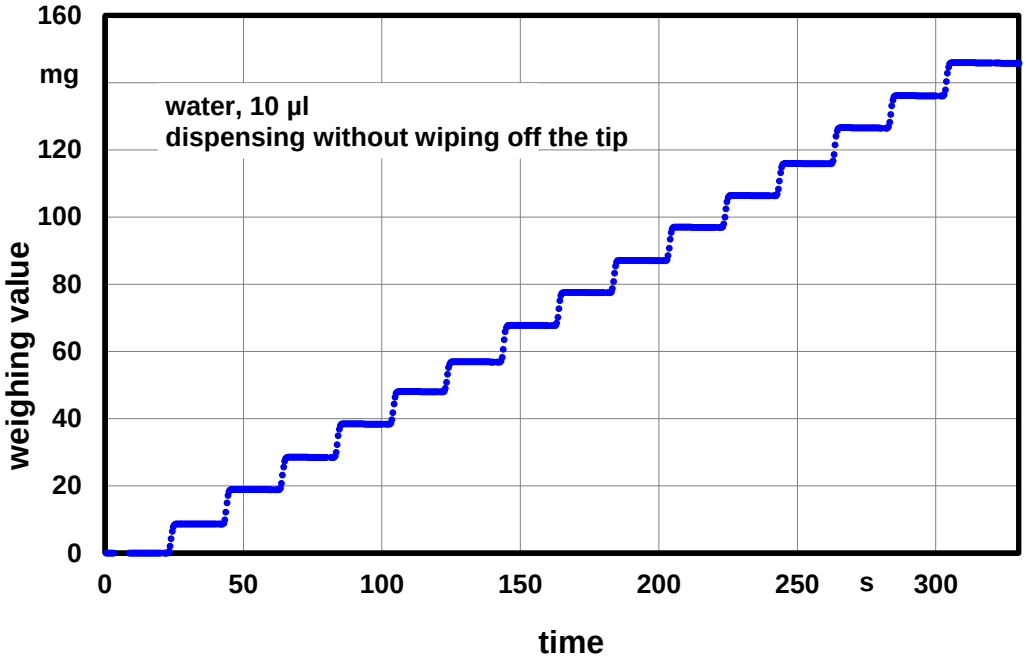


Fig. 4: Weighing value as a function of time for measurements including 15 dispensing operations of 10 µl water each, **without** wiping off the tip.
The time interval between the individual dispensing operations is reduced to 20 s. Each interval between the measurement points amounts to 0.4 s.

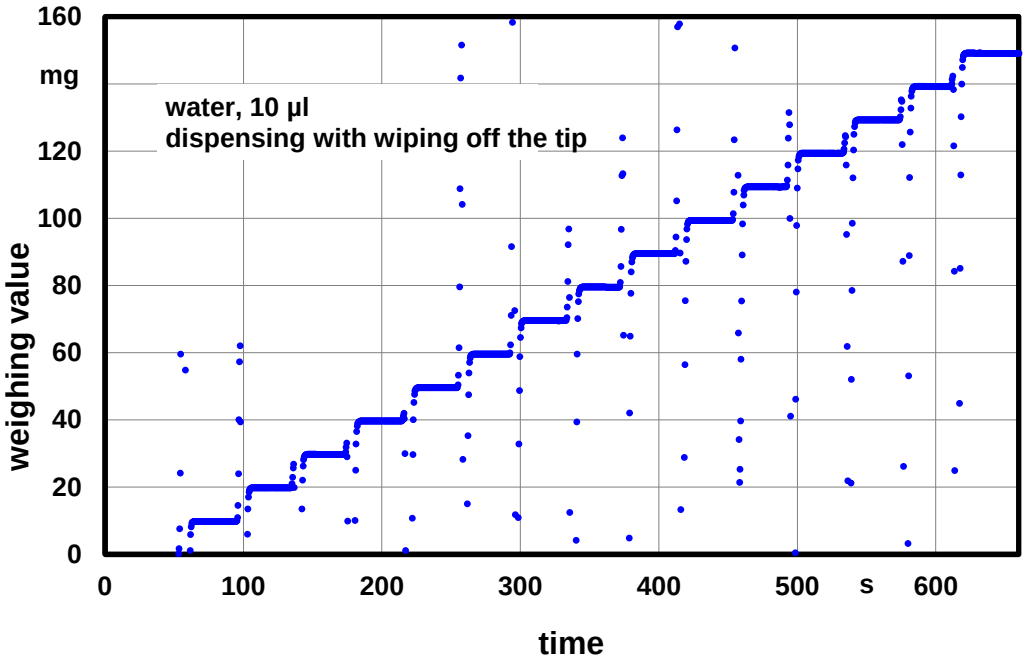


Fig. 5: Weighing value as a function of time for measurements including 15 dispensing operations of 10 µl water each, **wiping off** the tip.
The time interval between the individual dispensing operations amounts to 40 s. Each interval between the measurement points amounts to 0.4 s. The scattering of the measured values immediately after each dispensing operation is explained in the text.

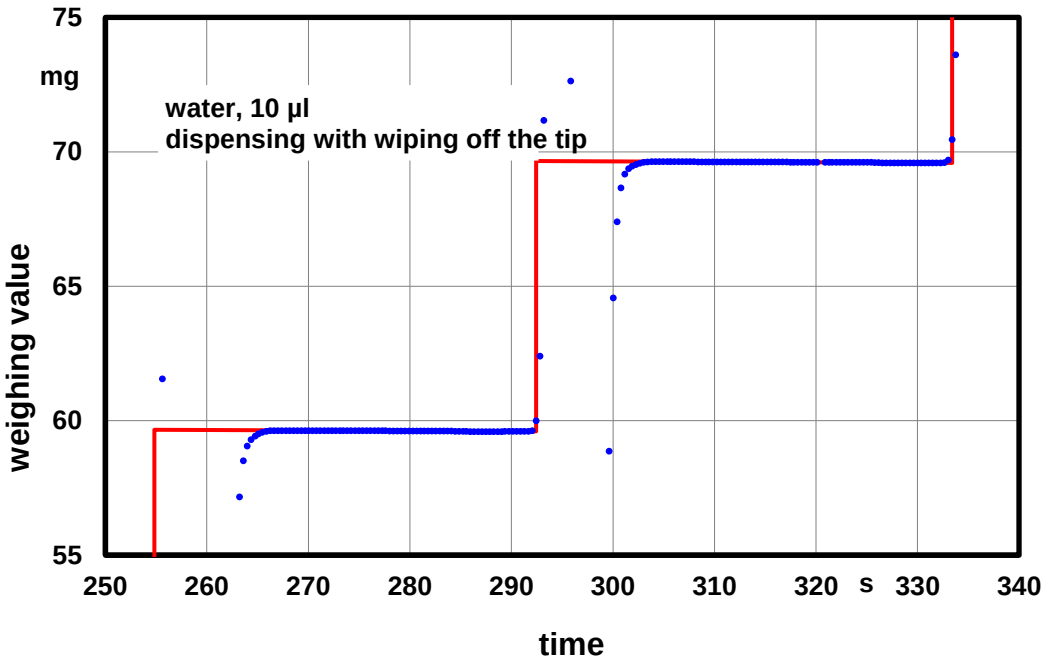


Fig. 6: Section of Fig. 5 with straight fitting lines. The height of the vertical sections is a measure of the weighing value of the dispensed liquid. The recovery time of the balance across which extrapolation must take place is clearly to be seen.

Since the mass of the dispensed liquid has decreased during this recovery time due to evaporation, which cannot be fully avoided, the weighing value must be extrapolated to the moment of the dispensing.

Figure 6 shows a graphic representation of an extrapolation. Extrapolation is performed by adjusting straight lines to the weighing values in the ranges with a stable readout below and above the time of dispensing. These straight lines are then prolonged up to the time of dispensing, and the difference between the straight lines at the time of dispensing correspond to the step height we are looking for. This step height reflects the weighing value of the dispensed liquid. The time of dispensing is defined as the beginning of the dispensing process, i.e. the application of the dispensing device to the wall of the weighing vessel. The dispensing and subsequent wiping-off take approx. 2 s to 3 s. Both straight lines are, however, nearly parallel; a change in the step height due to the shifting of the "time of dispensing" by 2 s, i.e. by the duration of the dispensing process, may therefore be neglected. Section 5.1 explains in more detail why it is admissible to use a linear extrapolation for simplification.

5 Fundamental investigations on evaporation

Dispensing liquids by means of piston-operated pipettes and microlitre syringes always implies open liquid surfaces. Such open liquid surfaces are prone to evaporation. This process influences and limits the measurement accuracy of dispensing in three different ways.

- Due to evaporation, the mass of the liquid in the weighing vessel decreases.
- The cooling caused by evaporation reduces the temperature of the reservoir, the weighing vessel and the dispensing device.
- The evaporated liquid remains in its gaseous form in the air above the liquid surface and thus has an influence on the density of the air which, in turn, enters in the air buoyancy correction.

5.1 Evaporation from weighing vessels

Due to the evaporation of liquid, the mass of the liquid present in the weighing vessel decreases. The readout indicated by the weighing instrument is therefore not stable: the indication drifts; the indicated values must be extrapolated back to the time of dispensing.

The evaporation of liquid may be reduced by increasing the liquid vapour fraction in the surrounding air. This is realized by means of a so-called evaporation trap (as described below). Another possibility to reduce evaporation is to select a suitable weighing vessel.

Evaporation was reduced by placing a circular vessel filled with liquid in the weighing chamber – an evaporation trap – which artificially increases the liquid vapour concentration of the air in the weighing chamber. When using water, it is possible to increase the relative humidity in the weighing chamber to reach 70 % to 85 % by enriching the air with water vapour. Stronger saturation is not possible since the weighing chamber is not hermetically closed and must additionally be opened for each new dispensing operation. When using other dispensing liquids, the evaporation trap must be correspondingly filled with this liquid. As a rule, saturation cannot be attained in this case either (the saturation degree can be determined by means of a simple measurement only when using water), but a clear decrease in the evaporation rate is observed.

The importance of an evaporation trap when using water can be demonstrated by measuring the evaporation rates at different relative humidity levels in the weighing chamber. The values listed in the following were measured on a beaker. When the weighing chamber is closed but no evaporation trap is used, the evaporation rate during the dispensing amounts to $38 \cdot 10^{-7}$ g/s. When an evaporation trap is used and the relative humidity thus obtained reaches approx. 90 %, the evaporation rate decreases down to $6 \cdot 10^{-7}$ g/s. When the lid of the weighing chamber (diameter: approx. 10 mm) is opened to introduce a pipette, the evaporation rate increases to reach $14 \cdot 10^{-7}$ g/s. When the lid of the weighing chamber is fully removed, this value even reaches $46 \cdot 10^{-7}$ g/s and is thus even higher than when operating the device without an evaporation trap, but keeping the weighing chamber closed. This is caused by the strong increase in convection.

The safest measure to reduce evaporation out of the weighing vessel is to close it by means of a lid. This lid, however, must be opened for each dispensing operation and then closed again. This implies manipulations in the weighing chamber and at the weighing vessel, which generates air convection and temperature variations that may affect the weighing result. Longer waiting times that are complied with in order to ensure stable conditions in the weighing chamber allow a larger zero drift of the weighing instrument but can thus also adulterate the weighing result. Furthermore, these waiting times are in contradiction with the requirement defined in the ISO 8655-6 standard stating that dispensing operations should take place at intervals of less than 60 s.

Removing the lid of the weighing vessel from the weighing platform during the dispensing operation also has a similar effect. In the case of very small volumes of liquid, such adulterations may no longer be neglected. The investigations described here were therefore conducted with an open weighing vessel that was firmly attached to the weighing platform.

But also the shape of the weighing vessel has an influence on the evaporation rate. Vessels with a smaller opening exhibit lower evaporation rates than vessels with larger openings – this was already described by Michel et al. [4] for the case in which the vessels were at equilibrium. In the case of a long-necked flask, the expected evaporation rate is smaller than in a beaker.

The present investigation first checked whether the behaviour described by Michel et al. [4] still applies if the vessels are not at equilibrium, as it is the case during a dispensing operation due to the fact that the weighing chamber has to be opened and a dispensing device introduced.

For this purpose, the evaporation of water was measured in a long-necked flask and in a beaker that are depicted in Fig. 3a.

The evaporation rate measured at equilibrium in the long-necked flask was $5 \cdot 10^{-8}$ g/s; in the beaker, this value amounted to $6 \cdot 10^{-7}$ g/s – a value 12 times as high. Equilibrium conditions were obtained by keeping the weighing chamber shut for at least 20 min. For these measurements, the air in the weighing chamber was nearly saturated with humidity (relative humidity: approx. 90 %). Dispensing processes disturb this state of equilibrium, since the weighing chamber has to be opened in order to introduce the pipette or the syringe into the weighing vessel. Convection occurs due to both removing the lid from the weighing chamber and by moving the pipette inside the weighing chamber. This influence is particularly pronounced in a long-necked flask. In the case of pipette tips whose diameter is only a little smaller than the inner diameter of the flask's neck, nearly all the air is expelled from the neck of the flask when introducing the pipette and then replaced by air with a lower saturation rate when removing the tip of the pipette. The evaporation rate therefore increases considerably. This effect is more pronounced when using a long-necked flask than when using a beaker. The latter having a wider opening, only a small fraction of the air present in the upper part of the weighing vessel is replaced. The measurement results confirm this. The evaporation rates measured after simulated dispensing operations increased to the same value for both vessels: $7 \cdot 10^{-7}$ g/s. The sequences of a dispensing process were merely simulated without actually injecting liquid in order to prevent the dispensed liquid from influencing the result.

For the dispensing itself, both vessels are thus equivalent, although the weighing beaker has an opening approx. 10 times as large as that of the long-necked flask. A weighing beaker should be preferred to a long-necked flask because the disturbance of the state of equilibrium caused by the dispensing process is considerably smaller, so that a much more reliable measurement can be expected.

Due to this result, a weighing beaker was used for the following investigations. In addition, the large opening of the weighing beaker makes it easier to introduce pipettes and to wipe them off against the wall of the vessel by holding them at an angle.

Besides these technical parameters, the evaporation rate also depends on the temperature and the vapour pressure of the liquid. Thus, the evaporation rate amounts to $2 \cdot 10^{-5}$ g/s for ethanol (vapour pressure: 52 mbar) and $2 \cdot 10^{-6}$ g/s for nonane (vapour pressure: 5 mbar). The

temperature during the dispensing operation was t = 20 °C for both measured values. The dispensing operation was conducted in a weighing beaker without an evaporation trap; the weighing chamber was closed immediately after the dispensing operation.

The evaporation rate is enhanced by yet another effect. The dispensing operation requires the tip of the dispensing device to be wiped off against the wall of the weighing vessel. This creates additional liquid surface. The evaporation rate is therefore higher than at equilibrium as long as liquid is present on the wall of the vessel above the liquid previously filled in.

The increase in the liquid surface is influenced by the surface tension and the interface tension of the liquid, by the shape of the dispensing device's tip, and by the volume of liquid expelled. It must therefore be expected that the evaporation rate differs not only when different liquids, but also different devices and different volumes are involved. Smaller volumes and smaller tip diameters lead to a smaller wet surface on the vessel's walls and thus to a smaller increase in the evaporation rate. The increase in the liquid surface is between 10 % and 50 % of the surface area (of the cross-sectional area of the weighing beaker).

To emphasize the effect of an increased evaporation rate, this effect was exaggerated for one of the measurements. Ethanol was selected as a liquid since the evaporation rate is particularly high due to its high vapour pressure. The liquid was injected against the wall of the vessel during the dispensing operation in order to achieve a particularly high increase in the liquid surface. The result of this demonstration measurement is shown in Fig. 7. The decrease in the weighing value when the liquid is dispensed directly into the liquid already available in the beaker without wetting the vessel's wall – i.e. when the liquid surface is not increased (red dots and red straight line) – is compared to the decrease in the weighing value when the liquid is injected against the vessel's wall during the dispensing process (blue dots and blue straight line).

To enable direct comparison, the two measurement curves are shifted in such a way that they overlap towards the end of the measurement. The blue curve indicates a stronger gradient (i.e. a larger evaporation rate) at the beginning of the measurement; however, it joins the slope of the red curve (which represents dispensing without increasing the surface of the liquid). The evaporation rate at the beginning is approximately twice as large as after 100 s. From that point on, both curves have the same slope[2].

Linearization of the curve, as already shown in Fig. 6 is, however, possible with sufficient

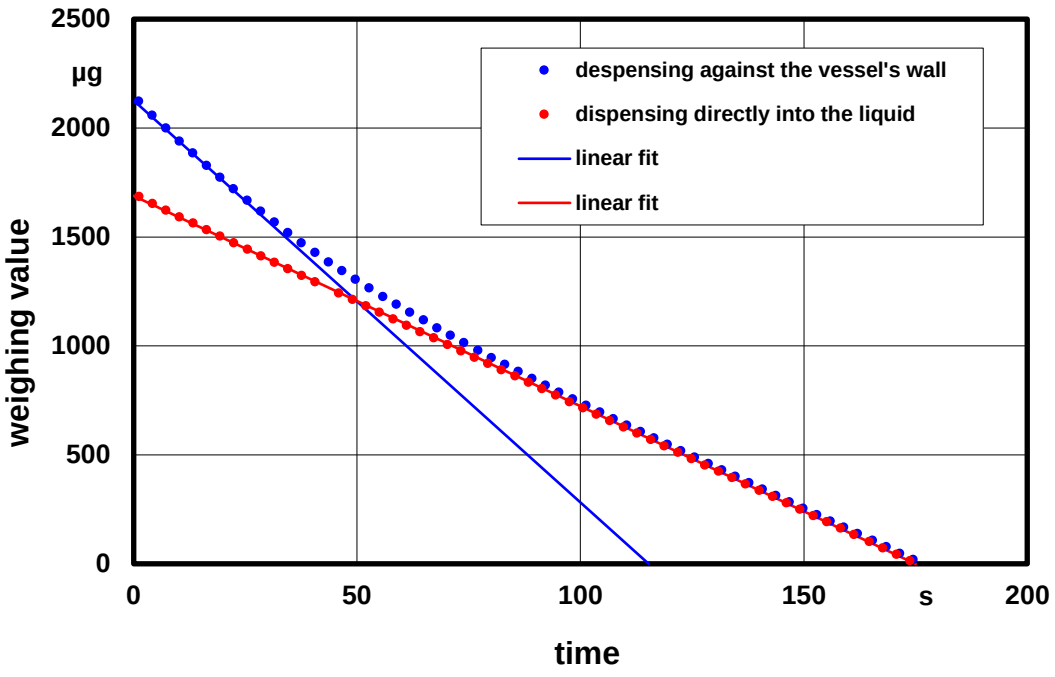


Fig. 7: Evaporation of ethanol
The results shown in the figure are those of the comparison between the decrease in the weighing value when dispensing directly into an existing volume of liquid (without increasing the liquid surface) and the same decrease when injecting the liquid against the vessel's wall (thereby increasing the liquid surface).

accuracy for small time values, so that it is also possible to extrapolate the measurement values to the time of dispensing[3]. This is shown by the blue straight line in Fig. 7. Since the increase in the liquid surface is much lower in real measurements than in this demonstration

[2] The measurements to determine the volume were carried out at 40 s intervals; the state of equilibrium for evaporation is therefore not reached during the measurement.
[3] Direct measurement at the moment of sampling is not possible due to the step response time of the weighing instrument (cf. Section 4.4).

measurement, linear extrapolation is more reliable for such measurements than in the example shown here.

5.2 Influence of evaporation on the temperature of the liquid in the reservoir and on the dispensing device

One of the quantities that has to be kept constant during a measurement is the temperature of the components involved. These are the liquid in the reservoir, the dispensing device, and the liquid in the weighing vessel. Due to thermal expansion, a temperature change leads to changes in the density/volume of the liquid and thus alters the measurement results.

The measurements are carried out at room temperature. The components mentioned are therefore first stored in the laboratory for long enough (at least one hour) for them to reach room temperature. However, all three of these components have liquid surfaces that are in contact with the ambient air so that they are subject to evaporation. This evaporation process causes the components to cool down. Evaporation must therefore be kept as small as possible.

5.2.1 Liquid in the reservoir

In the case of the liquid in the reservoir, evaporation is reduced by covering the container enclosing the liquid with a lid. This lid is only opened to remove liquid from the container. Without coupling to the copper ring, slight cooling by 0.2 K to 0.4 K would still occur due to the remaining evaporation. The warmth of the operator's hands transmitted when opening and closing the lid and when filling the pipette suffices to counteract this. This warming is in the order of 1 K to 2 K, which is sufficient to warm up the liquid in the reservoir. Both effects are considerably reduced due to coupling to the copper ring (cf. Section 4.2). However, temperature drifts during a measurement that takes approx. 11 min cannot be fully precluded. A limit value of max. 0.2 K for this warming was accepted within the scope of these investigations. If this value was exceeded, the measurement in question was rejected. For simplification purposes, the temperature of the liquid in the reservoir was only recorded at the beginning and after completion of a measurement. Each of the 15 individual dispensing operations of which one measurement consists can be assigned a temperature by means of linear interpolation between the temperature at the beginning and the temperature at the end of the measurement. Since it

cannot be proven by means of measurement results that the temperature drift is constant over time between the temperature at the beginning and the temperature at the end of the measurement, the maximum deviation of the liquid's temperature from the interpolated value at the moment of dispensing is assumed to be 0.1 K.

5.2.2 Liquid in the weighing vessel

The liquid in the weighing vessel also cools down due to evaporation. This effect, however, influences the determination of the dispensed volume only indirectly. The change in temperature of the liquid in the weighing vessel does not influence its mass, only its density, so that the air buoyancy and the air buoyancy correction change[4]. Related to the weighing value, this effect is in the order of 10^{-6} and is thus negligibly small.

The temperature of the weighing chamber also changes due to the cooling of the weighing vessel. It is further amplified by the cooling caused by evaporation in the evaporation trap. The temperature in the weighing chamber is therefore measured directly to be able to detect the influence of these temperature changes on the air buoyancy correction. The temperature change in the weighing chamber due to the effects described above amounts to a few 0.1 K.

[4] This is true regardless of the fact that the reason for the temperature change is obviously evaporation, which leads to a decrease in mass.

5.2.3 Dispensing device

Due to a sufficiently long tempering time prior to the measurement, the dispensing device is also at room temperature. The influence of the warmth of the operator's hands during use is reduced by means of structural means provided by the manufacturer (insulating plastic parts, large spacing between the part held by the operator and the temperature-critical parts) and by means of measures implemented by the operator (putting down the device between individual dispensing operations).

Unavoidable cooling occurs at the tip of the dispensing device. The tip of the dispensing device is plunged into the liquid for filling. Despite wiping off the tip, a thin film of liquid remains on the outside of the tip when removing the tip from the liquid. This thin film may also evaporate. In addition, a free liquid surface is present at the tip opening where evaporation may also occur. After expelling the liquid, the inside of the tip is also coated with a thin film of liquid. Due to the low thermal capacity of the tips, this evaporation leads to considerable cooling.

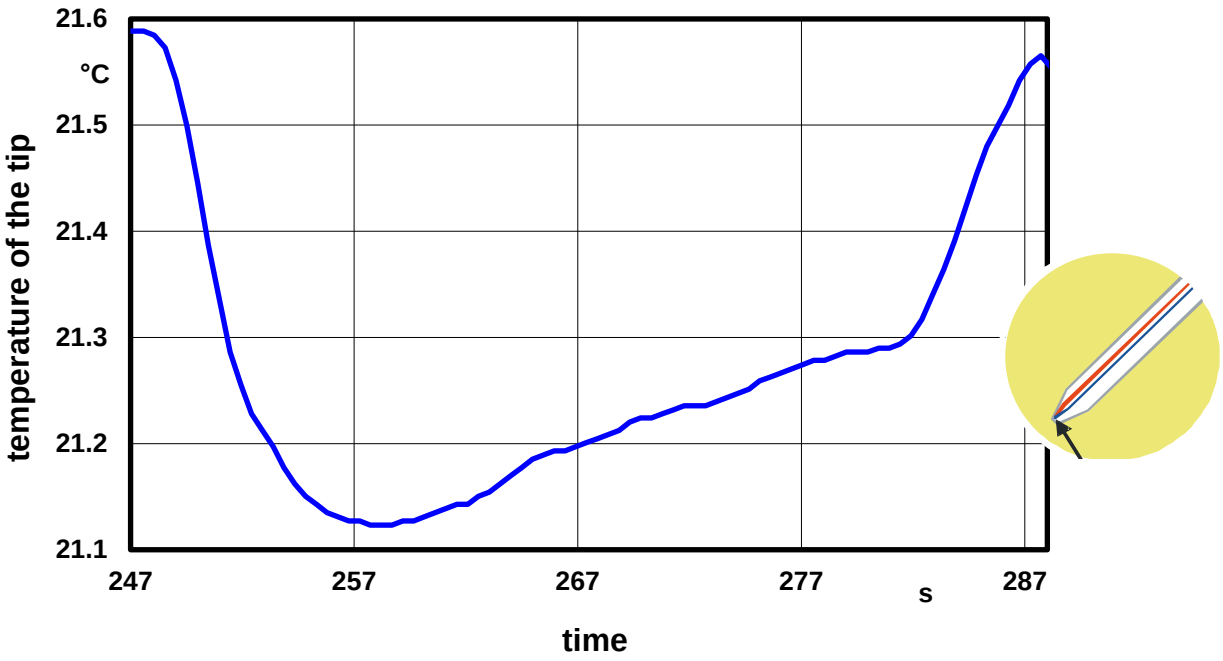


Fig. 8: Temperature behaviour of a pipette tip after filling it with water and after expelling the liquid.
The insert on the right is a schematic description of the arrangement of the temperature sensor in the pipette tip.

To measure the extent of this influence, the temperature of a pipette tip was determined by means of a copper-constantan thermocouple. The pipette used for the test was a positive displacement pipette with a nominal volume of 100 µl. The thermocouple was introduced into the tip of the pipette at the bearing point in such a way that the copper-constantan contact point within the tip was located near the outlet (see schematical representation in Fig. 8). For this purpose, the piston had to be removed. Due to the thermocouple wire, the connection between the pipette and the tip was not air-tight. In such a configuration, the pipette must not be used for dispensing purposes, but it can be filled like a real system and empties automatically due to lacking air-tightness. Several temperature evolution sequences were recorded in this constellation.

Figure 8 shows an example of the temperature evolution when filling and then emptying the pipette. At the beginning of the measurement, the thermocouple indicates the temperature of the liquid from the reservoir into which the tip is plunged for filling. After dispensing the liquid from the reservoir and after the liquid has run out of the pipette tip, the temperature considerably decreases within a few seconds. This drop in temperature amounts to approx. 0.5 K when the liquid used is water. After the liquid film having caused the cooling down has evaporated, the temperature in the tip slowly increases again. The case shown as an example is a simulation of a real dispensing cycle, which is why the tip was plunged into the liquid reservoir anew after 35 s. The temperature stabilizes to the temperature of the liquid reservoir within 4 s to 5 s.

The cooling process of the pipette tip shows good reproducibility. Figure 9 shows the temperature behaviour of a pipette tip during a full measuring cycle consisting of 14 individual dispensing operations. The figure shows the fast cooling by approx. 0.5 K and the slow warming up of the pipette tip when it is in contact with the surrounding air as well as the subsequent fast warming up when it is plunged into the reservoir of liquid again. The differences in the temperature cycle are caused by the different quantities of liquid remaining on the tip after it has been wiped off. Some temperature drops exhibit a slightly different temporal evolution and a somewhat larger temperature difference. This is particularly pronounced at the measuring time of 380 s. These deviations are possibly due to small water droplets that were not fully wiped off.

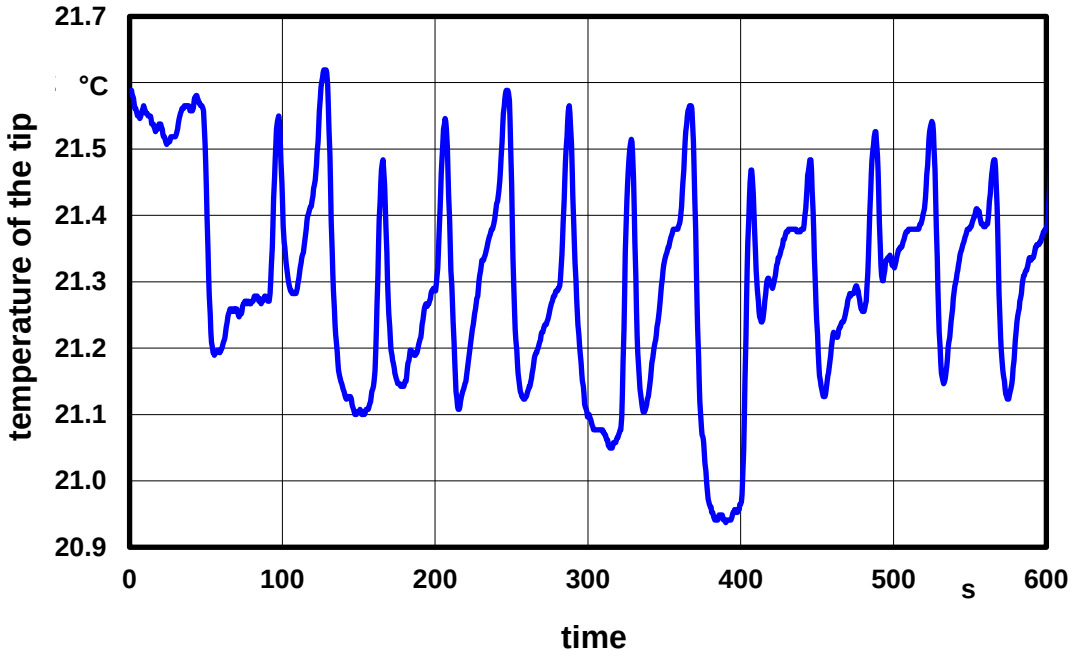


Fig. 9: Temperature behaviour of a pipette tip after filling it with water and after expelling the liquid; cycle consisting of 14 individual dispensing operations.

When using liquids other than water, the cooling due to evaporation may be considerably greater. The result shown in Fig. 10 is comparable to that shown in Fig. 9, where ethanol was used instead of water. Here, the temperature behaviour of the pipette tip is, as a rule, similar to that observed when using water. The individual sections that are characterized by fast cooling, slow warming up and then fast warming up are, however, not quite as pronounced. Due to the higher evaporation rate of ethanol, the temperature drop amounts to 5 K – i.e. it is approx. 10 times as high as when using water.

The time constants for the cooling and warming up as well as the extent of the cooling obviously depend on the thermal capacity of the pipette tip used. The values shown here are therefore merely guidance values. Based on the values mentioned, a pipette tip used several times should be plunged into the liquid reservoir for several seconds at a time for its temperature to align to that of the liquid. This particularly applies to the tips of air displacement pipettes which usually have to be humidified by dispensing and expelling liquid once prior to the actual dispensing operation.

Yet another problem occurs when using air displacement pipettes. When the tip cools down, the air interface also cools down, at least in the lower part of the tip. After plunging it into the liquid reservoir anew, the undercooled air interface is decoupled from the outer air due to the liquid. During the next warming up, the pressure in the air interface increases. Because the air interface is decoupled from the ambient air due to the liquid, its pressure cannot be equalized with that of the ambient air. Consequently, too little liquid is sucked into the pipette at the next dispensing. This effect occurs simultaneously with the increase in the air interface volume due to the absorption of liquid vapour described by Lochner et al. [5], and it is difficult to separate the two. This question could not be investigated within the scope of the tests described in this paper. Changing the prior humidification procedure could be a solution, namely by sucking air saturated with vapour rather than liquid into the pipette tip.

5.3 Change in the air density due to evaporating liquid

The third influence that evaporation has on the results merely regards measurements performed using the gravimetric method and their assessment.

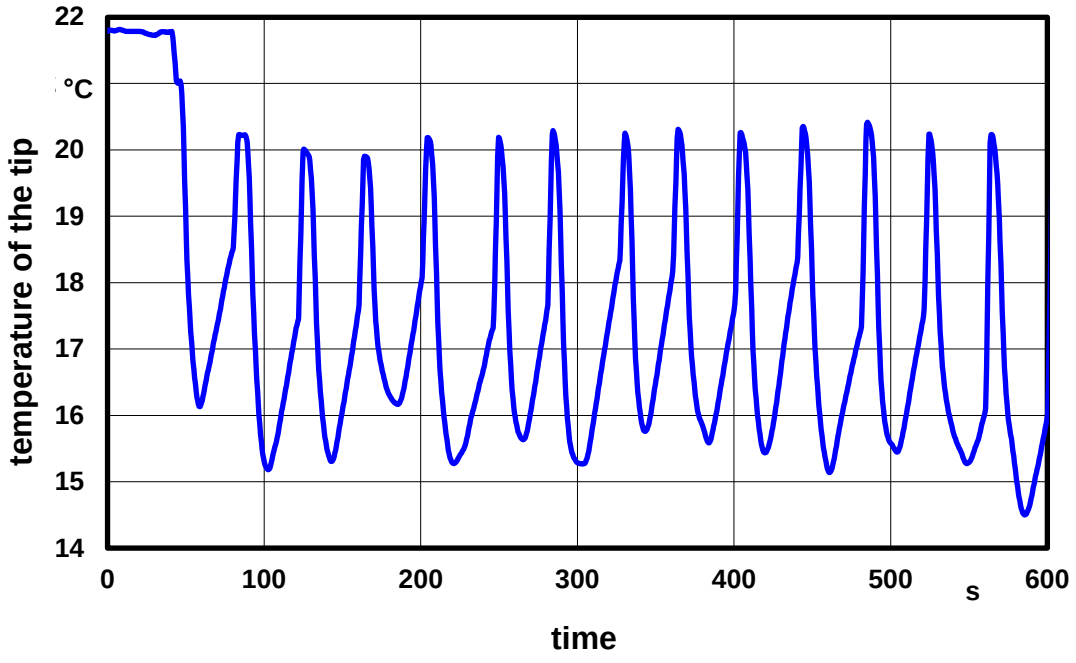


Fig. 10: Temperature behaviour of a pipette tip after filling it and expelling the liquid (using ethanol); cycle consisting of 14 individual dispensing operations.

The evaporating liquid changes the liquid vapour concentration of the air in the weighing chamber. This influences the density – and thus the buoyancy – of the air. Even with a constant mass, the balance reading will change (cf. Eq. (4-2)).

To be able to perform the duly adapted air buoyancy correction, it is necessary to know the changed air density. As explained in detail in Section 4.1, the influence of humidity on the air buoyancy correction is negligible. The following estimation aims to show that this also applies to other liquids than water.

For this purpose, we will consider a gas mixture consisting of several types of gases. The pressure, volume, temperature and number of gas molecules are assumed to be constant. The gas molecules may not interfere with each other. Thus, the system can be described by means of the general equation of state.

(5-1) $$pV = Nk_{\mathrm{B}}T$$

The mass of the gas in the volume V is yielded by

(5-2) $$m = \sum_{\mathrm{i}} N_{\mathrm{i}} M_{\mathrm{i}} \quad , N = \sum_{\mathrm{i}} N_{\mathrm{i}} = \mathrm{const}$$

where

p is the pressure;
V is the volume;
N is the number of particles (gas molecules);
k_{B} is the Boltzmann constant;
T is the absolute temperature;
m is the mass;
N_{i} is the number of type-i particles, and
M_{i} is the mass of a type-i particle.

(4-1), (5-1), and (5-2) allow the following formula to be stated for the density of the gas:

(5-3) $$\rho = \frac{m}{V} = \frac{p}{k_{\mathrm{B}}T} \sum_{\mathrm{i}} \frac{N_{\mathrm{i}} M_{\mathrm{i}}}{N} = \frac{p}{k_{\mathrm{B}}T} \sum_{\mathrm{i}} \frac{p_{\mathrm{i}} M_{\mathrm{i}}}{p} = \frac{1}{k_{\mathrm{B}}T} \sum_{\mathrm{i}} p_{\mathrm{i}} M_{\mathrm{i}}$$

where p_{i} is the partial pressure of the type-i gas, $p = \sum_i p_i = \mathrm{const}$, $\frac{p_i}{p} = \frac{N_i}{N}$.

The density of a gas mixture is thus only determined by the sum of the products of the partial pressures and of the molecular masses if the pressure, the volume and the temperature are constant. This relation becomes even more simple when considering the ratio of the densities of two gas mixtures.

(5-4) $$\frac{\rho_{\mathrm{gas1}}}{\rho_{\mathrm{gas2}}} = \frac{\sum_{\mathrm{i}} p_{\mathrm{i},1} M_{\mathrm{i},1}}{\sum_{\mathrm{j}} p_{\mathrm{j},1} M_{\mathrm{j},1}}$$

In particular when comparing the density of air saturated with water vapour to that of air that is free of water vapour (called *dry air* in the following), the following applies:

(5-5) $$\rho_{\mathrm{rel}} \equiv \frac{\rho_{\mathrm{air,saturated}}}{\rho_{\mathrm{air,dry}}} = \frac{p_{\mathrm{vapour}} M_{\mathrm{vapour}} + p_{\mathrm{air}} M_{\mathrm{air}}}{p_{\mathrm{air,dry}} M_{\mathrm{air}}}$$

and

(5-6) $$p_{\mathrm{air,dry}} = p_{\mathrm{vapour}} + p_{\mathrm{air}} = const$$

For the subsequent consideration, the molar masses of the gases will be used[5]:

M_{air} = 28.8 g (mixture of 80 % nitrogen and 20 % oxygen)

M_{water} = 16 g

M_{ethanol} = 60 g

M_{nonane} = 128 g

$p_{\mathrm{air,\ dry}}$(20°C) = 1013 hPa (normal pressure)

$p_{\mathrm{vapour,\ water}}$(20°C) = 23 hPa (vapour pressure at 20 °C)

$p_{\mathrm{vapour,\ ethanol}}$(20°C) = 51 hPa (vapour pressure at 20 °C)

$p_{\mathrm{vapour,\ nonane}}$(20°C) = 4.8 hPa (vapour pressure at 20 °C)

According to (4-3), the density of dry air under normal conditions (1013 hPa, 20 °C) is 1.204 kg/m^3.

[5] Since relative quantities are determined, the units may be chosen arbitrarily.

Comparing air saturated with water vapour to dry air:

$$\rho_{rel}(\text{water}, 20°C) = \frac{990\ \text{hPa} \cdot 28.8\ \text{g} + 23\ \text{hPa} \cdot 16\ \text{g}}{1013\ \text{hPa} \cdot 28.8\ \text{g}} = 0.9899$$

Comparing air saturated with ethanol vapour to dry air:

$$\rho_{rel}(\text{ethanol}, 20°C) = \frac{962\ \text{hPa} \cdot 28.8\ \text{g} + 51\ \text{hPa} \cdot 60\ \text{g}}{1013\ \text{hPa} \cdot 28.8\ \text{g}} = 1.0545$$

Comparing air saturated with nonane vapour to dry air:

$$\rho_{rel}(\text{nonane}, 20°C) = \frac{1008.2\ \text{hPa} \cdot 28.8\ \text{g} + 4.8\ \text{hPa} \cdot 128\ \text{g}}{1013\ \text{hPa} \cdot 28.8\ \text{g}} = 1.0163$$

The density of air saturated with water vapour is lower than that of dry air! The reason for this is the lower molar mass of water compared to that of nitrogen and oxygen.

The density of air saturated with ethanol vapour is higher by 5.5 % than that of dry air. The density of air saturated with nonane vapour is higher by 1.6 % than that of dry air!

Inserting into Equation (4-2)for the air buoyancy correction yields the following values:

Water in dry air: $m/W = 1.001057$

Water in air saturated with water vapour: $m/W = 1.001046$

Ethanol in dry air: $m/W = 1.001377$

Ethanol in air saturated with ethanol vapour: $m/W = 1.001452$

Nonane in dry air: $m/W = 1.001529$

Nonane in air saturated with nonane vapour: $m/W = 1.001554$

The respective differences between the air buoyancy corrections in "dry" air and in air saturated with the respective vapour amount to[6]:

Deviation in the case of water: $-1.1 \cdot 10^{-5}$

Deviation in the case of ethanol: $+7.5 \cdot 10^{-5}$

[6] Absolute and relative differences are the same in this case, since the values are very close to 1.

Deviation in the case of nonane: $+2.5 \cdot 10^{-5}$

Since full saturation of air in the weighing chamber cannot be achieved in practice (from experience with water, a saturation value of 70 % can be assumed), the actual deviation is correspondingly lower.

Based on these estimations, neglecting the change in density of air due to evaporating liquid may therefore lead to relative deviations in the order of $5 \cdot 10^{-5}$. These deviations are tolerable; as contributions to the measurement uncertainty, they must, however, be included in the measurement uncertainty budget.

6 Measurement uncertainty budget for the measurement of a volume of liquid with the gravimetric measuring device

When drawing up a measurement uncertainty budget, it is necessary to differentiate between the measurement uncertainty that is associated with the gravimetric determination of a volume of liquid located on the balance and the measurement uncertainty that is associated with the dispensing of this volume of liquid by the dispensing device. When setting up a measuring device, the aim is to keep the uncertainty of the volume determination using this device clearly lower than would be the case when dispensing the same volume with a dispensing device. The measurement uncertainty of the standard measuring device set up should therefore not exceed one tenth of the maximum permissible errors indicated in the standards.

The measurement uncertainty budget comprises uncertainty contributions that are associated with the weighing operation, with the determination of the air buoyancy correction and with the determination of the density of the liquid. The uncertainty contribution associated with the weighing operation can be found in the calibration certificate of the weighing instrument (if available). To facilitate comparison between the individual uncertainty contributions, all individual contributions to the measurement uncertainty are listed here.

The measurement uncertainty associated with the weighing operation consists of contributions due to the readability (smallest digitizing step), reproducibility, nonlinearity and sensitivity drift. Modern weighing instruments perform self-calibration that traces the weighing result to integrated calibration weights. This also compensates for influences due to the place of installation of the weighing instrument (location-dependent gravity) and for influences due to the ambient temperature (here, as uncertainty contribution remains the drift of the ambient temperature during the measurement). The measurement uncertainty budget includes the calibration uncertainty of the mass of the above-mentioned calibration weights as an uncertainty contribution.

A weighing operation always consists in weighing the weighing vessel before and after the dispensing of the liquid to be measured (tare weighing and gross weighing), i.e. it consists of two weighing steps. The weighing result is the difference between the weighing value of those two weighing steps. This is also true if the tare weighing is replaced by a zero adjustment operation; in this case, the value of the tare weighing is merely subtracted from all other values.

The contributions due to reproducibility and readability must therefore be specified twice in a measurement uncertainty analysis. The sensitivity drift only includes the drift of the ambient temperature during the measurement (see above). The nonlinearity specified for the weighing instrument holds true if a large part of the measuring range is used by the measuring instrument for the weighing. If the difference in the weighing value is much smaller than the measuring range of the weighing instrument, then nonlinearity affects both weighing values roughly to the same extent; it is thus nearly fully compensated for by computing the difference. The value for nonlinearity can therefore be described by a value reduced by the ratio of the weighing value difference to the measuring range if this difference is small compared to the measuring range. This is the case for the weighing operations at hand.

Contributions of the air buoyancy correction to the measurement uncertainty result from uncertainties in the determination of the air temperature, ambient pressure and relative humidity as well as from the uncertainty associated with the formula used to calculate the air density. The change in the air density due to the liquid vapour from the dispensed liquids was estimated in Section 5.3. The values of this estimation are not used for the air buoyancy correction, but taken into account in the measurement uncertainty budget as uncertainty contributions.

Uncertainty contributions when determining the density of the liquids involved in the measurement result from the uncertainties of the temperature determination and from the uncertainty of the equation describing the dependence of density on temperature. Other influences such as the gas concentration in the liquid and the influence of the ambient pressure on the liquid's density are negligibly small (smaller than $5 \cdot 10^{-6}$) and are therefore not considered.

The influences of evaporation described in Section 5 require another contribution to be included in the measurement uncertainty budget for the extrapolation of the weighing value to the time of dispensing, which is made necessary by the decrease in the liquid mass inside the weighing vessel. The influence of the temperature change due to evaporation in the reservoir is directly acquired by measuring the temperature. The influence of the change in temperature in the weighing vessel may be neglected. The influence of the change in temperature of the pipette tips has no influence on the measurement uncertainty of the measurement using the gravimetric device, but it does influence the dispensed volume. It is therefore part of the measurement result

and not a contribution to the measurement uncertainty of the measurement using the gravimetric device.

The sensitivity coefficients that describe the conversion of the measurement uncertainties of the units of the respective measuring quantity into the unit of volume are, in part, dependent of the volume. They also contain the density of the liquid as a parameter, so that they change with the temperature and when a different liquid is used. The measurement uncertainty budget is therefore only applicable to the specified liquid, the specified volume and the specified temperature.

Table 2 represents, as an example, the measurement uncertainty budget for the measurement of 1 µl water, and Table 3 the measurement uncertainty budget for the measurement of 50 µl ethanol. A detailed measurement uncertainty budget for the measurement of 20 µl water with the gravimetric device is given in Annex 1.

Figure 11 compiles the expanded measurement uncertainties that are associated with the measurement of liquid volumes using the gravimetric device for water, nonane, and ethanol in the volume range between 1 µl and 100 µl. The figure shows that the measurement uncertainty for small volumes is nearly constant and only starts increasing at volumes larger than 10 µl. Values for volumes smaller than 1 µl are therefore not provided. The constant uncertainty at small volumes is due to the volume-independent uncertainty contributions. In the case of water and nonane, these contributions are mostly the uncertainty of the weighing instrument's reproducibility and the uncertainty of the numerical compensation for evaporation. The prevailing contribution to the measurement uncertainty of the volume determination of ethanol is the uncertainty of the numerical compensation for evaporation. In the case of large volumes, the uncertainty of the density determination also considerably contributes to the measurement uncertainty. This contribution depends on the volume: in the case of water, it is lower than for other liquids due to water's lower thermal expansion coefficient. In the case of ethanol, the uncertainty of the density determination considerably contributes to the total uncertainty at large volumes: at 50 µl, it reaches 30 %, and at a volume of 100 µl, its share amounts to approx. 50 %[7].

[7] This share is calculated by dividing the squared uncertainty contribution by the squared total uncertainty, i.e. as the quotient of variances.

Table 2: Measurement uncertainty budget for the measurement of 1 µl water

	Parameter	Value	Standard measurement uncertainty	Sensitivity coefficient	Uncertainty contribution
Weighing instrument	Readability for W_1	0.0 kg	$28.9 \cdot 10^{-12}$ kg	$-1.0 \cdot 10^{9}$ nl/kg	$-29 \cdot 10^{-3}$ nl
	Readability for W_2	0.0 kg	$28.9 \cdot 10^{-12}$ kg	$1.0 \cdot 10^{9}$ nl/kg	$29 \cdot 10^{-3}$ nl
	Reproducibility for W_1	0.0 kg	$577 \cdot 10^{-12}$ kg	$-1.0 \cdot 10^{9}$ nl/kg	$-580 \cdot 10^{-3}$ nl
	Reproducibility for W_2	0.0 kg	$577 \cdot 10^{-12}$ kg	$1.0 \cdot 10^{9}$ nl/kg	$580 \cdot 10^{-3}$ nl
	Nonlinearity for W_2 - W_1	0.0 kg	$23.1 \cdot 10^{-12}$ kg	$1.0 \cdot 10^{9}$ nl/kg	$23 \cdot 10^{-3}$ nl
	Calibration of the internal weights	1	$577 \cdot 10^{-9}$	$1.0 \cdot 10^{3}$ nl	$580 \cdot 10^{-6}$ nl
	Sensitivity drift	0.0 °C	$289 \cdot 10^{-3}$ °C	$1.0 \cdot 10^{-3}$ nl/°C	$290 \cdot 10^{-6}$ nl
	Correction for evaporation	0.0 kg	$1.15 \cdot 10^{-9}$ kg	$1.0 \cdot 10^{9}$ nl/kg	$870 \cdot 10^{-3}$ nl
Liquid density	Temperature t_F	20 °C	$57.7 \cdot 10^{-3}$ °C	$210 \cdot 10^{-3}$ nl/°C	$12 \cdot 10^{-3}$ nl
	Approximation formula	0.0 kg/m³	$5.77 \cdot 10^{-3}$ kg/m³	-1.0 nl · m³/kg	$-5.8 \cdot 10^{-3}$ nl
Air density	Temperature t_A	20 °C	$57.7 \cdot 10^{-3}$ °C	$-8.3 \cdot 10^{-3}$ nl/°C	$-480 \cdot 10^{-6}$ nl
	Pressure p_A	$1.013 \cdot 10^{3}$ hPa	1.15 hPa	$2.1 \cdot 10^{-3}$ nl/hPa	$2.4 \cdot 10^{-3}$ nl
	Relative humidity φ	70.0 %	11.5 %	$-180 \cdot 10^{-6}$ nl/%	$-2.1 \cdot 10^{-3}$ nl
	Influence of other liquids on the air density	1.0	0.0	0.0 nl	0.0 nl
Volume			**1.20 nl**		

Expanded measurement uncertainty: 2.4 nl; coverage factor: 2

Table 3: Measurement uncertainty budget for the measurement of 50 µl ethanol

	Parameter	Value	Standard measurement uncertainty	Sensitivity coefficient	Uncertainty contribution
Weighing instrument	Readability for W_1	0.0 kg	$28.9 \cdot 10^{-12}$ kg	$-1.3 \cdot 10^{9}$ nl/kg	$-37 \cdot 10^{-3}$ nl
	Readability for W_2	0.0 kg	$28.9 \cdot 10^{-12}$ kg	$1.3 \cdot 10^{9}$ nl/kg	$37 \cdot 10^{-3}$ nl
	Reproducibility for W_1	0.0 kg	$577 \cdot 10^{-12}$ kg	$-1.3 \cdot 10^{9}$ nl/kg	$-730 \cdot 10^{-3}$ nl
	Reproducibility for W_2	0.0 kg	$577 \cdot 10^{-12}$ kg	$1.3 \cdot 10^{9}$ nl/kg	$730 \cdot 10^{-3}$ nl
	Nonlinearity for W_2 - W_1	0.0 kg	$23.1 \cdot 10^{-12}$ kg	$1.3 \cdot 10^{9}$ nl/kg	$29 \cdot 10^{-3}$ nl
	Calibration of the internal weights	0.0 °C	$289 \cdot 10^{-3}$ °C	$51 \cdot 10^{-3}$ nl/kg	$15 \cdot 10^{-3}$ nl
	Sensitivity drift	1	$577 \cdot 10^{-9}$	$51 \cdot 10^{3}$ nl	$29 \cdot 10^{-3}$ nl
	Correction for evaporation	0.0 kg	$5.77 \cdot 10^{-9}$ kg	$1.3 \cdot 10^{9}$ nl/kg	4.4 nl
Liquid density	Temperature t_F	20 °C	$57.7 \cdot 10^{-3}$ °C	-51 nl/°C	-2.9 nl
	Approximation formula	0.0 kg/m³	$57.7 \cdot 10^{-3}$ kg/m³	-65 nl · m³/kg	-3.7 nl
Air density	Temperature t_A	20 °C	$57.7 \cdot 10^{-3}$ °C	$-550 \cdot 10^{-3}$ nl/°C	$-32 \cdot 10^{-3}$ nl
	Pressure p_A	$1.013 \cdot 10^{3}$ hPa	1.15 hPa	$140 \cdot 10^{-3}$ nl/hPa	$160 \cdot 10^{-3}$ nl
	Relative humidity φ	70.0 %	11.5 %	$-12 \cdot 10^{-3}$ nl/%	$-140 \cdot 10^{-3}$ nl
	Influence of other liquids on the air density	1.0270	$15.6 \cdot 10^{-3}$	70 nl	1.1 nl
Volume			**6.65 nl**		

Expanded measurement uncertainty: 13 nl; coverage factor: 2

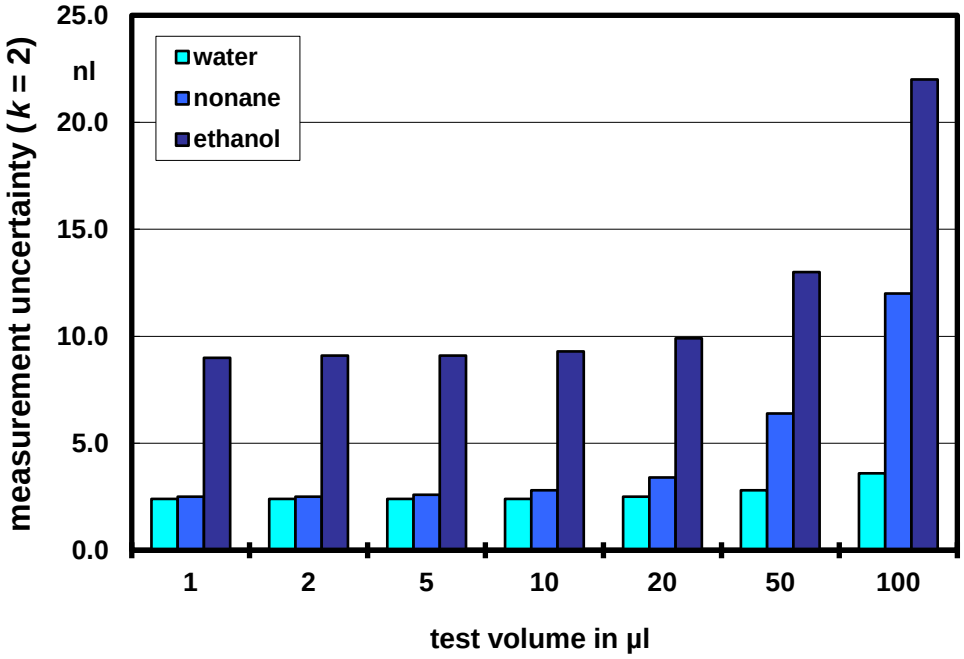


Fig. 11: Expanded measurement uncertainty (k = 2) for the measurement of liquid volumes using the gravimetric measuring setup for selected test volumes.
In the case of volumes smaller than 10 µl, the measurement uncertainty is practically independent of the volume measured.

In Table 4, the measurement uncertainty associated with the gravimetric volume determination is compared with the maximum permissible errors specified in Part 2 of the ISO 8655 standard.

The table shows that the requirement expressed at the beginning of the current Section – according to which the measurement uncertainty of the measurement performed with the gravimetric setup may not exceed one tenth of the maximum permissible errors – is more than complied with in the case of water. The values for nonane are very similar to those and are therefore not listed in the table. In the case of ethanol, this requirement is not fully met. When measuring volumes of 1 µl and 2 µl with the gravimetric setup, the measurement uncertainty amounts to one fifth of the maximum permissible errors. For the maximum permissible error of the systematic errors, the above-mentioned requirement (one tenth of the maximum permissible errors) is only met with volumes of 5 µl and larger; for the maximum permissible error of random errors, this is the case only from volumes of 20 µl on. The weaker requirement usually placed on such measurements, which specifies that the ratio of the measurement uncertainty to the maximum permissible error may not exceed a third, is met at all volumes.

Table 4: Measurement uncertainties associated with the gravimetric measurement setup compared to part 2 of the EN ISO 8655 standard [8]

Test volume	Measurement uncertainty (k = 2) of the measurement performed with the gravimetric measurement setup		maximum permissible errors for the deviation according to ISO 8655, part 2	
	Water	Ethanol	systematic	random
1 µl	2.4 nl	9.0 nl	50 nl	50 nl
2 µl	2.4 nl	9.1 nl	80 nl	40 nl
5 µl	2.4 nl	9.1 nl	125 nl	75 nl
10 µl	2.4 nl	9.3 nl	120 nl	80 nl
20 µl	2.5 nl	9.9 nl	200 nl	100 nl
50 µl	2.8 nl	13.0 nl	500 nl	200 nl
100 µl	3.6 nl	22.0 nl	800 nl	300 nl

[8] The comparison has not been performed for test volumes of 0.2 µl since no maximum permissible errors are specified in this standard for this volume.

7 Comparing the dispensing of water, ethanol, and nonane

In order to answer the question posed in Section 3 as to whether calibration using water as a calibration liquid can be transferred to the dispensing of other liquids, measurements were first performed using water, ethanol, and nonane as liquids. The volume range investigated extended from 0.2 µl to 50 µl. The dispensing devices used for this comparison were commercially available positive displacement piston-operated pipettes (also called positive displacement pipettes in the following) and microlitre syringes of various manufacturers. Both devices with a fixed volume and devices with a variable volume were used. Some of the microlitre syringes and piston-operated pipettes with a variable volume were used for several test volumes. They were applied in a range from 5 % to 100 % of their nominal volume[9].

The dispensing devices used differed with regard to their design and to the materials the expelling piston and the cylinder were made of (plastic cylinders are called "tips", especially in the case of air displacement pipettes). The devices compared consisted of:

4 piston-operated pipettes with a glass cylinder and a steel piston (5 test volumes
2 piston-operated pipettes with a glass cylinder and a steel piston with an additional plastic seal (3 test volumes)
4 piston-operated pipettes with a plastic cylinder and a plastic piston (9 test volumes), and
4 microlitre syringes with a steel cylinder and a steel piston (4 test volumes).

As far as possible, devices from several manufacturers were used. Due to the large number of measurements, only one specimen of each type of device was investigated. In the figures below, the different types of positive displacement piston-operated pipettes are designated as: positive displacement pipettes glass/steel, positive displacement pipettes glass/steel with plastic seal, and positive displacement pipettes plastic/plastic. With all devices, the dispensed volume of water, ethanol, and nonane was compared without changing the pipette tip to prevent influencing the dispensing results by different tips.

As described in Sections 4.3 and 4.4, one measurement consisted of 15 individual dispensing operations, of which only Nr. 3 to 12 were evaluated and used for averaging and for determining

[9] **The nominal volume** is the maximum utilizable volume of a volumetric sampling device as specified by the manufacturer. In the present paper, the volume used for a measurement is called **test volume**, as specified in ISO 8655-6. In the case of devices with a fixed volume, the nominal volume and the test volume are identical.

the repeatability standard deviation. These measurements were carried out with each of the dispensing devices for the liquids listed above and then compared with each other.

Examples of such dispensing operations are shown in Figs. 12 to 15 (p. 50ff) for diverse test volumes. Dispensings of water, ethanol, and nonane are represented in each of the figures. Figure 12 shows the dispensing of 50 µl using a 100 µl piston-operated pipette with variable volume. The tip and the piston of this device are made of plastic. Figure 13 shows the dispensing of 10 µl with a fixed-volume pipette with a glass cylinder and a steel piston, whereas Fig. 14 shows the dispensing of 1 µl with a 20 µl variable-volume pipette similar to that used in Fig. 12 (a system with a tip and a piston both made of plastic). Figure 15 shows the dispensing of 0.2 µl with a 0.5 µl microlitre syringe whose cylinder and piston are both made of metal.

These figures are a good illustration of the dependence of the evaporation rate both on the test volume and on the dispensing device which was postulated in Section 5.1. As described there, the loss due to evaporation depends not only on the vapour pressure, but also on the additional surface created when wiping off the tip – and thus on the surface tension of the liquid and on the shape of the dispensing device's tip as well as on the volume of liquid expelled. That is the reason why the evaporation rate is different depending on the devices used and on the volumes involved. Smaller volumes and smaller tip diameters lead to a smaller wet surface on the vessel's wall and thus to a smaller increase in the evaporation rate, compared to the stationary value that is achieved when no additional liquid is present on the walls of the vessel anymore.

These differences also occur in the measurements shown in Figs. 12 to 15. Table 5 lists the loss due to evaporation determined from the above-mentioned figures for these measurements. The table states the volumes of liquid that evaporate within a period of 40 s, i.e. in the period between two dispensing operations. In the grey-shaded columns next to the values, it is indicated whether the liquid vapour concentration of the air in the weighing chamber was increased by means of an evaporation trap. This was always the case for water; for ethanol, an evaporation trap was used only for small test volumes. Nonane is (very slightly) toxic. For this reason, no evaporation trap was used.

Table 5: Volume of liquid having evaporated from the weighing vessel in the interval of time between two dispensing operations[10]. In the grey-shaded columns, it is indicated whether an evaporation trap was used or not.

Test volume	Liquid	Use of an evaporation trap				
	Water		Ethanol		Nonane	
0.2 µl	19 nl	yes	304 nl	yes	76 nl	no
1 µl	17 nl	yes	279 nl	yes	72 nl	no
10 µl	46 nl	yes	720 nl	no	92 nl	no
50 µl	68 nl	yes	913 nl	no	139 nl	no

For the test volumes 1 µl, 10 µl, and 50 µl, the volume of liquid evaporating increases with increasing test volume, as expected. The evaporated volume is, however, larger at a test volume of 0.2 µl than at a test volume of 1 µl. This is due to the behaviour of the different dispensing devices used when wiping the tip off. To measure 0.2 µl, a microlitre syringe with a very thin, flexible steel cylinder and a very small outlet was used. This steel cylinder, however, is not rigid and therefore bends slightly when drawn against the wall of the vessel for wiping off the tip. The angle it forms with the wall of the vessel is thus very small, which may lead to a different wiping-off behaviour. The piston-operated pipette used for the test volume of 10 µl has a rigid plastic cylinder that does not bend when wiping off the tip.

Although the vapour pressure – and thus the evaporation rate – of nonane is very low, the evaporated volume is larger than in the case of water. The reason for this is the larger wet surface which is itself due to the lower surface tension.

Due to the different evaporation behaviours, the four figures cannot be "scaled". For instance, the curve for ethanol lies above that of nonane at a test volume of 50 µl, which is due to the

[10] The losses caused by evaporation are compensated for arithmetically. The sole values stated here are thus **no** indicator for the measurement uncertainty contributions due to evaporation.

larger density of ethanol. At a test volume of 10 µl, the larger evaporation rate already has such a strong effect that these two curves are very close to each other. At smaller test volumes, the measured values for ethanol lie below those of nonane. What is particularly remarkable is the value obtained at a test volume of 0.2 µl. In the case of ethanol, the evaporated volume is larger than the volume added by means of the dispensing device, which means that the quantity of liquid in the weighing vessel decreases although liquid is added to it. Moreover, the evaporation rate is not constant over time: the curve forms a slight bend. Yet, it is possible to assess these curves successfully if the losses due to evaporation are measured and arithmetically compensated for. The dispensed volumes calculated based on the curves shown have a coefficient of variation of only 3 %.

Figs. 16 and 17 show the comparison between the dispensed volumes of water and of ethanol. These figures show the difference between water and ethanol for the averaged values obtained from 10 individual dispensing operations. Since the volumes measured range across more than two decades, Fig. 17 also shows the relative deviation in addition to the difference shown in Fig. 16 to facilitate comparison. The measurement uncertainty bars that are also plotted indicate the uncertainty of the difference for the coverage factor $k = 2$ which was calculated from the repeatability standard deviation of the measurements.

The dispensed volume of ethanol is, apart from one exception, slightly smaller than the volume of water to which it is compared. For volumes of 2 µl and more, this underfeeding amounts to 0.5 % to 1.5 % for all devices (there is one exception as well). In the case of smaller volumes, differences of more than 10 % were found. One essential reason for the underfeeding is the large evaporation rate that already starts taking effect on the way from the reservoir to the weighing vessel. Here, the liquid evaporates on the outer surface of the pipette tip and from its orifice. The evaporation out of the orifice of the pipette tip is so high in the case of ethanol that it is visible under a microscope.

The differences between the dispensing of water and that of nonane are shown in Figs. 18 and 19. Here again, the relative deviation is shown in Fig. 19 in addition to the absolute difference shown in Fig. 18. The dispensed volumes of nonane are mostly lower than those of water. Contrary to ethanol, inverse deviations were, however, also observed, i.e. the dispensed volume of nonane is in a few cases larger than the dispensed volume of water. In many cases, the order of magnitude of these deviations is comparable to that of the measurement uncertainty.

The quantitative indications provided for the deviations are therefore less reliable and cannot be applied to other devices. The influence of the evaporation of liquid from the device tip is not observed here – which was expected, since nonane has a much lower vapour pressure than ethanol.

Apart from one exception, the results do not allow any conclusions to be drawn as to the best or least suited device for dispensing, either for ethanol or for nonane. Differences rather depend on the individual properties of the devices than on general design properties. Piston-operated pipettes with a glass cylinder and a steel piston with a plastic seal are the only exception. This type of device is designed for larger volumes. In the present investigation, one of these devices was used for volumes of 10 µl and 20 µl and another for a volume of 50 µl. The device for 10 µl and 20 µl shows no abnormalities when ethanol is used. The deviations in the dispensed volume are comparable to the values obtained using other devices. The device for 50 µl, in contrast, shows a much higher deviation. In the case of nonane, both devices show clear underfeeding. A visual check of the devices showed that the liquid had passed the seals and reached the area above the seals. The devices were significantly stiffer and more difficult to operate after the measurements than before. Obviously, the seal materials were damaged by the organic liquids and had therefore become porous. The seals might also have absorbed some liquid and swollen. The effect was much more significant with nonane than with ethanol. However, since the measurements with nonane were always performed after those with ethanol, the seals might also have been previously damaged by ethanol[11]. Due to the damage to the seals, these devices were not submitted to further investigations.

[11] It is important to bear in mind that all measurements were carried out without changing the pistons and cylinders.

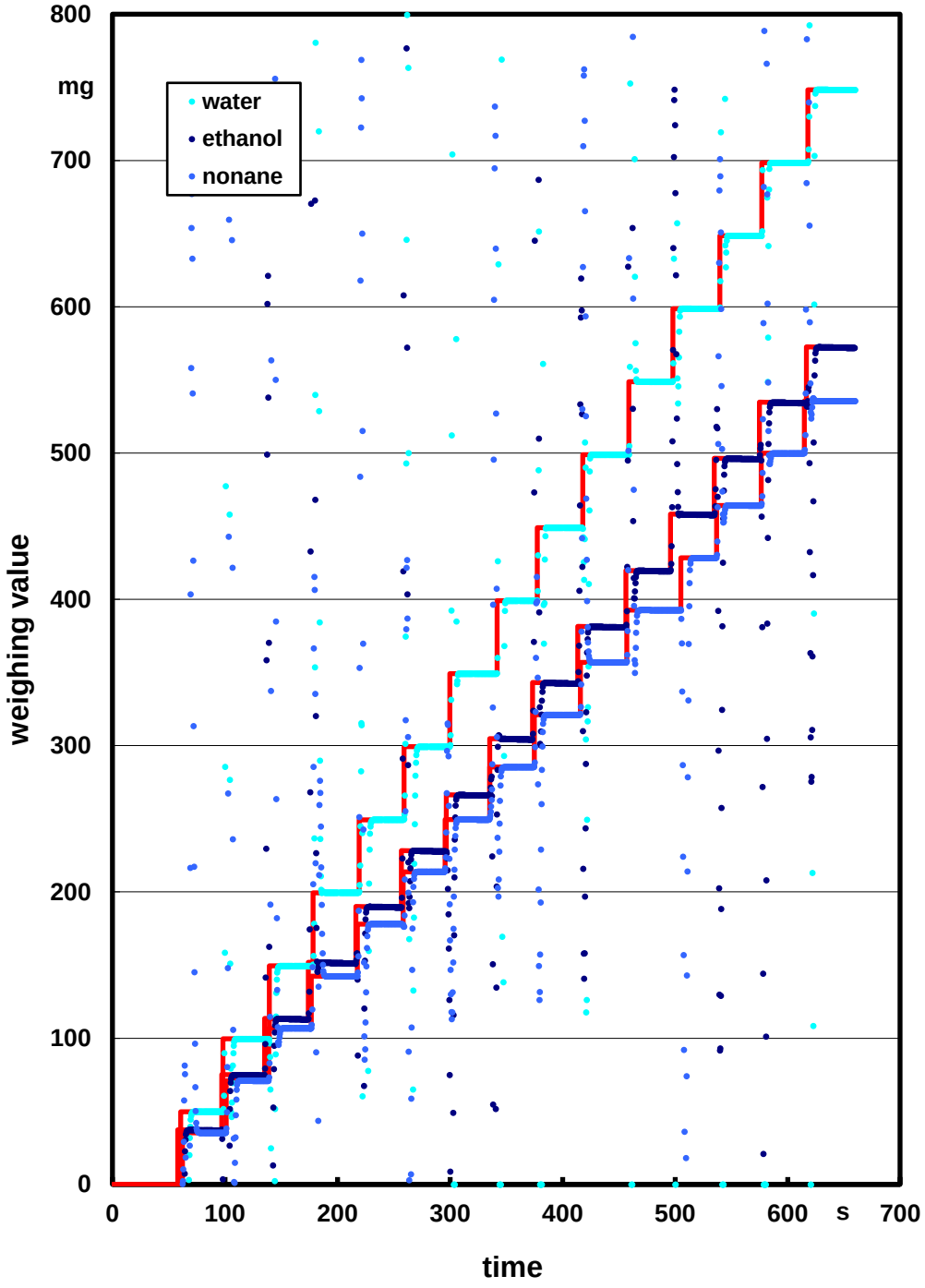


Fig. 12: Weighing value for 15 dispensing operations of 50 µl water, ethanol and nonane, respectively, with a positive displacement piston-operated pipette with a plastic piston and a plastic cylinder. The continuous step curves are composed of regression lines. The leap onto the next line occurs at the moment of each dispensing operation. The height of the steps is a measure of the respective weighing value of the dispensed volume.

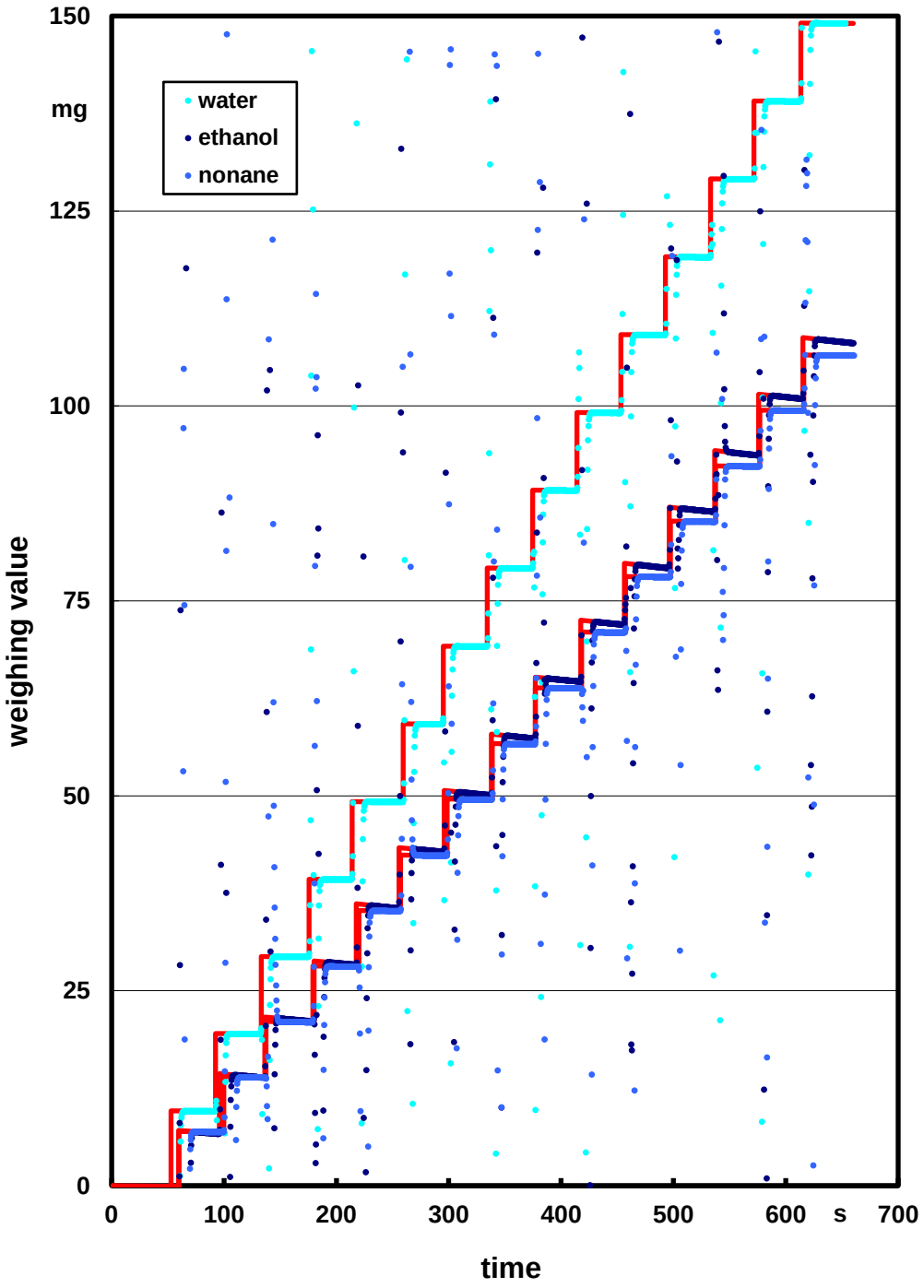


Fig. 13: Weighing value for 15 dispensing operations of 10 µl water, ethanol and nonane, respectively, with a positive displacement piston-operated pipette with a steel piston and a glass cylinder. The continuous step curves are composed of regression lines. The leap onto the next line occurs at the moment of each dispensing operation. The height of the steps is a measure of the respective weighing value of the dispensed volume.

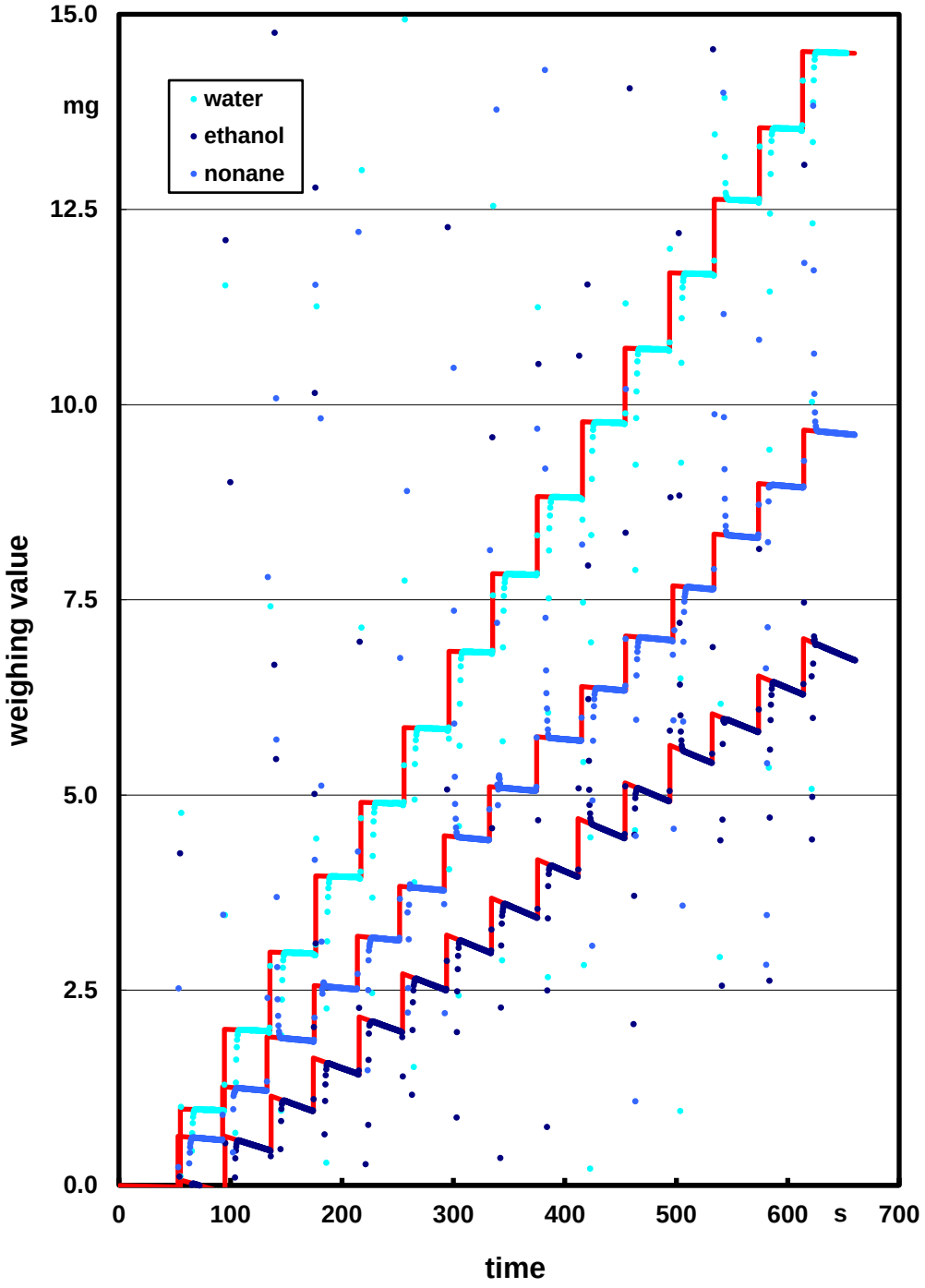


Fig. 14: Weighing value for 15 dispensing operations of 1 µl water, ethanol and nonane, respectively, with a positive displacement piston-operated pipette with a plastic piston and a plastic cylinder. The continuous step curves are composed of regression lines. The leap onto the next line occurs at the moment of each dispensing operation. The height of the steps is a measure of the respective weighing value of the dispensed volume.

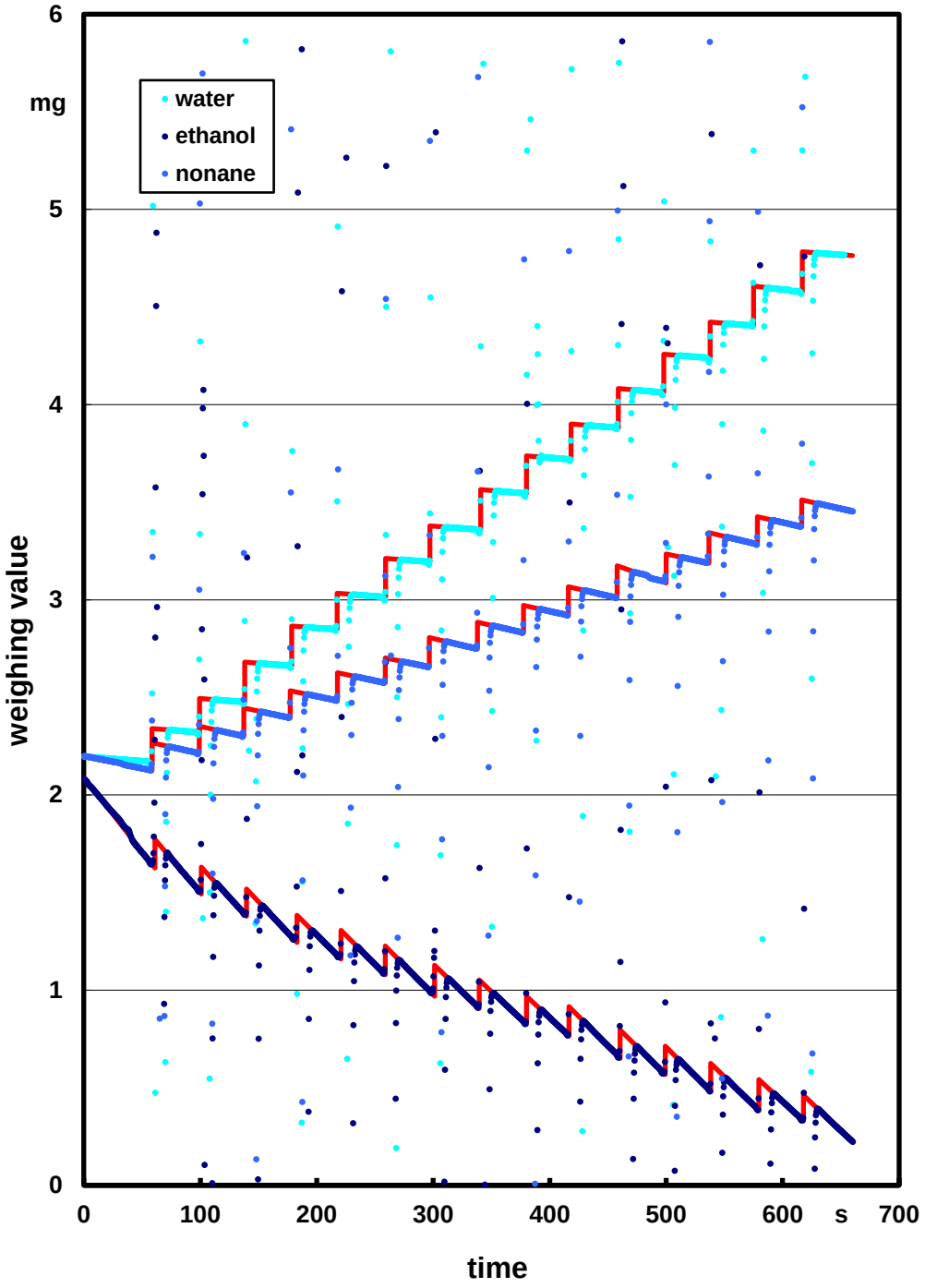


Fig. 15: Weighing value for 15 dispensing operations of 0.2 µl water, ethanol and nonane, respectively, with a microlitre syringe. The continuous step curves are composed of regression lines. The leap onto the next line occurs at the moment of each dispensing operation. The height of the steps is a measure of the respective weighing value of the dispensed volume.

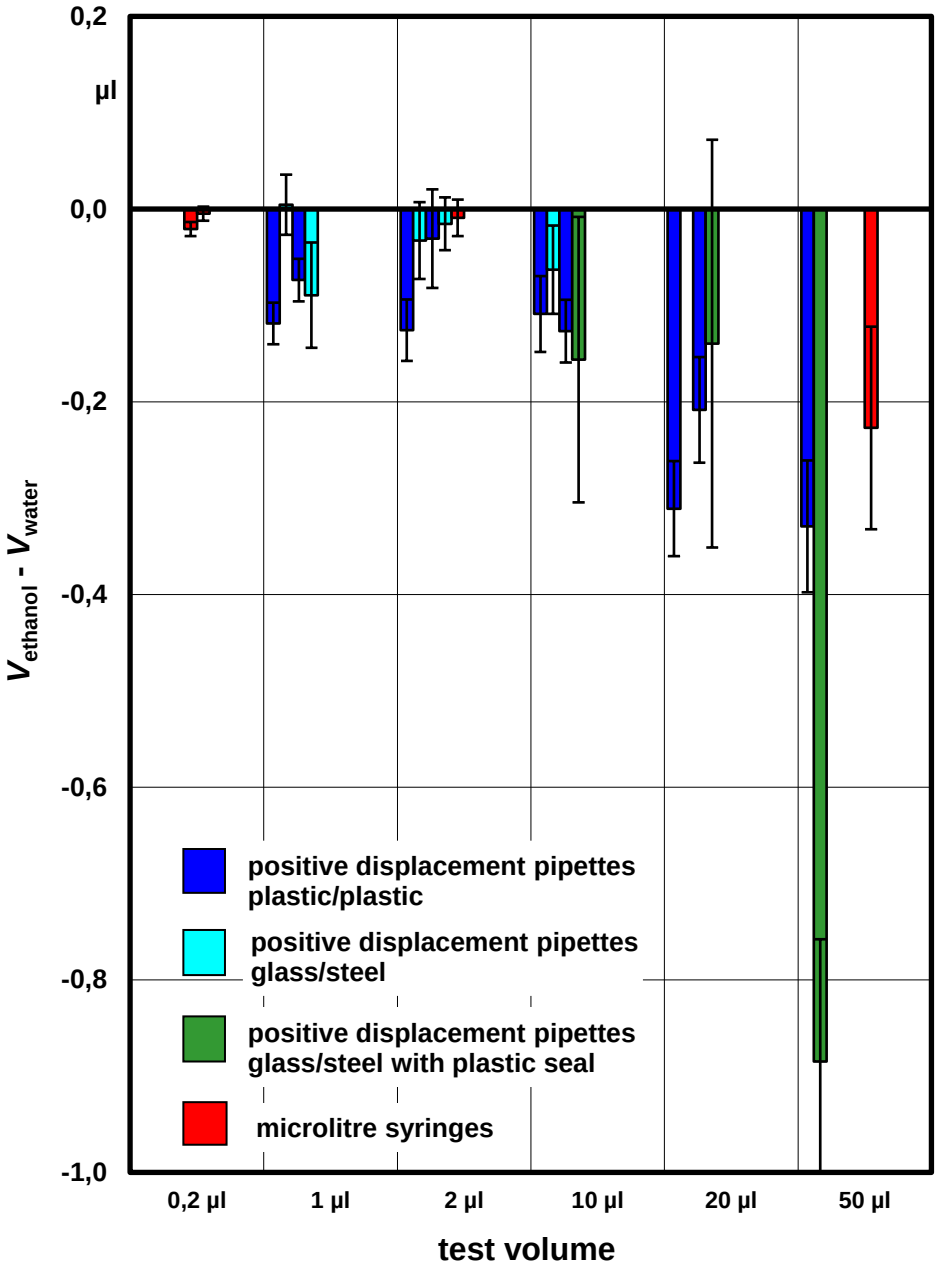


Fig. 16: Difference between the dispensed volume of water and ethanol.
The figure shows the absolute difference of the values averaged from 10 dispensing operations for water and for ethanol, respectively. Each bar represents the results for one dispensing device. The measurement uncertainty bars indicate the uncertainty of the difference which was calculated from the repeatability standard deviation of the difference (coverage factor $k = 2$).

In order to allow the individual dispensing devices to be compared considering several liquids, the position of each of the devices in this diagram and the following ones has been kept the same. Positions in the diagram therefore remain empty if no measurement is available for the device and volume considered.

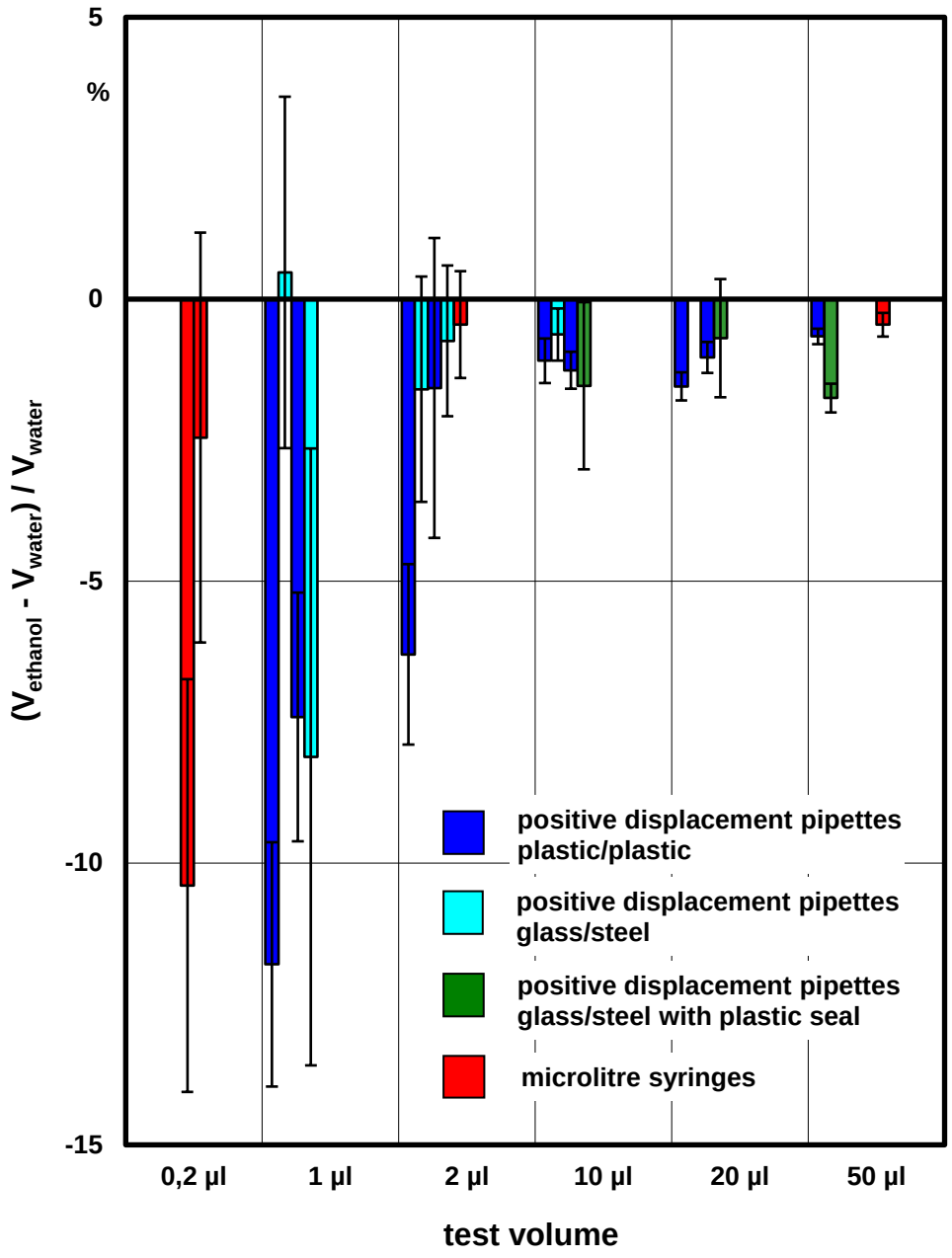


Fig. 17: Difference between the dispensed volume of water and ethanol.
The figure shows the relative difference of the values averaged from 10 dispensing operations for water and for ethanol, respectively. Each bar represents the results for one dispensing device. The measurement uncertainty bars indicate the uncertainty of the difference which was calculated from the repeatability standard deviation of the relative difference (coverage factor k = 2).

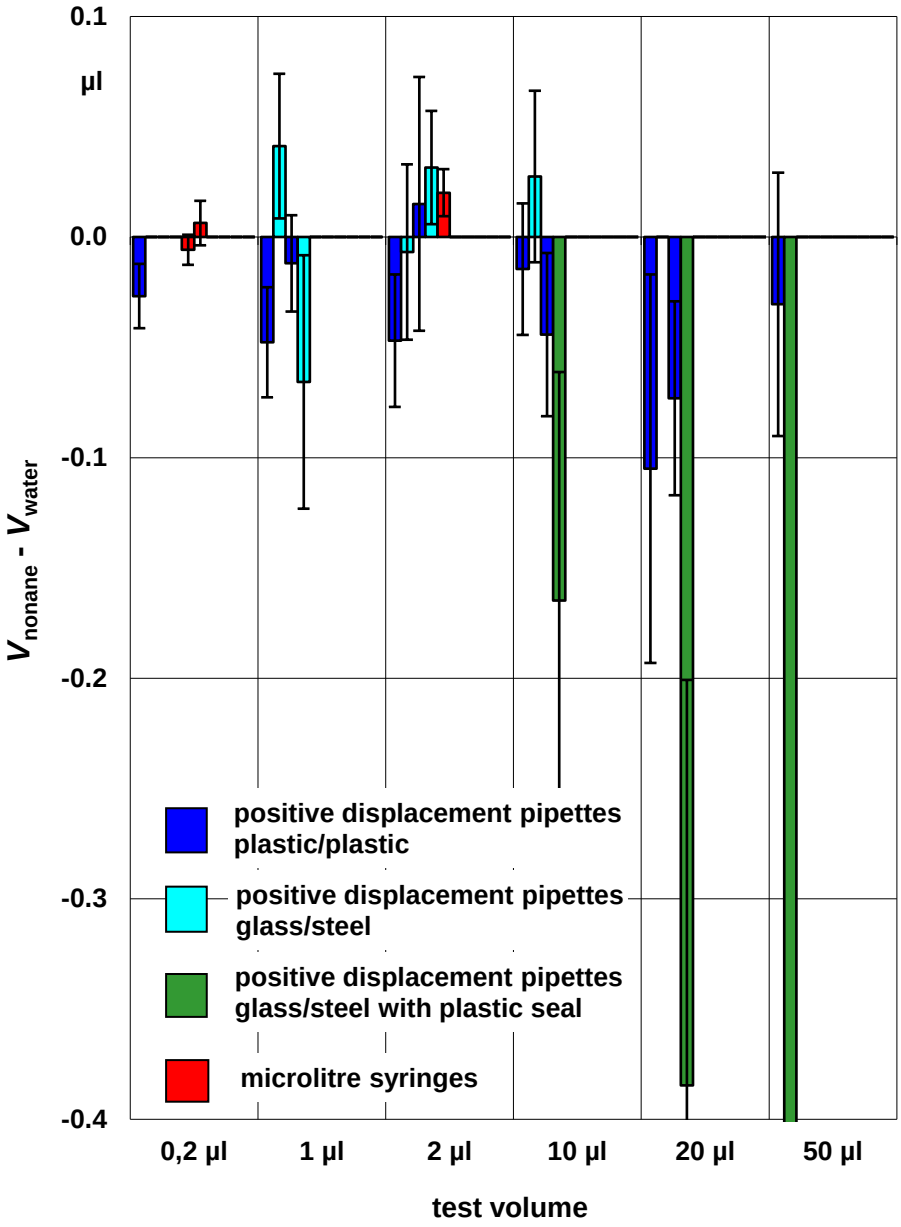


Fig. 18: Difference between the dispensed volume of water and nonane.
The figure shows the absolute difference of the values averaged from 10 dispensing operations for water and for nonane, respectively. Each bar represents the results for one dispensing device. The measurement uncertainty bars indicate the uncertainty of the difference which was calculated from the repeatability standard deviation of the difference (coverage factor k = 2).

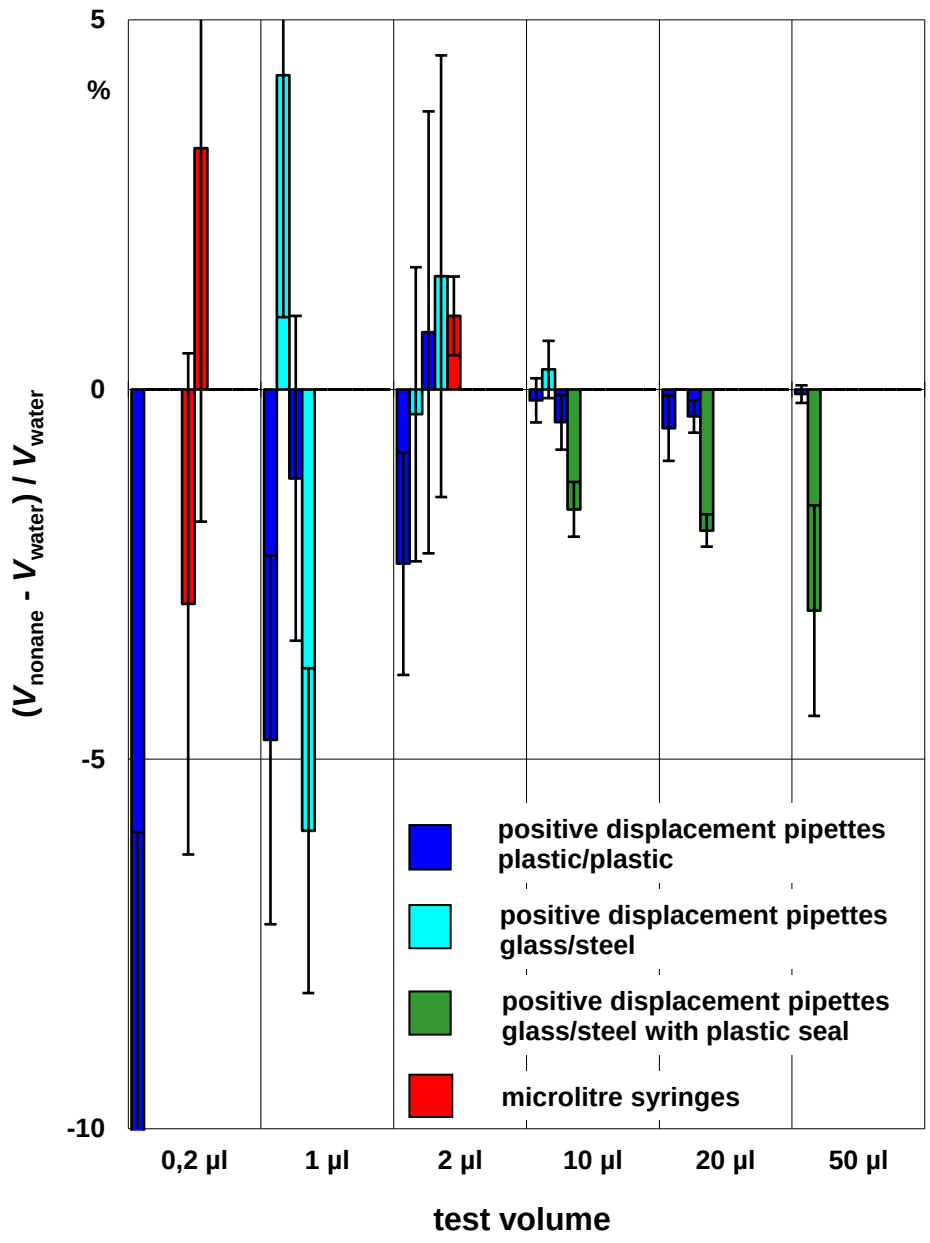


Fig. 19: Difference between the dispensed volume of water and nonane.
The figure shows the relative difference of the values averaged from 10 dispensing operations for water and for nonane, respectively. Each bar represents the results for one dispensing device. The measurement uncertainty bars indicate the uncertainty of the difference which was calculated from the repeatability standard deviation of the relative difference (coverage factor $k = 2$).

Water and the two organic liquids (ethanol and nonane) differ in particular with regard to their vapour pressure. The high vapour pressure was already mentioned as a reason for the underfeeding in the case of ethanol. This effect does not occur with nonane, since its vapour pressure is lower than that of water (cf. Table 1). Another considerable difference is the surface tension. The surface tension of water is more than three times that of ethanol and nonane – which both have similar values. This leads to a completely different wettability behaviour of the cylinders by ethanol and nonane. Whereas water hardly wets the outer surface of the device's cylinder, the other two liquids "creep" up the outer surface and wet a large part of it. Figure 20 demonstrates the wettability of the outer surface of the pipette tip. The figure shows a shadow

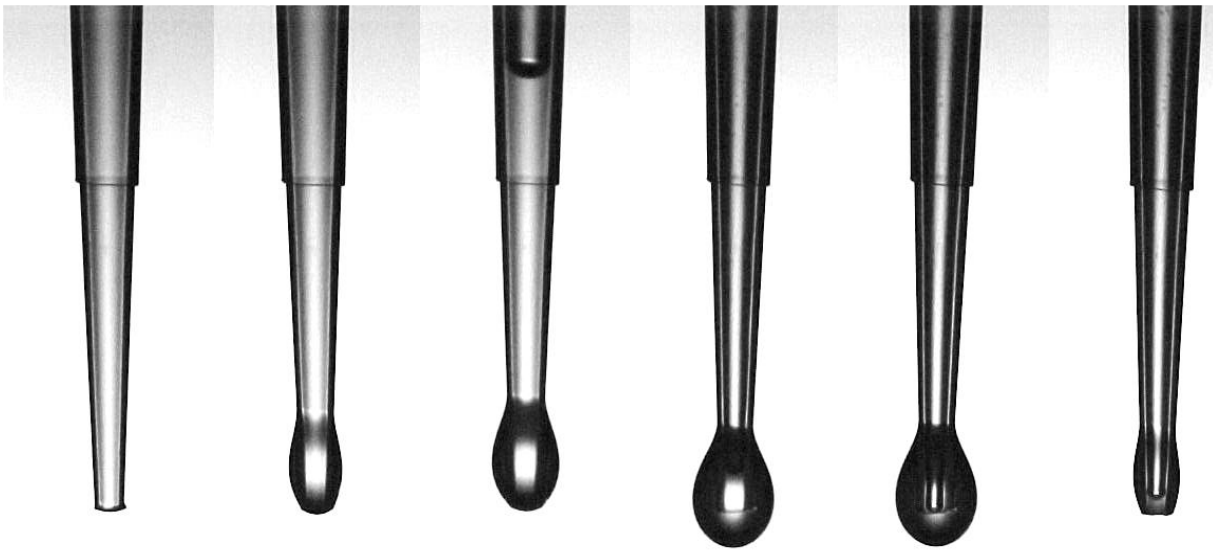


Fig. 20: Wettability of the pipette tip while expelling nonane.
For the purpose of these photos, the pipette tip was not drawn against the wall of the vessel.

projection of a pipette tip filled with nonane while the liquid is being expelled. This photo was taken using an air displacement pipette as a dispensing device. There is therefore no piston to be seen in the photo. Instead of the cylinder, the pipette tip is visible.

In the image sequence, one sees how liquid leaves the pipette tip and first wets the outer surface of the pipette, until a drop forms that is heavy enough to drop off. After that, this procedure

repeats. The liquid rises several millimetres[12] on the outer surface of the pipette. The remaining liquid must be brought into the weighing vessel by means of a wipe-off procedure. The efficiency of this procedure is not sufficient because the liquid wets the tip all around. The roughness of the outer surface of the cylinder's surface as well as ridges and uneven intersections at the tip of the cylinder[13] have a considerable influence on the success of the wipe-off procedure. The liquid adheres to such surfaces, thus preventing it from flowing out. These are very probably essential reasons for the deviations in the dispensed volume of water for one thing and ethanol and nonane for another. Water hardly wets the outer surface of the tip while being expelled, which explains why no water remains on the outer surface.

The tip of the devices analogously becomes wet during the filling procedure; this liquid can as well only be partly removed by wiping off the tip. In this context, a difference is expected between ethanol and nonane. Whereas ethanol largely evaporates on the outer surface when the dispensing device is moved from the reservoir to the weighing vessel due to its high vapour pressure, nonane remains there. This causes the dispensed volume to be larger in the case of nonane than when using ethanol, but also partly larger than when water is used.

[12] Cf. the photos in Fig. 21 which show the behaviour of water when being expelled.
[13] Such an uneven intersection with ridges is visible in the partial image in Fig. 20.

8 Comparing the dispensing of water and human serum

As described in Section 7, the dispensed volume of water was compared to the dispensed volume of human serum. Serum consists of 90 % water. Many physical properties of serum are therefore similar to those of water. Its density, for example, is merely 2 % to 3 % larger, its viscosity approx. 15 % larger than that of water. Its vapour pressure is also determined by the vapour pressure of water. With approx. 56 mN/m, its surface tension is, however, much lower than that of water. Stronger wettability of the dispensing device tips is therefore expected than when dispensing water. One clear difference is the biological-chemical behaviour of serum. In particular the proteins contained in serum cause it to feel "sticky". Even after serum has been expelled, a liquid film that is difficult to remove remains on the walls of the vessel.

Apart from the dispensing devices used in Section 7, investigations on human serum were also carried out with air displacement piston-operated pipettes. The differences in the behaviour of the liquids when dispensing water and serum are demonstrated in Figs. 21 and 22. As in Fig. 20, the figures show a shadow projection of a filled pipette tip while the liquid is being expelled. The device used is an air displacement pipette; the dispensed test volume amounts to 2 µl.

The three individual pictures in Fig. 21 were taken consecutively; they show the moments 1) prior to expelling the liquid, 2) after pressing the pipetting pushbutton to the first stop, and 3) after pressing it down to the final stop. Prior to expelling, the pipette is filled with water; in this silhouette projection, the tip filled with liquid is light-coloured. The second picture shows the pipette tip immediately after pushing the button down to the first stop. The liquid has been expelled; the last drop is still hanging at the end of the pipette tip. The pipette tip's wettability by water is minimal. It may, however, be increased due to impurities in the tip and due to unevenness and ridges. In the third picture, the pushbutton was pressed down to the final stop, pushing the air interface of the pipette further downwards in order to blow out the last remnants of liquid. This removes the last drop of water from the tip. The tip is fully emptied, except for a thin water film inside it.

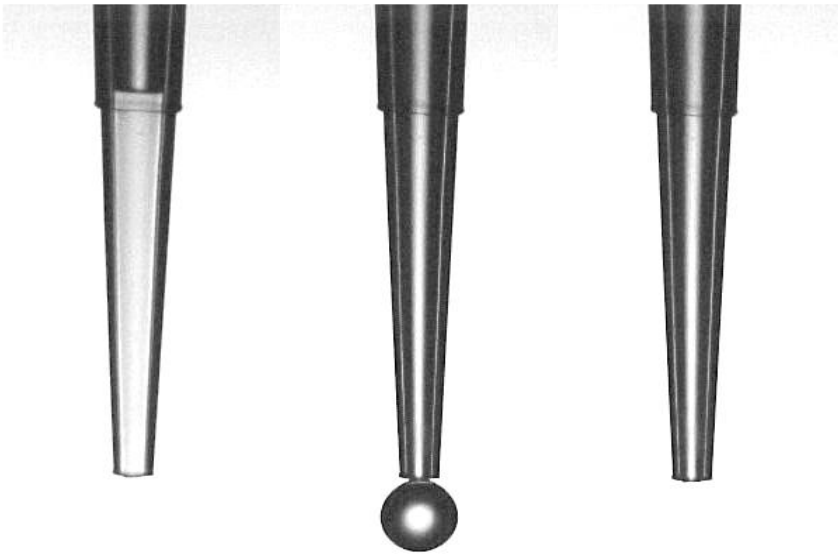


Fig. 21: Sequence of pictures showing the tip of an air displacement pipette filled with **water** being emptied. The quantity of liquid is 2 µl.
For the purpose of these photos, the pipette tip was not drawn against the wall of the vessel.

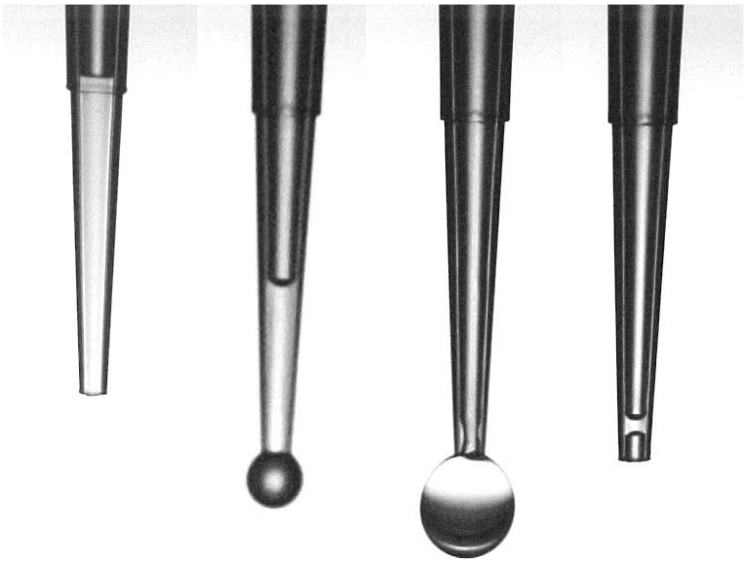


Fig. 22: Sequence of pictures showing the tip of an air displacement pipette filled with **serum** being emptied. The quantity of liquid is 2 µl.
For the purpose of these photos, the pipette tip was not drawn against the wall of the vessel.

The expelling behaviour is completely different when the dispensing liquid used is human serum. Fig. 22 first shows the same picture sequence as Fig. 21. The first picture of the sequence shows the pipette tip filled with serum. The second picture shows the situation after pressing the pushbutton down to the first stop. The liquid adheres so much to the inner wall of the tip that only a small amount is pushed out. Not until the pushbutton is pressed down to the final stop is the liquid pressed out of the pipette tip. A bubble of serum partly filled with air forms at the outlet of the tip (in the silhouette representation, the area of the bubble that is filled with air is light-coloured). Serum can still be seen in the lower part of the inner tip. The serum bubble can be wiped off by drawing the tip against the inner wall of the vessel (4th picture of the sequence). The liquid remaining in the tip gathers to form a liquid membrane. This membrane can no longer be expelled, so that a small quantity of liquid remains in the pipette tip.

As shown in Fig. 23, the volume of liquid remaining in the pipette tip is not easily reproducible. It is possible to reduce this left-over quantity by drawing the tip against the inner wall of the vessel while expelling the liquid (3rd picture in the sequence of Fig. 23).

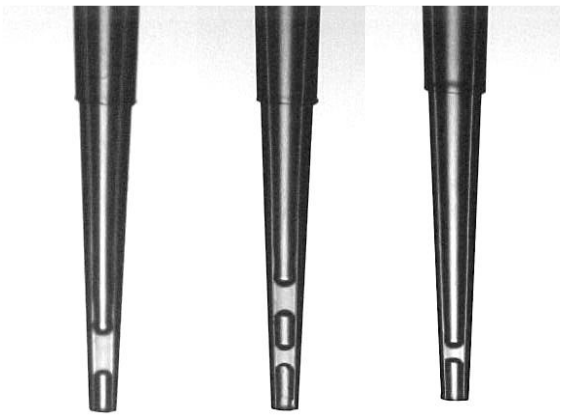


Fig. 23: Serum remaining in the pipette tip after the dispensing. The original quantity of liquid was 2 µl.
During the dispensing in the picture on the right, the pipette tip touched the vessel inner wall; this was not the case in the picture on the left and in the one in the middle.

The quantity of serum remaining in the pipette tip considerably reduces the volume dispensed by air displacement pipettes. In contrast, this dispensing loss can be fully avoided or at least considerably reduced when using positive displacement pipettes, due to the forced expelling of the liquid by the plunger.

The wettability of the outer surface of the tip is higher than with water, but not as high as with nonane[14]. This effect must be expected both in air displacement pipettes and positive displacement pipettes. In the latter case, however, the influence of this effect has a much lesser influence on the dispensed volume than the liquid remaining in the tips of air displacement pipettes.

Figs. 24 to 27 show the comparison between dispensed volumes of water and serum for positive displacement pipettes and for microlitre syringes. Since different dispensing devices exhibit considerable differences in the dispensed volumes, only test volumes from 10 µl to 50 µl are plotted in Figs. 24 and 25 which are enlarged sections of Figs. 26 and 27. The deviations between the dispensed volumes of water and serum shown here are very small. They are often below 0.1 %; deviations of 0.14 % and 0.29 %, respectively, were measured with two devices. There is only one microlitre syringe that exhibits a striking deviation of 1 %. No clear trend towards dispensing of excessive or insufficient volumes could be made out. Both occur roughly equally frequently. In the selected volume range and for the devices tested, calibration with water is rather well transferable to dispensing serum. This statement cannot be applied to any random device. The 1 % deviation exhibited by one of the syringes, however, shows that it makes sense to check each device separately.

In Figs. 26 and 27, the values for air displacement pipettes as well as the test volumes of 0.2 µl, 1 µl, and 2 µl are additionally plotted. In the case of small test volumes, the relative deviations for positive displacement devices and microlitre syringes are clearly larger than at test volumes of more than 10 µl. They increase to up to 2 %, and in one case even to 3.6 %. Two microlitre syringes show particularly good agreement at a test volume of 0.2 µl. The deviations are below 2 %!

[14] The surface tension of nonane is approx. 22 mN/m, i.e. still much lower than that of serum which amounts to approx. 56 mN/m. It must, however, be pointed out that in this case, not only surface tensions, but also (quantitatively not known) interface tensions have to be considered.

As expected, air displacement pipettes show large deviations. For all test volumes of more than 1 µl, the values of underfeeding vary between 3.4 % and 10.5 %. An average underfeeding of 5 % can be indicated as a general statement. At test volumes of 0.2 µl, in contrast, deviations of more than 30 % were found. At such volumes, air displacement pipettes must by no means be used to dispense serum.

Even at larger volumes, it is preferable not to use air displacement pipettes to dispense serum if quantitatively exact specified dispensing is required. The reason for underfeeding, as described above, is the remaining liquid that adheres to the tip's wall. The liquid expelling process is not well reproducible, so that even calibrating the air displacement pipette with serum as a calibration fluid will not yield reliable results. If the pipette tip needs replacing, a positive displacement piston-operated pipette with interchangeable cylinders and plunger may be used instead of an air displacement pipette. Investigated systems of this type are the devices designated as "positive displacement devices plastic/plastic" in Fig. 24 and the following. Positive displacement piston-operated pipettes and microlitre syringes are well suited for dispensing serum. Calibration of these devices with water is, under certain conditions, transferable to serum. However, since considerable deviations were observed between the dispensing of water and the dispensing of serum with certain devices, calibrating with serum is recommended.

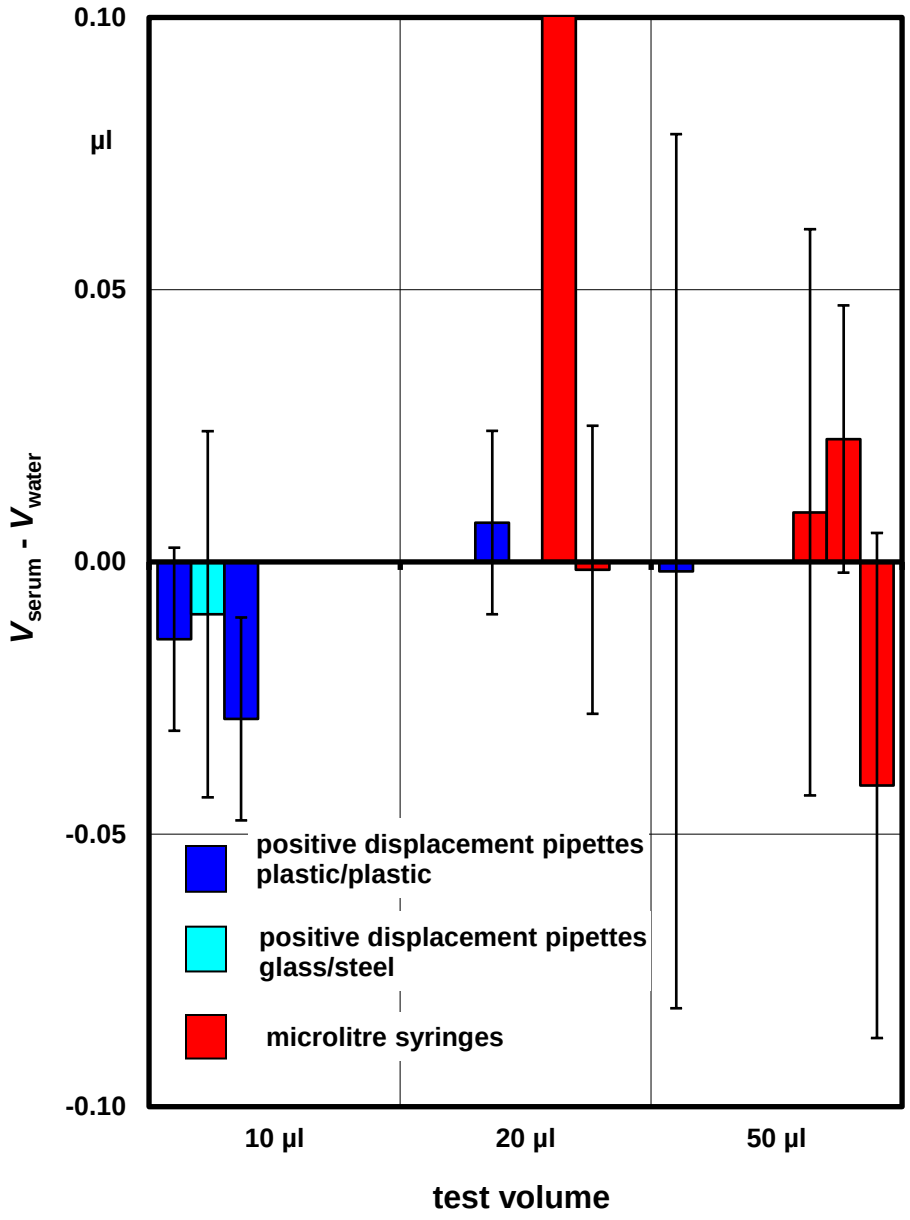


Fig. 24: Difference between the dispensed volume of water and serum for test volumes of 10 µl, 20 µl, and 50 µl.
The figure shows the absolute difference of the values averaged from 10 dispensing operations for water and for serum, respectively. Each bar represents the results for one dispensing device. The measurement uncertainty bars indicate the uncertainty of the difference which was calculated from the repeatability standard deviation of the difference (coverage factor k = 2).

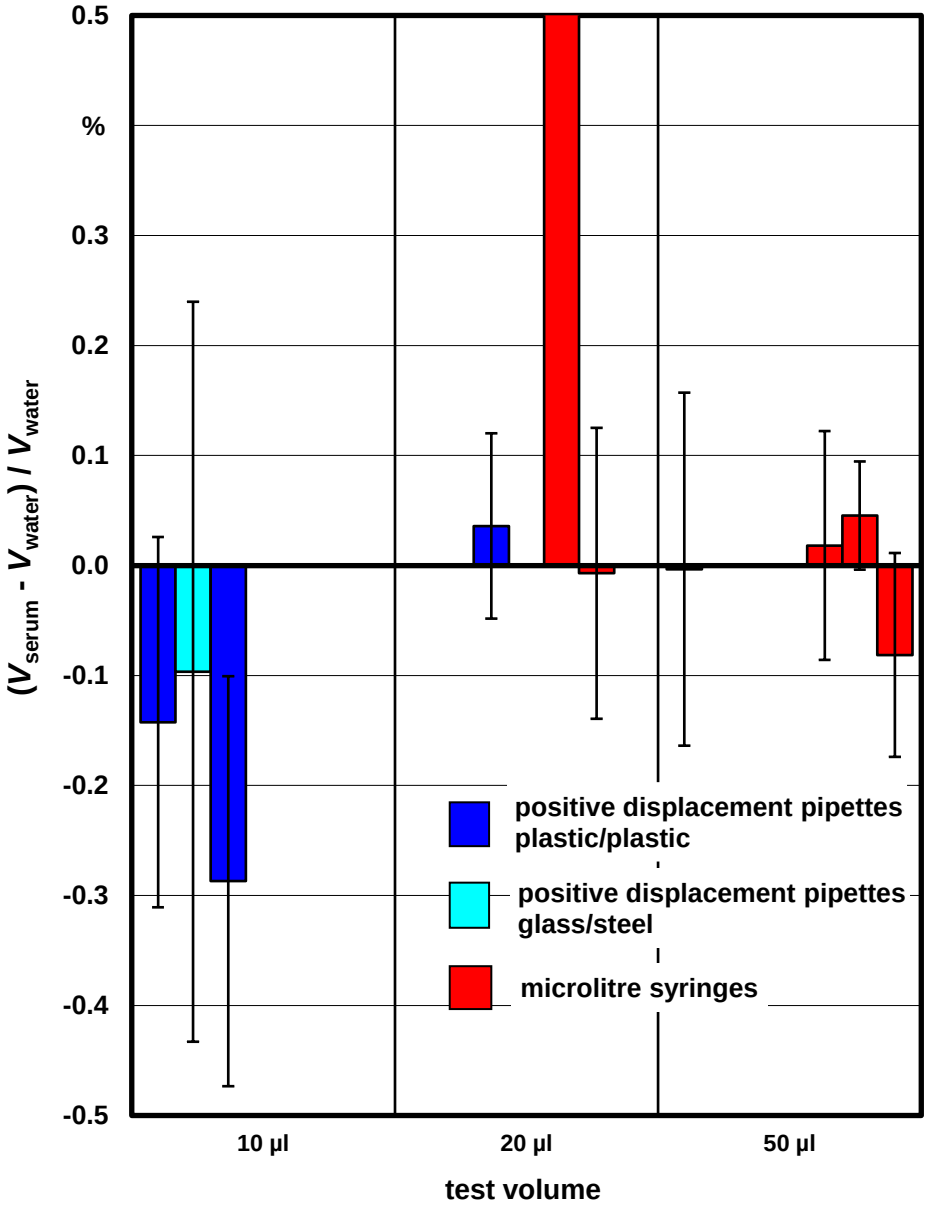


Fig. 25: Difference between the dispensed volume of water and serum for test volumes of 10 µl, 20 µl, and 50 µl.
The figure shows the relative difference of the values averaged from 10 dispensing operations for water and for serum, respectively. Each bar represents the results for one dispensing device. The measurement uncertainty bars indicate the uncertainty of the difference which was calculated from the repeatability standard deviation of the relative difference (coverage factor k = 2).

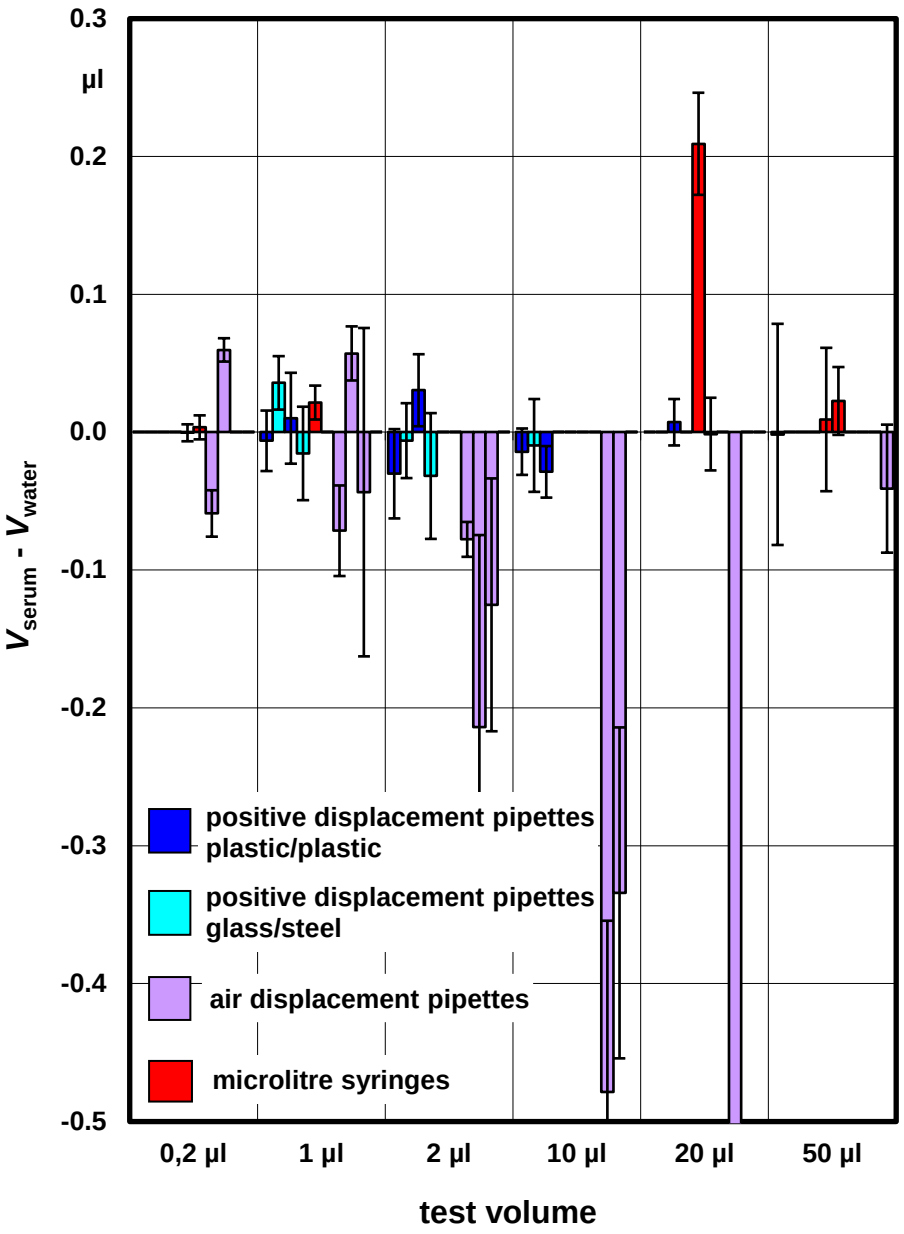


Fig. 26: Difference between the dispensed volume of water and serum for test volumes from 0.2 µl to 50 µl.
The figure shows the absolute difference of the values averaged from 10 dispensing operations for water and for serum, respectively. Each bar represents the results for one dispensing device. The measurement uncertainty bars indicate the uncertainty of the difference which was calculated from the repeatability standard deviation of the difference (coverage factor k = 2).

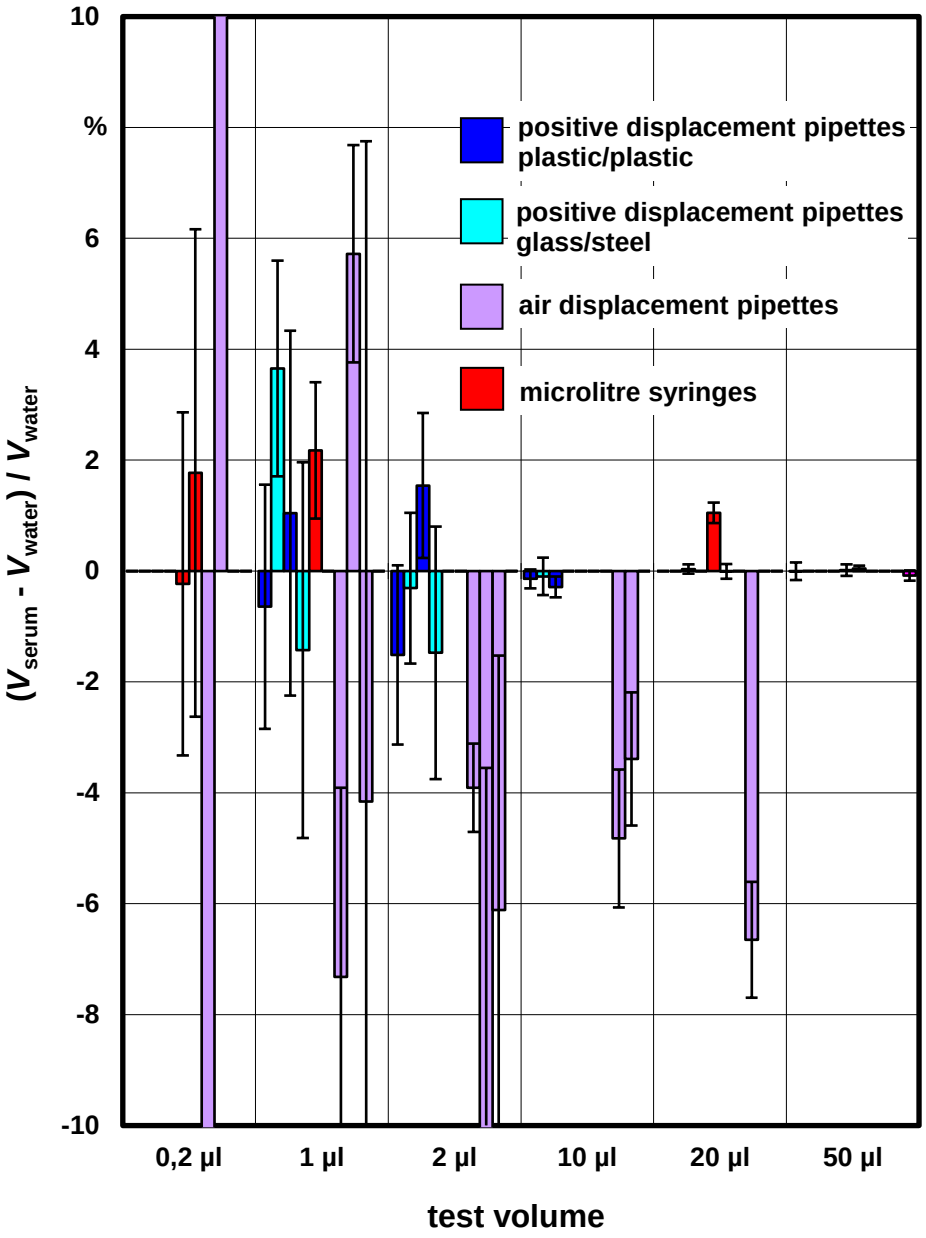


Fig. 27: Difference between the dispensed volume of water and serum for test volumes from 0.2 µl to 50 µl.
The figure shows the relative difference of the values averaged from 10 dispensing operations for water and for serum, respectively. Each bar represents the results for one dispensing device. The measurement uncertainty bars indicate the uncertainty of the difference which was calculated from the repeatability standard deviation of the relative difference (coverage factor k = 2).

9 Possibility of replacing water as a calibration liquid

The results described in sections 7 and 8 show that the results obtained when calibrating liquid dispensing devices with water are not always transferable to other liquids. Calibrating with the liquid that will be used later is more reliable. In the case of human serum, calibration with human serum is therefore preferable. But gravimetric calibration will generally fail since the density of serum is not known with sufficient accuracy. Determining density conventionally by means of a pycnometer is experimentally very demanding; especially filling the pycnometer with serum, which tends to froth, without air bubbles forming is very difficult. This measurement also necessitates more serum than the quantities normally available. Determining density with a densimeter according to the oscillator principle, for which approx. 1.5 ml serum are needed, is not feasible in many laboratories due to the high costs of such devices. When sending serum to another laboratory to determine its density, one runs the risk of the serum changing during transport, since uninterrupted cooling often cannot be guaranteed.

It is therefore necessary to find ways to determine the density of the calibration liquid without risking its changing during transport or to determine the density in a calibration laboratory reliably, but at reasonable costs.

Both ways were pursued in the present investigation. The first method consists in calibrating so-called control material as a replacement liquid. It is described in section 9.2. The second method describes a possibility of determining the density of serum based on analytical data. This method is described in section 10. Prior to these descriptions, section 9.1 contains a report on investigations concerning the stability of serum. From these investigations into stability, it is possible to draw conclusions as to the required accuracy with which the serum density and the density of replacement liquids must be determined.

9.1 Stability of serum

One important aspect for the accuracy required for density determination is the stability of the density of serum. To obtain reference values of the density of serum, both the short-term stability of the density of serum and the long-term stability of frozen serum were investigated.

For these investigations, density was determined by means of a densimeter working according to the oscillator principle. The volume of liquid required for one measurement amounts to approx. 1.5 ml. The measurement uncertainty of the device is smaller than $5 \cdot 10^{-2}$ kg/m^3; its reproducibility is better than $1 \cdot 10^{-2}$ kg/m^3. The device allows the temperature dependence of density to be measured. The uncertainty of the temperature determination amounts to 10 mK. During density determination, the liquid is located in the measuring tube of the device, nearly cut off from the ambient air. Evaporation of liquid components into the ambient air may therefore be neglected.

The short-term stability of the density of serum can be investigated in two cases. In the one case, the serum is kept in a closed container; in the other, it is stored in an open vessel. The conditions prevailing during real dispensing are a mixture of these two cases. The serum may be kept in a closed reservoir while the dispensing operations are taking place, but this reservoir must be opened to fill the dispensing device.

To determine the short-term stability in a closed container, the measurements that had been carried out to determine the dependence of the density on the ambient temperature can be used. As mentioned above, the serum is basically cut off from the environment during the measuring time. There is no evaporation. To check reproducibility, two opposite temperature curves were measured (i.e. the temperature was first progressively increased from the lowest to the highest measurement temperature; after that, a temperature curve was measured where the temperature was progressively reduced, starting from the highest measurement temperature to the lowest). The deviations of the data obtained in this way are clearly smaller than $1 \cdot 10^{-2}$ kg/m^3; they are thus within the reproducibility range of the measuring instrument. It takes up to 2 h to measure the temperature dependence of density. Within this period of time, the density of serum thus remains stable if the serum is placed in a closed container.

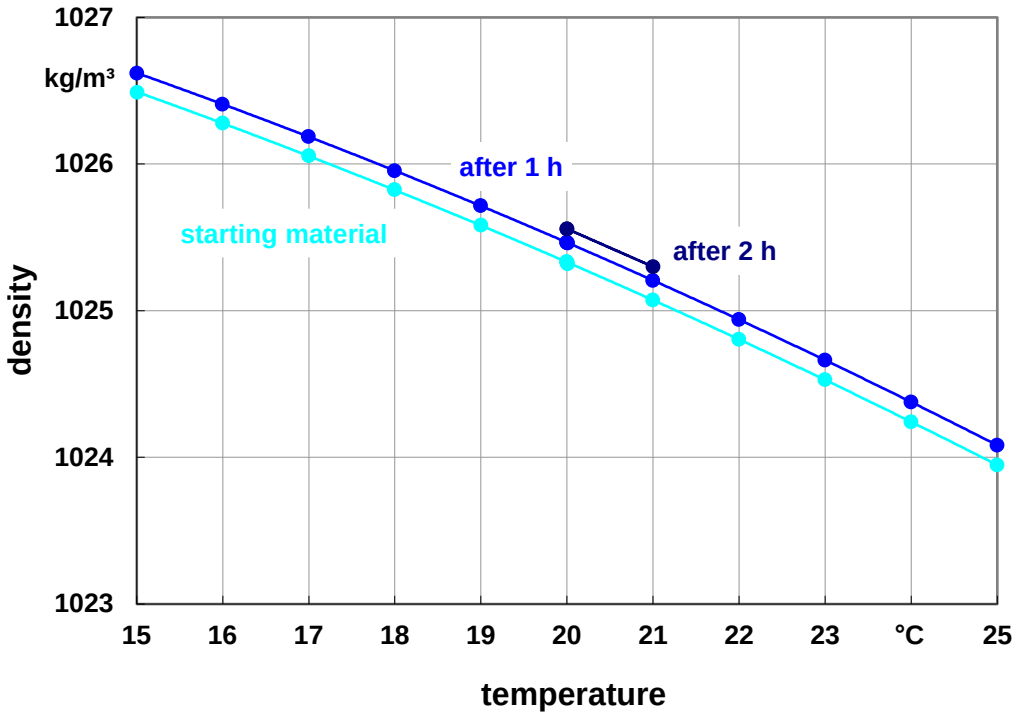


Fig. 28: Short-term behaviour of the density of serum
During the measurements performed to determine the density, the liquid (approx. 3 ml) was stored in an open beaker, as shown in Fig. 3, at room temperature.

In the second case, the measurements were performed on serum that was kept in an open beaker at room temperature between measurements. The measurement results are shown in Fig. 28. This figure shows the deviations for several temperatures to emphasize the reliability of this measurement. Serum is a multi-component mixture. The composition – and thus the density – of this mixture changes due to evaporation. In the case of serum, it is mainly water that will evaporate. This leads to an increase in density. In the present measurement, this increase amounted to 0.13 kg/m^3 after the liquid was stored in an open vessel for 1 h and another 0.10 kg/m^3 after another hour.

As described above, the change in density due to evaporation is reduced by covering the reservoir while performing the dispensing. The change in density of serum during the dispensing operations is therefore estimated to be 0.05 kg/m^3 at the most.

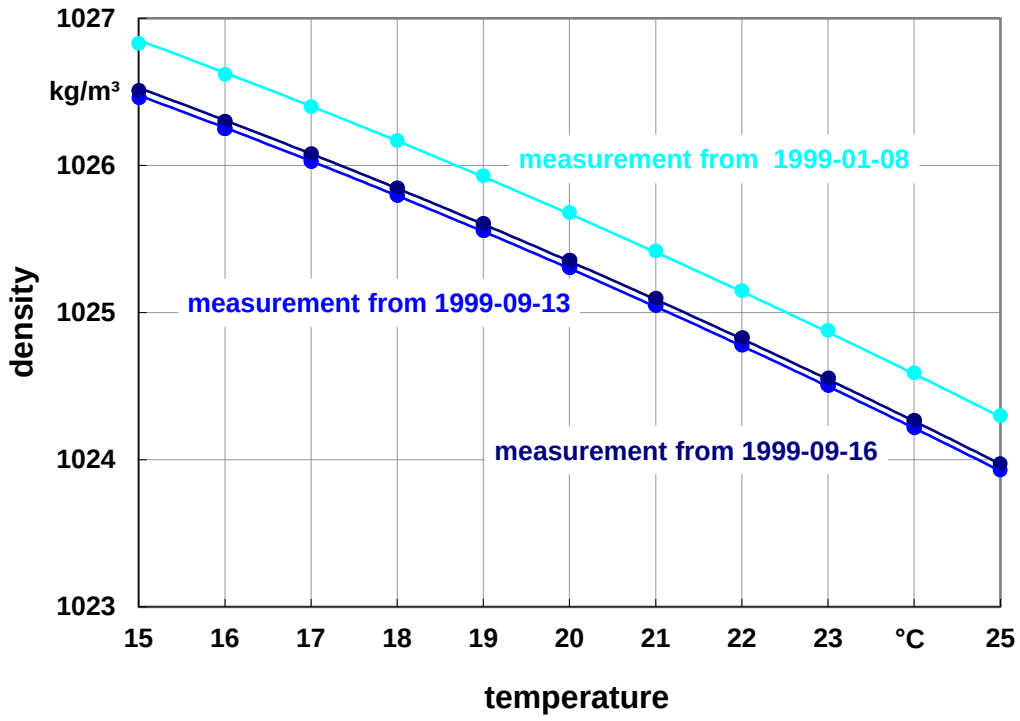


Fig. 29: Long-term behaviour of the density of serum
Between the density measurements, the liquid was stored in a freezer at -18 °C. Density was determined immediately after thawing each time.

As a biologically active liquid, serum does not remain stable for a very long time. For storage purposes, the material was therefore deep-frozen and kept at -18 °C. Even when stored in this way, changes in density occur. This is shown in Fig. 29. In the figure, two density measurements are plotted that were performed at an interval of 8 months as well as a third measurement that was performed only 3 days after the second measurement. Whereas a clear difference appears between the first two measurements (density has decreased by 0.37 kg/m^3), the deviation between the last two measurements is in the order of the measurement accuracy of the densimeter, i.e. not deemed significant. The density loss during the 8 months the serum was frozen cannot be explained. The freezing and thawing processes are evidently not involved in the change: such an influence would have also been visible in the measurements carried out at an interval of 3 days, since the serum was also frozen in this interval.

For the short-term, the possible change in density of the serum due to freezing, thawing, transferring into other vessels, and evaporation should thus be estimated to be in the order of 0.1 kg/m^3. The density indication for serum must therefore only be accurate to 0.1 kg/m^3.

9.2 Control material as a replacement liquid for serum

So-called control material is a serum-like product that is provided by pharmaceutical companies to check analysis results in medical analysis. It is a standard product in medical analysis laboratories. This material is obtained from human serum. A salt solution is added to the serum to stabilize it. It may also be enriched with serum components in order to adjust certain concentrations. Control material is provided both at "normal" and at pathological concentrations. The concentration of the individual components is determined by several laboratories. The material comes with a list of these results.

The control material is obtained from a serum pool (the size of one batch is in the order of approx. 1 m^3). This ensures that a large quantity of the same material is available. This serum pool is distributed as a lyophilized (freeze-dried) powder filled in flasks. Flasks that must be reconstituted by adding 5 ml of water are a common size. Storing and transporting it as lyophilized material ensures high durability against ageing. Only when water is added for reconstitution does the material become as active as serum from a chemical and biological point of view.

Once it has been reconstituted, this control material may be used to calibrate dispensing devices. Since it is manufactured from real serum, it is expected that its dispensing behaviour will be very similar to that of serum. Since a pool of approx. 1 m^3 is available in one batch, it is possible to stock enough control material of the same batch to carry out frequent calibrations.

Density can be determined once by an authorized laboratory. To determine density, the serum may be reconstituted on site, so that it does not have to be transported once reconstituted. It must merely be ensured that all the material produced by reconstitution always has the same density. This was checked by means of several samples. In Fig. 30, three samples reconstituted from material of the same batch are compared. At 20 °C, these samples have a density of 1029.377 kg/m^3, 1029.392 kg/m^3, and 1029.440 kg/m^3, respectively. At 20 °C, the maximum relative difference between the three samples is $6 \cdot 10^{-5}$. The value of 0.1 kg/m^3 can thus be stated as the reproducibility of the density of control material when using material from the same batch (i.e. from the same serum pool). Comparisons using control material from different batches showed slightly larger deviations. Here, the value of 1 kg/m^3 can thus be stated as the reproducibility of the density.

The density reproducibility of control material from the same batch is similar to the value that was stated for the short-term stability of the density of serum. Since the same short-term stability can be assumed for control material as for serum, this reproducibility of the density of control material is therefore sufficient to be used for gravimetric calibration.

Figs. 31 to 32 show the comparison between dispensed volumes of control material and serum. For this purpose, the same representation was chosen as in Figs. 26 and 27. The results of the comparison often show a small deviation against the comparison between serum and water. This is, however, not always the case. The result is therefore not satisfactory. Since the dispensing properties of serum and control liquid are assumed to be very similar as they are so closely related, so that no differences in the dispensed volumes are expected, these deviations are more likely to have been caused by other factors.

Comparing the deviation with the measurement uncertainty indicates that the measured deviation is significant. When comparing serum to water, the measurement uncertainty bars often do not cross the zero line: there are thus clearly measurable differences that are different from zero in the dispensed volumes of serum and of water. In contrast, when comparing control liquid with serum, the measurement uncertainty bars all cross the zero line apart from very few exceptions. The value of the difference in the dispensed volume of control liquid and serum may, within the limits of the measurement uncertainty, thus be zero. The deviations actually measured are then caused by the lack of dispensing reproducibility for serum or control liquid. In particular when using air displacement pipettes, section 8 emphasized the poor reproducibility of the quantity of serum remaining in the tip. This can be deemed an essential reason for the remaining deviations.

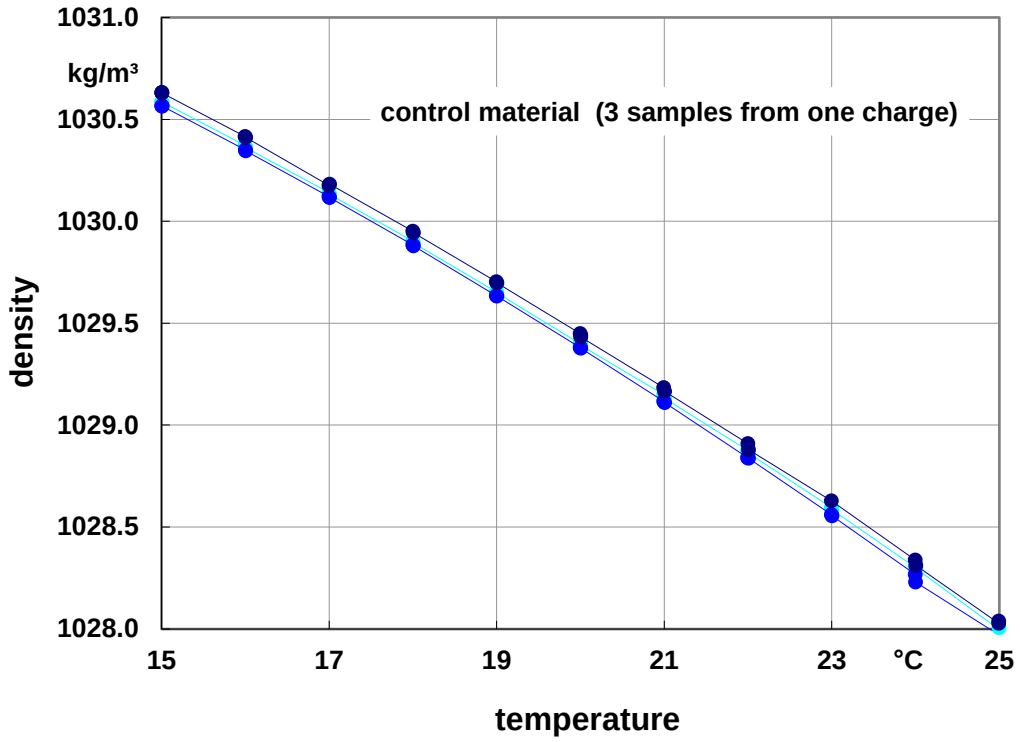


Fig. 30: Temperature-density curve for three control materials reconstituted from material from the same batch (from the same pool)

The still insufficient agreement between the results for serum and control material raise the question as to whether 10 individual dispensing operations on which the averaging is based are sufficient. Section 11 looks into this in more detail.

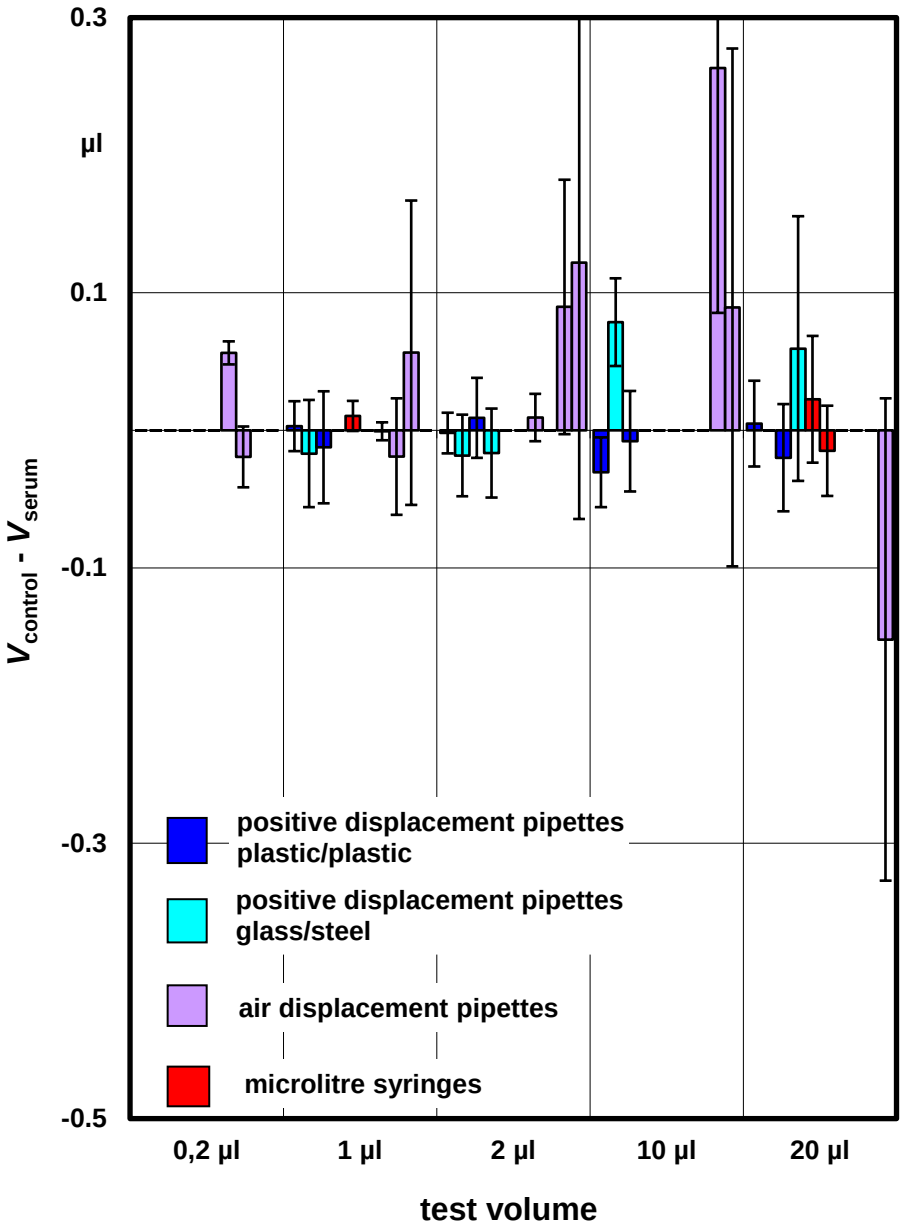


Fig. 31: Difference between the dispensed volume of control material and serum for test volumes from 0.2 µl to 20 µl.
The figure shows the absolute difference of the values averaged from 10 dispensing operations for control material and for serum, respectively. Each bar represents the results for one dispensing device. The measurement uncertainty bars indicate the uncertainty of the difference which was calculated from the repeatability standard deviation of the difference (coverage factor k = 2).

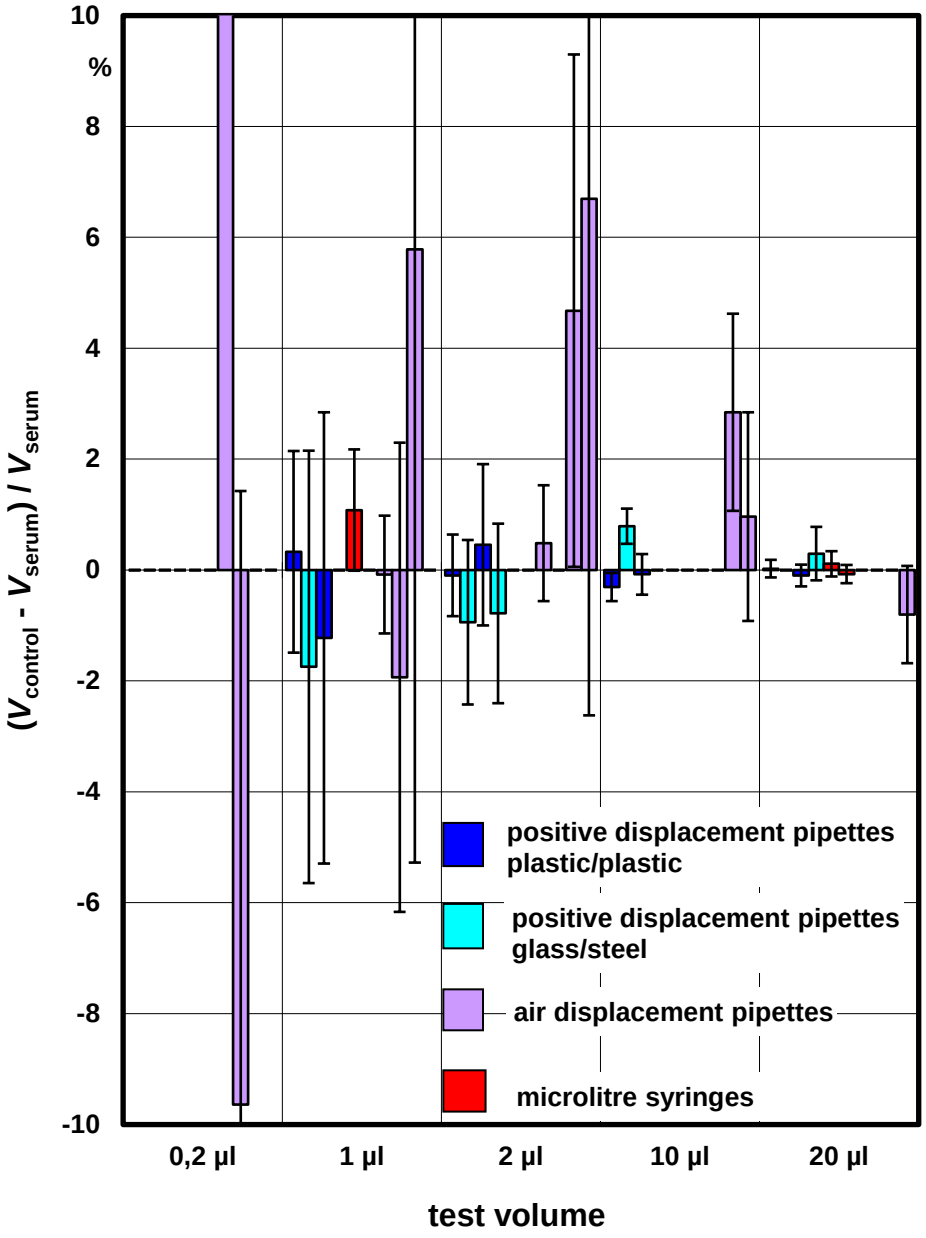


Fig. 32: Difference between the dispensed volume of control material and serum for test volumes from 0.2 µl to 20 µl.
The figure shows the relative difference of the values averaged from 10 dispensing operations for control material and for serum, respectively. Each bar represents the results for one dispensing device. The measurement uncertainty bars indicate the uncertainty of the difference which was calculated from the repeatability standard deviation of the relative difference (coverage factor k = 2).

10 Density of human serum

10.1 Serum density at 20 °C

In the introduction to section 9, it was already pointed out that the density of human serum as a biological fluid is not sufficiently well known to be able to use it for gravimetric calibrations. On the other hand, determining density on site with conventional methods – such as pycnometers, densimeters according to the oscillator principle – is too complex or too cost-intensive.

Within the scope of the present investigation, we therefore determined the density of 69 individual samples. Here, the term of 'individual sample' means serum from a single person that was obtained with only one blood collection. The measurements were performed using a densimeter according to the oscillator principle, as described in section 9.1.

For the investigation, serum was provided by the Medizinische Hochschule Hannover (MHH) and by the University of Bonn. These serums are redundant material that was no longer needed for examinations at the central chemical laboratory of the hospitals. No further selection of the

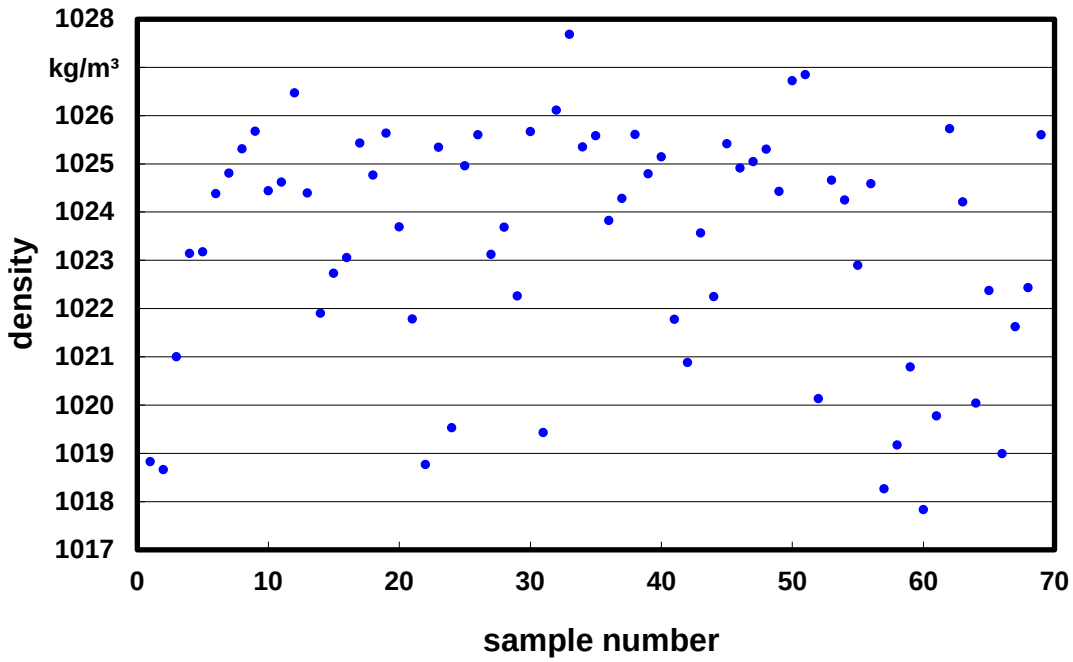


Fig. 33: Measured density of 69 individual serum samples

material was performed. The samples are therefore representative. They might include serum from persons whose blood has been subjected to modification due to diseases.

The results of the investigation are shown in Fig. 33. The density of the samples investigated vary between 1017 kg/m^3 and 1028 kg/m^3. A cluster is observed in the range around 1025 kg/m^3. A simplified statement for the serum density without taking this cluster into account consists in stating the interval (1022 ± 6) kg/m^3.

Serum consists of 90 % water. Serum therefore has a density that is close to that of water. The changes in density due to the other components of serum only account for approx. 2.5 %. Splitting serum density into the density of water on the one hand and the density of the other components on the other to simplify matters yields:

(10-1) $\rho_{serum} = \rho_{water} + \rho_{components} = 998.2\ kg/m^3 + (24 \pm 6)\ kg/m^3$.

The bandwidth of the interval in which all serum densities lie thus amounts to 50 % of the additional density fraction due to the components other than water. Due to the reference to the total density, which includes the water fraction, the value stated in the previous paragraph is possible, while indicating a rather small uncertainty of 0.6 %.

A more detailed description of the density of serum requires a detailed consideration of all the components of serum. Table 6 lists the most important components of serum (other than water – only those are listed below, ignoring that water is the main component of serum). The limits stated in the table include the so-called reference range of concentration – the minimum and maximum concentration of these components in the serum of a healthy person. The values were taken from a brochure of the Medizinische Hochschule Hannover [15].

The table shows that only few components of serum may have an influence on density. These are proteins, which account for approx. 80 % of the serum components, and common salt, which accounts for approx. 10 % of the serum components. If common salt, which is a polar substance, is diluted in water, the volume of salt water thus created does not increase by the volume of diluted salt, but by a much smaller amount. The increase in density of the common salt solution in water is therefore very large.

Table 6: Serum components according to [15]

Component	Concentration reference range min	max	Density of the pure substance
	kg/m^3	kg/m^3	kg/m^3
Total protein	65.0	80.0	?
NaCl	8.1	8.6	2160
Cholesterol $C_{27}H_{46}O$	1.2	3.1	1070
Triglycerides	0.5	5.4	900
Glucose	0.7	1.0	1525
Urea	0.2	0.4	1340
KCl	0.3	0.4	1980
$CaCl_2$	0.2	0.3	2150
$MgCl_2$	0.1	0.1	2320

Cholesterol and triglycerides, as other serum components, exhibit far lower concentrations and densities. Their densities are very close to that of water. Since these two components are nonpolar, it is expected that they will not dissolve without a volume increasing. Their influence on the serum density is therefore very small (these components of serum do not dissolve in pure water). The density of glucose and urea is very different to that of water, but their concentration in serum is very low. This also applies to all other components of serum. The interactions between the individual components are very complex. The influence on density may, however, also be neglected due to the low concentration of most of these components.

An approach to the traceability of the density of serum to the concentration of each of the serum components may thus also be limited to the components protein and common salt. Here, the interaction between the two components is also neglected. The density of serum ρ_{serum} is therefore considered as a linear function of the density fractions of the three components water (ρ_{water}), common salt ($\Delta\rho_{NaCl}$), and protein ($\Delta\rho_{protein}$). A constant term $\Delta\rho_{res}$ is introduced to allow for the influence of the neglected serum components and for the possible influence of interactions. The following equation is then obtained:

(10-2) $\rho_{serum} = \rho_{water} + \Delta\rho_{NaCl} + \Delta\rho_{protein} + \Delta\rho_{res}$

The change in the water density due to the addition of common salt is easy to measure. It was determined experimentally with a densimeter working according to the oscillator principle as being:

(10-3) $\Delta\rho_{NaCl} = (0.7132 \pm 0.0036) \cdot K_S$

K_S is the concentration of common salt in kg/m^3. The maximum uncertainty of density due to the uncertainty stated in (10-3) amounts to 0.03 kg/m^3. This uncertainty is neglected in the following.

To determine the unknown influence of the protein concentration and of the additive residue term on density, the following equation can be evaluated:

(10-4) $\rho_{measured\ value} - \rho_{water} - \Delta\rho_{NaCl} = \rho_{diff} = \Delta\rho_{protein} + \Delta\rho_{res}$

For this purpose, the (measured) density of the sample, its common salt concentration and its protein concentration must be known. For evaluation according to Equation (10-4), 27 pool serums were produced. Each of the pool serums consisted of 10 individual serums. The pools were chosen so as to cover a wide range of protein concentrations. In particular, a pool with an

Table 7: Concentration span of the serum components in the pool serums investigated

	Protein	Sodium	Cholesterol	Triglycerides
Maximum concentration in kg/m^3	82.9	8.85	2.21	2.05
Minimum concentration in kg/m^3	46.3	7.94	1.75	1.34
Average concentration in kg/m^3	66.1	8.26	2.07	1.58
Standard deviation in kg/m^3	6.7	0.17	0.11	0.19

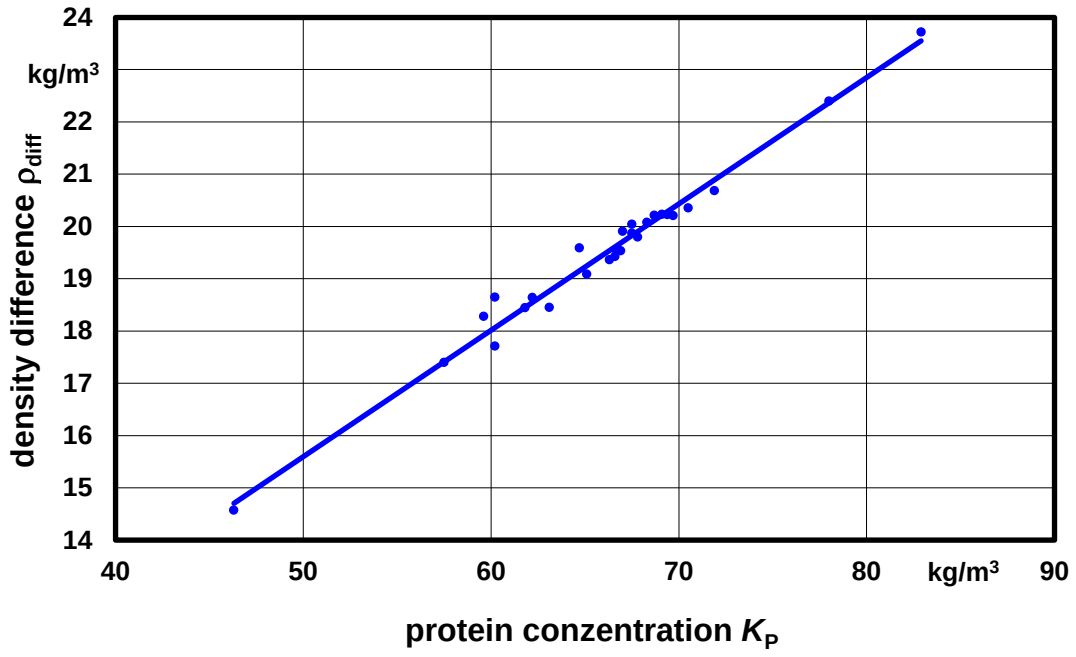


Fig. 34: Density difference ρ_{diff} of the pool serums investigated as a function of the protein concentration, and accordingly fitted straight lines. See text for the uncertainty of the straight lines.

extremely low protein concentration and a pool with an extremely high protein concentration were produced. On the contrary, when selecting the individual serums, attention was paid to the cholesterol and triglyceride concentrations being in a medium range for the influence of the remaining components gathered as a residue to vary as little as possible. Pooling the samples led to another averaging, so that samples with average concentrations in all serum components (except for protein) were available.

The concentrations of the most relevant serum components were determined both in the individual serums and in the pool serums formed using the individual serums. The minimum and maximum concentrations of these serum components in the pool serums are compiled in Table 7. The uncertainty of the analytical determination of the protein and sodium concentrations[15] amounts to approx. 1 % to 1.5 %. This uncertainty may be neglected within the scope of the present consideration.

15 The sodium concentration is usually stated in the analysis. Here, it is assumed that the sodium concentration entirely comes from common salt.

The measured densities of the pool serums are plotted in Fig. 34 as a function of the protein concentration. The plotted straight line confirms that the linear approximation is applicable with good accuracy. It was determined using the least squares method. The serums with extreme protein concentrations can also be described using this straight line. The slope of the straight line[16] is 0.2417; the value of the axis intercept 3.508 kg/m^3.

Thus, the following applies:

(10-5) $\rho_{diff} = 0.2417 \cdot K_P + 3.508$, K_P: protein concentration in kg/m^3

The uncertainty consideration must include both the uncertainty of the slope and that of the axis intercept of the straight line. When fitting a straight line, the axis intercept and the slope are generally correlated quantities. This must be taken into account when stating the uncertainty. The uncertainty statement can, however, be considerably simplified by means of a simple mathematical transform. The axis intercept and the slope are not correlated if the sum across all x-values (here: K_P) becomes 0. This can be achieved by shifting the x-values of the data pairs by the average value of all x-values $x_{average} = \Sigma_i\, x_i/N$, N is the number of data pairs, another notation is $\Sigma_i\, (x_i - x_{average}) = 0$ (cf. [15], Annex H3).

In the present case, the average value of all protein concentrations amounts to 66 kg/m^3, and the uncertainty of the correspondingly shifted pairs of measurement values is:

u(axis intercept) = 0.044 kg/m^3

u(slope) = 0.0067

The equation thus obtained for the uncertainty is:

(10-6) $u^2(\rho_{diff}) = (0.044\ kg/m^3)^2 + (K_P - 66\ kg/m^3) \cdot (0.0067)^2$

or, with the coverage factor $k = 2$:

(10-7) $U(\rho_{diff}) = 2 \cdot \{(0.044\ kg/m^3)^2 + (K_P - 66\ kg/m^3)^2 \cdot (0.0067)^2\}^{1/2}$, $k = 2$.

16 The unit of the slope is 1 because density and concentration formally have the same unit, namely kg/m^3.

The full equation required to compute the serum density from the analytical values of common salt and protein is thus:

(10-8) $$\rho_{poolserum} = \rho_{water} + \Delta\rho_{NaCl} + \Delta\rho_{protein} + \Delta\rho_{res}$$

$$\rho_{poolserum} = 998.2\ kg/m^3 + 0.7132 \cdot K_S + 0.2417 \cdot K_P + 3.508\ kg/m^3$$

$$U(\rho_{poolserum}) = 2 \cdot \{(0.044\ kg/m^3)^2 + (K_P - 66\ kg/m^3)^2 \cdot (0.0067)^2\}^{1/2},\ k = 2$$

K_S: common salt concentration in kg/m^3; K_P: protein concentration in kg/m^3

Some of the uncertainty values for the determination of ρ_{serum} from the analytical values are listed in Table 8. As mentioned above, uncertainties associated to the analytical determination of the common salt and protein concentrations are neglected. The uncertainty of the equation describing the conversion of the common salt concentration in a density fraction is also neglected.

If the protein concentration of the serum is in the normal range from 65 kg/m^3 to 80 kg/m^3, the expanded uncertainty $u(\rho_{normal\text{-}poolserum}) = 0.2$ kg/m^3 ($k = 2$) may be stated as a simplified value for the determination of $\rho_{Poolserum}$ from the analytical values. The relative expanded uncertainty for this range is thus $2 \cdot 10^{-4}$.

This provides the analytical laboratory with a tool to determine the serum density from data that are easily accessible for the laboratory. This allows the analytical laboratory to perform the gravimetric calibration of volumetric dispensing devices on site. The uncertainty stated for the serum density is in the range from 60 kg/m^3 to 70 kg/m^3 for protein concentrations, and thus similar to the uncertainty that must be assumed to apply due to the changes in the serum density

Table 8: Uncertainties of the pool serum density determination

Protein concentration in kg/m^3	40	45	50	55	60	65	70	75	80	85
$U(\rho_{poolserum})$ ($k = 2$) in kg/m^3	0.36	0.29	0.23	0.17	0.12	0.09	0.10	0.15	0.21	0.27

Table 9: Concentration variation of the serum components in the individual serums investigated

	Protein	Sodium	Cholesterol	Triglycerides
Maximum concentration in kg/m^3	80.0	8.42	3.10	1.55
Minimum concentration in kg/m^3	56.0	7.71	1.50	0.70
Average concentration in kg/m^3	70.3	8.17	2.13	1.16
Standard deviation in kg/m^3	6.2	0.17	0.47	0.27

caused by storage and evaporation (cf. section 9.1). It is therefore sufficiently small for calibration.

The results shown were obtained using pool serum. In Fig. 35, the density values of individual serums are compared with the values of the pool serums as a function of the protein concentration. The figure shows the difference between the measured density and the density computed by means of Equation (10-8). The standard deviation of the pool serums plotted in this straight line amounts to 0.22 kg/m^3; the deviation of the individual serums is 0.46 kg/m^3 – and thus approximately twice as much as that of the pool serums. This illustrates clearly that the parameters of the individual serums that were selected to form pools only deviated very little from average values. In the case of the individual serums shown here for comparison purposes, the concentrations of the main serum components are considerably more scattered. For the individual serums, the maximum and minimum concentrations of protein, sodium, cholesterol and triglycerides are listed in Table 9.

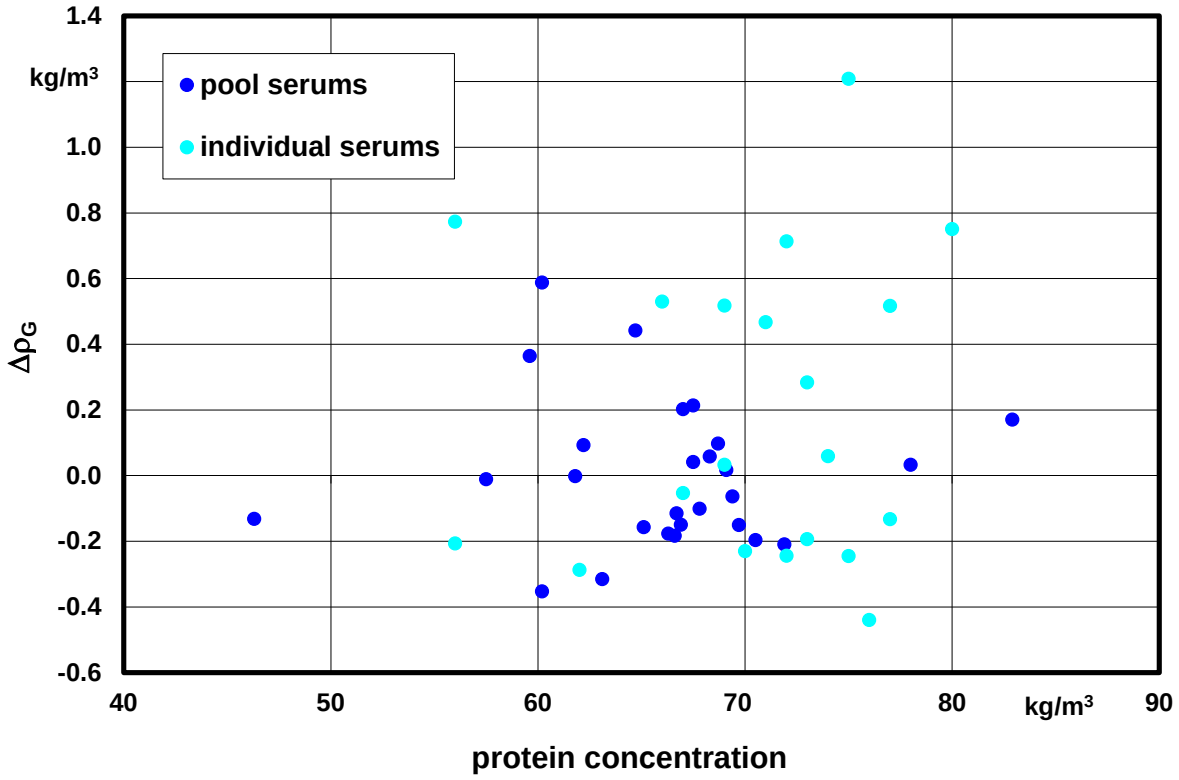


Fig. 35: Comparison of the density of individual serums and of pool serums
The diagram shows the difference $\Delta\rho_G$ between the measured density and the density computed by means of Equation (10-8). The comparison between the individual and the pool serums shows the stronger scattering of the density of individual serums.

10.2 Temperature dependence of the serum density

When determining the serum density, the temperature dependence of density was measured on several serum samples. For this purpose, six samples were measured in the temperature range from 15 °C to 25 °C; sample no. 7 was measured in the temperature range from 15 °C to 40 °C. The measurements were carried out at temperature intervals of 1 °C each. For each measurement series, at least 11 pairs of measurement values $\rho_i(t_i)$ are available; for the measurement series up to 40 °C, 26 pairs of measurement values are available. The protein concentration of the investigated samples varied from 56 kg/m^3 to 76 kg/m^3. Figure 36 shows

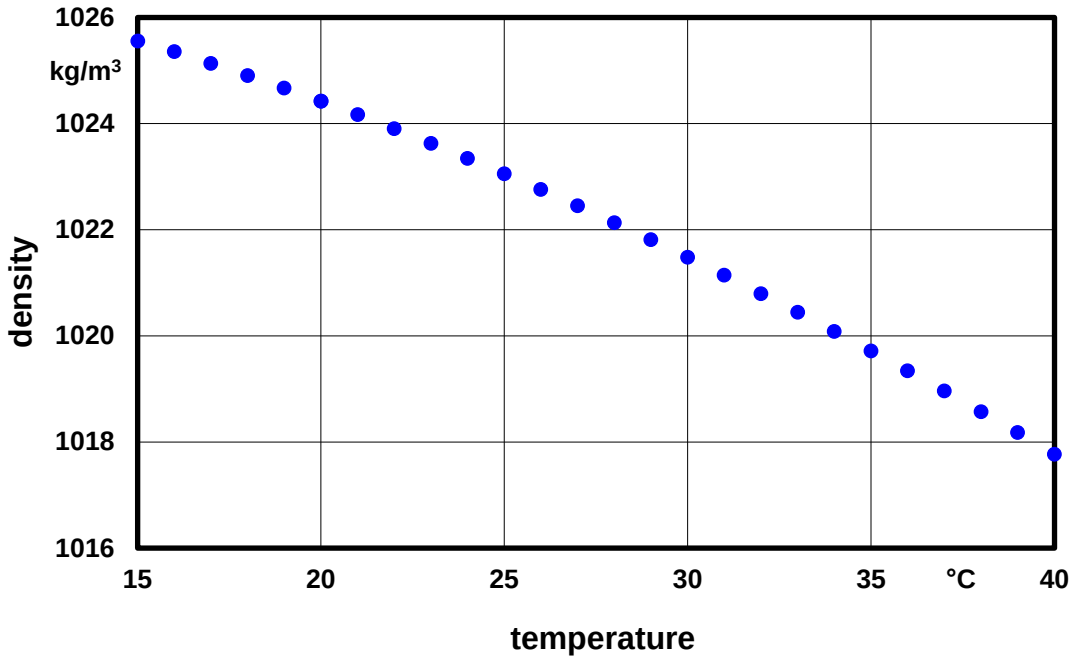


Fig. 36: Temperature dependence of the density of human serum. The shape of the curve is described quantitatively in Equation (10-10).

an example of temperature dependence of the serum density for sample no. E25, an individual serum with a protein concentration of 69 kg/m^3.

For each measurement series, a square polynomial was fitted to the measurement data according to the least squares method. To facilitate comparison of the curves, an equation of the form below was chosen:

(10-9) $\rho_{serum}(t) = \rho_{serum}(20\ °C) \cdot (1 + a_1 \cdot (t - 20\ °C) + a_2\,(t - 20\ °C)^2)$

Since the coefficients do not differ much for different serums, the fitting coefficients of all 7 polynomials were subsequently averaged.

(10-10) $\rho_{serum}(t) =$
$\rho_{serum}(20\ °C) \cdot (1 - 2.438 \cdot 10^{-4}\ K^{-1} \cdot (t - 20\ °C) - 4.460 \cdot 10^{-6}\ K^{-2}\ (t - 20\ °C)^2)$

The standard deviation of all measurement data from the function (10-10) obtained in this way amounts to $8 \cdot 10^{-6}$ in the temperature range from 15 °C to 25 °C (since the function values are close to 1, this numerical value can be used both absolutely and relatively). The standard deviation does not appear to be dependent on the temperature. The equation can also be applied to the temperature range from 25 °C to 40 °C. Here, the deviation of the measured values from

function (10-10) increases steadily with increasing temperature from $8 \cdot 10^{-6}$ at 25 °C to $1.7 \cdot 10^{-4}$ at 40 °C. A statistical statement has little weight of evidence here since there is only one measurement series available.

11 Reproducibility of dispensing results

In the present investigation, many measurements were carried out several times. In these repeated measurements, certain ambient parameters such as the relative humidity in the weighing chamber were varied. However, several measurements were also reiterated under repeatability conditions with a very short time interval between the individual measurements.

Many of these repeated measurements yield measurement results that are not in good agreement within the scope of the measurement uncertainty, i.e. these measurement results deviate from each other by more than the value of the measurement uncertainty.

A quantitative criterion to analyse measurement results is provided in EA 2-03 [14] in Annex H. According to this criterion, the deviation between two measurement results is normalized against the measurement uncertainty.

(11-1) $$E_{\mathrm{n}} = \frac{x_1 - x_2}{\sqrt{U_1^2 - U_2^2}}$$

where x_1 and x_2 are the measurement results (here the values averaged from 10 dispensing operations), and U_1 and U_2 are the associated uncertainties with the coverage factor $k = 2$. If the normalized value E_{n} is smaller than 1, then both results (x_1 and x_2) are acceptable.

Many measurements do not meet this criterion.

A result with a measurement uncertainty that is stated as an expanded uncertainty with the coverage factor $k = 2$ has a coverage probability of 95 % for the corresponding measurement interval. For statistical reasons, it is also possible that 5 % of the measurement results do not lie in this interval and thus do not meet criterion (11-1). In the case of the measurements carried out with piston-operated pipettes, this is, however, more often the case (in 10 % to 20 % of all measurements). This frequency of occurrence depends on the device and on the liquid measured. This suggests that poorly reproduced parameters or additional influences that remain undiscovered do play a role. To verify such an influence, two measurement series consisting of 100 individual measurements instead of the usual 10 individual measurements were carried out. One measurement was carried out using water as a dispensing liquid; another was carried out with serum. The dispensing device used was a positive displacement piston-operated pipette;

the test volume for the dispensing was 10 µl. The pipette tip was not changed during the measurements in order to preclude an influence on the measurement result due to different tips.

The individual measurements were performed according to the same principle as when the measurements were repeated 10 times: all marginal parameters are kept as constant as possible (dispensing procedure, time interval between the individual dispensing operations, room temperature, liquid temperature). The time between two consecutive dispensing operations was 40 s (the same as for the series of 10 measurements); the total measuring time for a series was thus 4000 s. The result of the measurement series using water as a dispensing liquid is shown in Fig. 37. Here the difference between the measured volume and the average of all of the 100 measurements is plotted for each measured value. In addition, a dashed line and a solid line show the repeatability standard deviation for the coverage factors $k = 1$ and $k = 2$. The individual values of this measurement series are scattered around the average value; 31 out of the 100 values are outside the interval for the repeatability standard deviation with $k = 1$; four values are outside the interval for $k = 2$. The statistic of this result is therefore the expected one for a measurement series with values with a normal distribution.

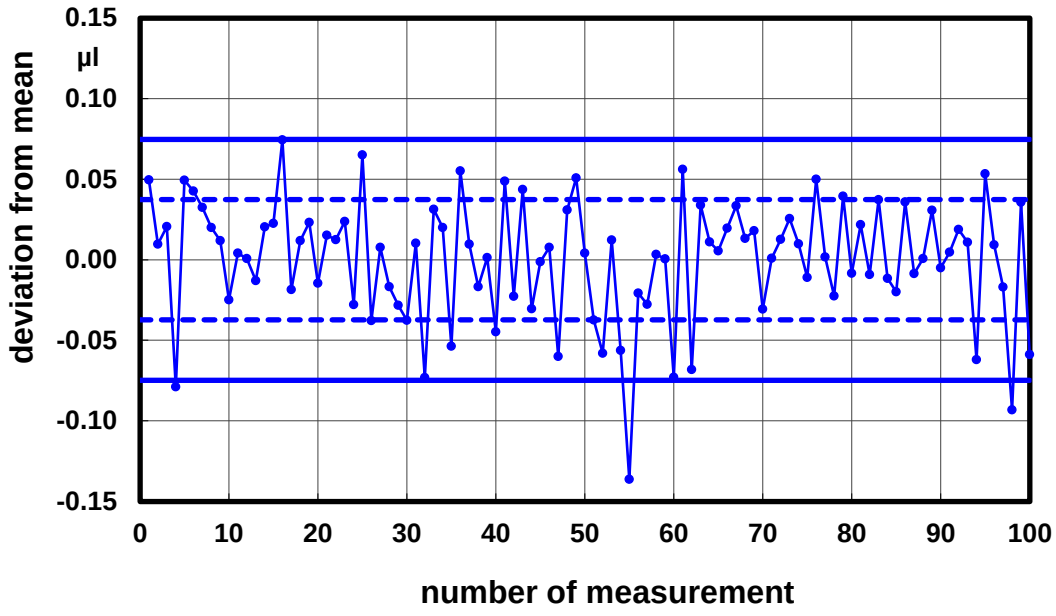


Fig. 37: Dispensing of water repeated 100 times. Positive displacement piston-operated pipette with a test volume of 10 µl. The values plotted are the deviation of the individual measurements from the average of all measurements.
The dashed line shows the simple standard deviation determined for all of the 100 measurements ($k = 1$); the solid line shows the expanded standard deviation ($k = 2$).

However, there are a few peculiarities:

In the case of the measurement values 5 to 12, a drift towards smaller volumes is observed. This drift is larger than the repeatability standard deviation.

The first 25 results are mostly above the average value; the same applies to the last 40 results.

The values between the measurements no. 25 and no. 50 are scattered around the average value, whereas the values between the measurements no. 50 and no. 60 are below the average value. Here, "changes" occur that can apply over a period of more than 10 measurements and then disappear again.

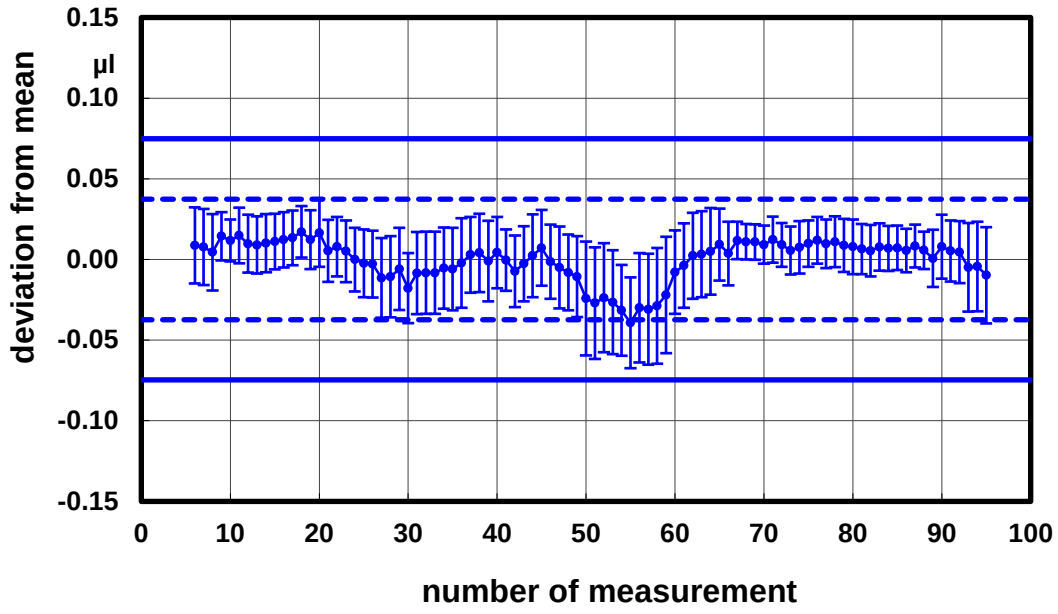


Fig. 38: Dispensing of water repeated 100 times. Floating average with averaging across 10 individual dispensing operations each time. The values plotted are the deviation of the 10 average values from the average of all measurements. The data of the individual measurements are plotted in Fig. 37.
The dashed line shows the simple standard deviation determined for all of the 100 measurements (k = 1); the solid line shows the expanded standard deviation (k = 2).

In a measurement series with 100 individual measurements, these results may vary within the bandwidth that is usually admitted in statistics. If, however, only the usual 10 individual measurements are carried out (this corresponds to the arbitrary selection of 10 consecutive measurement values from this series of 100), then average values and standard deviations may occur for these series of 10 that do not meet the criterion (11-1) mentioned earlier. This is shown in Fig. 38. Here, the floating average value is formed across a series of 10 consecutive

measurement values of the measurement shown in Fig. 37, i.e. the average value across the measurement values 1 - 10, then across the measurement values 2 - 11, 3 - 12, etc. The uncertainty bar plotted here is the uncertainty of the average value (coverage factor $k = 2$) computed from the repeatability standard deviation of the respective series of 10 values[17]. In this representation, the peculiarities described above are easier to be observed. In particular in the middle of the measurement series where the average values of the series of 10 measurements are clearly below the total average value is conspicuous. That these values are below the total average value in this section is **not only** due to the very small measurement value no. 55; all measurement values in this area are too low.

Average values from this part of the curve are not in agreement with values from other parts of the series of 100 measurements. A look at Fig. 38 makes it clear that the measurement uncertainty bars do not always overlap. Criterion (11-1) is even more stringent since in (11-1), the measurement uncertainties are summed up quadratically, whereas linear summation of the measurement uncertainties is performed for the "optical check" in Fig. 38.

These deviations cannot be explained. No changes in the measurement and ambient parameters nor an influence due to the operator could be observed. One possibility could be impurities of the air (dust) that collected at the moist tip of the pipette and influenced the dispensing. These are, however, too small to be detected with the naked eye. Such accretions could possibly be wiped off only after several dispensing operations. The "recovery time" in which the influence of such effects is compensated for lies between approx. 20 to 30 individual measurements.

Fig. 39 shows the results of the second measurement series, which was conducted with serum as the dispensing liquid. The results of the individual dispensing operations are considerably more scattered than when conducting the measurements with water as the dispensing liquid. The number of individual values lying outside the intervals around the average value that are prescribed by the repeatability standard deviation with the coverage factors $k = 1$ and $k = 2$ is higher. The differences between two consecutive measurements are also larger than when using water. From measurement value nos. 40 to no. 60, there is a drift towards smaller values; between measurement values nos. 60 and 80, in contrast, there is a drift towards larger

17 It must be kept in mind that the measurement uncertainty contributions associated with volume measurement using the gravimetric device are negligible.

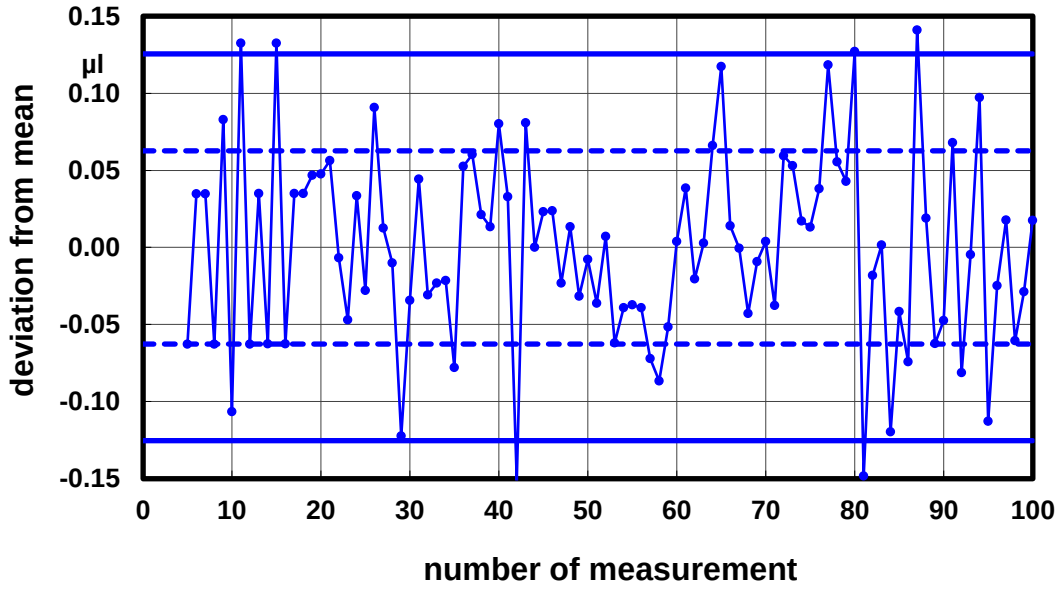


Fig. 39: Dispensing of serum repeated 100 times. Positive displacement piston-operated pipette with a test volume of 10 µl. The values plotted are the deviation of the individual measurements from the average of all measurements.
The dashed line shows the simple standard deviation determined for all of the 100 measurements (k = 1); the solid line shows the expanded standard deviation (k = 2).

measurement values. There are also larger areas where all measured values lie above or below the average value.

The representation in Fig. 40, by analogy with Fig. 38, shows this behaviour more clearly. There are strong changes in the floating average values. Here too, the intervals prescribed by the average value and the measurement uncertainty often do not overlap. The measurement uncertainty is up to three times as high as the measurement uncertainties observed when measuring with water. One noteworthy aspect is the low measurement uncertainty in the area surrounding measurement point no. 50. Here, a strong drift becomes evident, both for individual measurements and for the average value. However, the measurement uncertainty computed from the repeatability standard deviation is as high as the smallest measurement uncertainties observed when measuring with water. Here, the "recovery time" mentioned when measuring with water amounts to approx. 30 measurements.

Serum contains impurities in the form of coagulated proteins. If these coagulations adhere to the pipette tip, they can have a similar influence on the measurement as dust particles. The

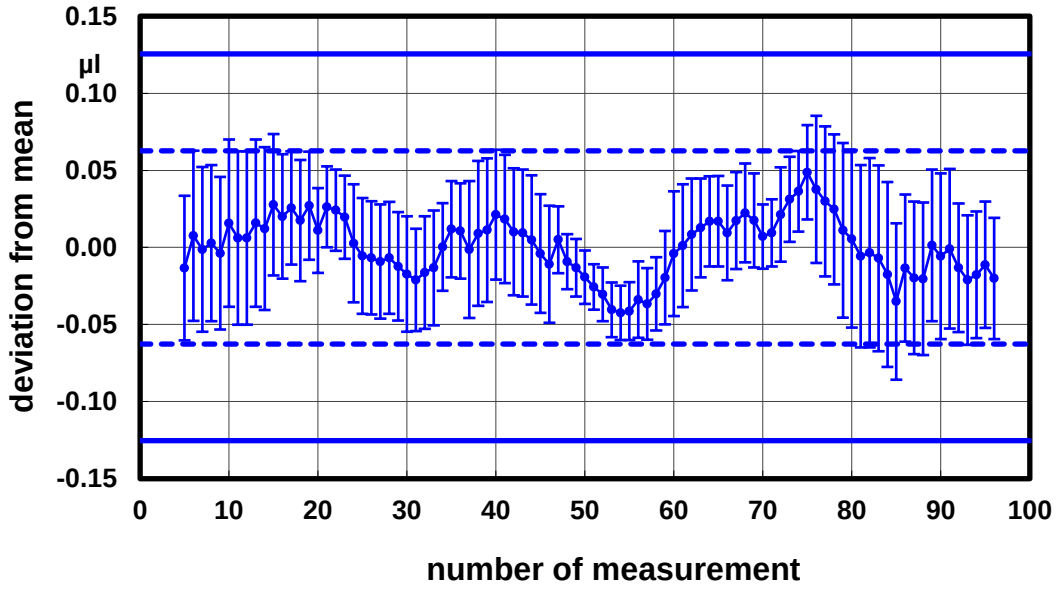


Fig. 40: Dispensing of serum repeated 100 times. Floating average with averaging across 10 individual dispensing operations each time. The values plotted are the deviation of the 10 average values from the average of all measurements. The data of the individual measurements are plotted in Fig. 39.
The dashed line shows the simple standard deviation determined for all of the 100 measurements (k = 1); the solid line shows the expanded standard deviation (k = 2).

result of the measurement with serum thus seems to confirm the assumption according to which dust particles adhering to the pipette tip could be responsible for the deviations.

The main difference in the floating average values amounts to 0.056 µl with water as a dispensing liquid and to 0.091 µl with serum. Differences of that order must be taken into account in repeat measurements and may therefore also occur when comparing the dispensed volumes of different liquids.

Apart from three exceptions, the differences between the dispensing of serum and the dispensing of control liquid shown in Fig. 31 lie below this span of 0.09 µl. The values for positive displacement pipettes and microlitre syringes are even well below these values: apart from one exception, all of the differences lie below 0.03 µl in this case. This comparison makes it clear that the usual measurement procedure consisting of 10 individual measurements does not allow improved accuracy. If the above-mentioned "recovery time" is taken into account, a

series of at least 50 individual measurements seems necessary to obtain a reliable average value. This corresponds to approximately twice the "recovery time"[18].

These results show that indicating the repeatability standard deviation based on a series of 10 dispensing operations often yields an uncertainty that is too small. The question as to how the statement of the uncertainty must be modified to enable more reliable uncertainty statements cannot be answered based on the two-measurement series shown here.

18 This is only necessary for calibrations with high accuracy requirements. This is shown by a comparison between the measurement values obtained with water as a sampling liquid and the requirements laid down in the ISO 8655-2 standard, which apply to water as a sampling liquid. For a test volume of 10 µl, the maximum admissible systematic deviation amounts to 0.12 µl, and the maximum admissible repeatability standard deviation to 0.08 µl. The difference between the minimum and the maximum value measured here amounts to 0.056 µl; the maximum repeatability standard deviation is 0.057 µl (with the coverage factor $k = 1$!). These two extreme values thus still are within the limits defined by the standard. The maximum admissible repeatability standard deviation is, however, sometimes clearly exceeded when using serum as a sampling liquid.

12 Alternative volume determination method: photometry

As already mentioned in section 3.1, another method that is often used to determine volume is based on the photometric measurement of the absorption of a contrast agent. When using the photometric method, a contrast agent solution with known contrast agent concentration is dispensed into a liquid of known volume and known absorbance. From the change in absorbance measured, it is possible to determine the volume of the contrast agent solution added to the mixture.

12.1 Physical fundamentals of photometry

Light absorption by a contrast agent solution can be used as a measure for the concentration of the contrast agent in the solution. For many contrast agents and not too high concentrations of contrast agent, the extinction coefficient (also called absorptivity) of the light that traverses the contrast agent solution is proportional to the contrast agent concentration. This relation is known as Beer's law (or Beer-Lambert-Bouguer law).

(12-1) $k = k_c \cdot c$

k is the extinction coefficient of the contrast agent solution; k_c is the molar extinction coefficient, and c is the concentration.

To determine the extinction coefficient of light through a liquid, the light is sent through a spectrophotometric cell in the photometer, and the ratio of the intensity of incident radiation to outgoing radiation is determined. The correlation between the incident and the outgoing radiation intensity is described by the Lambert-Bouguer law. The ratio of the incident to the outgoing radiation intensity depends exponentially on the layer thickness of the liquid under test and on the extinction coefficient k characterizing the liquid.

(12-2) $I_o = I_i \cdot e^{-k \cdot d}$

I_i, I_o Intensity of the incident and of the outgoing radiation, respectively

d Thickness of the liquid layer

k Extinction coefficient (absorptivity) of the liquid

An equation using a decadic base rather than the natural base e is often used for technical applications.

(12-3) $I_o = I_i \cdot 10^{-k' \cdot d} = I_i \cdot 10^{-A}$

k' decadic extinction coefficient (absorptivity) of the liquid

$A = k' \cdot d$ absorbance of the liquid

The measurement result usually indicated by measuring instruments is absorbance *A*, i.e. the dimensionless product of the layer thickness by the decadic extinction coefficient[19].

(12-4) $A = k' \cdot d = - \lg(I_o/I_i)$

The layer thickness of the measured liquid is determined by the spectrophotometric cell and is, in principle, known. Calculating the extinction coefficient from the absorbance is therefore simple, but an operation that is often not performed. In comparison measurements carried out with spectrophotometric cells of the same layer thickness, the absorbance values obtained are compared instead.

The absorption of light by contrast agents generally depends on the wavelength. Absorbance should therefore be measured at a wavelength at which absorption reaches its maximum.

Beer's law (Equation (12-1)) can also be expressed as the relation between the contrast agent concentration and absorbance.

(12-5) $c = k_c' \cdot A$

If the straight lines described by (12-5) are known, it is possible to determine the concentration *c* of a contrast agent solution by measuring the absorbance.

To be able to use photometry in order to calibrate volumetric dispensing devices, it is necessary to spike the calibration liquid with contrast agent. At least one dilution (in water) is produced from this original contrast agent solution. This dilution is then used to determine the increase of the straight lines described by (12-5) – i.e. the relation between the absorbance and the concentration of the contrast agent solution. Due to the linearity between the contrast agent solution and absorbance, **one** dilution is sufficient. However, **several** different dilutions are required to check the validity range of Beer's law. Using several dilutions also reduces the

[19] Absorbance is dimensionless. The "unit" used is, however, often A.

uncertainty when indicating a numerical value for the increase. Here, the reference quantity for the concentration of the original contrast agent solution can be arbitrarily[20] chosen as 1, since it is merely used as a comparison value in other calculations. The numerical value equation determined in this way is called *calibration curve* in the following.

The original contrast agent solution is also the liquid used for dispensing with the dispensing device to be calibrated. This dispensing operation is performed directly into a vessel with an existing volume of liquid whose absorbance is known. The existing volume of liquid must be the same liquid as that selected as a dilution to measure the calibration straight lines – water in the present case. After the liquid has been dispensed and thoroughly mixed with the previously existing volume of liquid, the solution's absorbance can be measured. Based on the calibration curve, the change in absorbance allows the change in the contrast agent concentration to be calculated, so that the volume added by dispensing can also be calculated.

(12-6) $$c = \frac{c_1 V_1 + c_2 V_2}{V_1 + V_2}$$

(12-7) $$V_2 = V_1 \frac{c_1 - c}{c - c_2}$$

where c is the contrast agent concentration after dispensing; V_1 is the existing volume of liquid; c_1 is the contrast agent concentration in the existing volume; V_2 is the volume (to be determined) added by dispensing, and c_2 is the contrast agent concentration of the volume added by dispensing. In the above-described measurements, the existing volume was pure water without contrast agent, so that $c_1 = 0$. (The absorption of light by the air in the beam path, by the walls of the photometric cell and the pure water contained in the cell as well as the reflected fractions of light are compensated for by the measuring instrument by comparison with a reference photometric cell.) If the concentration of the original contrast agent solution is set as 1 when determining the calibration curve, then $c_2 = 1$ applies. Thus, (12-7) can be simplified as follows:

(12-8) $$V_2 = V_1 \frac{c}{1 - c}$$

If a calibration curve is measured with the photometer with which the volumetric dispensing devices have been photometrically calibrated, then it is easy to trace the photometric measurements since one and the same measuring instrument is used to carry out merely one comparison measurement. The comparison measurement ensures traceability of the volume V_2

[20] This is possible since the calibration curve must be measured directly. If reference to existing calibration data is required, then the concentration must be indicated in absolute units of the amount of substance.

to be determined to other volumes. These other volumes are the volume V_1 and the volumes of the original contrast agent solution and of the dilution that are used to determine the calibration curve. These volumes must therefore be traceable. In the present case, traceability of these volumes was ensured by gravimetric volume determination.

12.2 Producing the contrast agent solutions to calibrate volumetric dispensing devices

Calibrating volumetric dispensing devices using the photometric method first requires a calibration contrast agent solution to be produced and the validity range of Beer's law to be verified. For this purpose, it is necessary to select a contrast agent that will dissolve in the liquid to be dispensed. In the investigation at hand, the contrast agent ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) was used. The contrast agent dissolves both in water and in serum and has proved stable in both liquids. An aqueous solution of ABTS is of a very light green colour; the absorption maximum is in the near UV range at 340 nm (Fig. 41).

To verify the validity range of Beer's law, solutions of the contrast agent in water and in serum were produced with various contrast agent concentrations. These solutions were used to measure the absorbance. Figs. 42 and 43 show the results obtained. On the axis of ordinate, "volume concentration" designates the concentration of the diluted contrast agent solution in relation to the original contrast agent solution. When performing the measurement with water, this solution consisted of 28.6 mg of ABTS dissolved in 11.937 g of water; when performing the measurement with serum, it consisted of 32 mg of ABTS dissolved in 12.07 g of serum. In Fig. 42, the linear area at small contrast agent concentrations is clearly visible. In the present measurements, the intermediate area, in which the calibration curve is nonlinear, was not used to calibrate volumetric dispensing devices. The "saturation range" at absorbance values of more than 4 (which corresponds to an intensity of the light coming out of the photometric cell of less than 10^{-4} of the intensity of the incident light) is an artefact of the measuring device that is not able to resolve absorbances of more than 4.

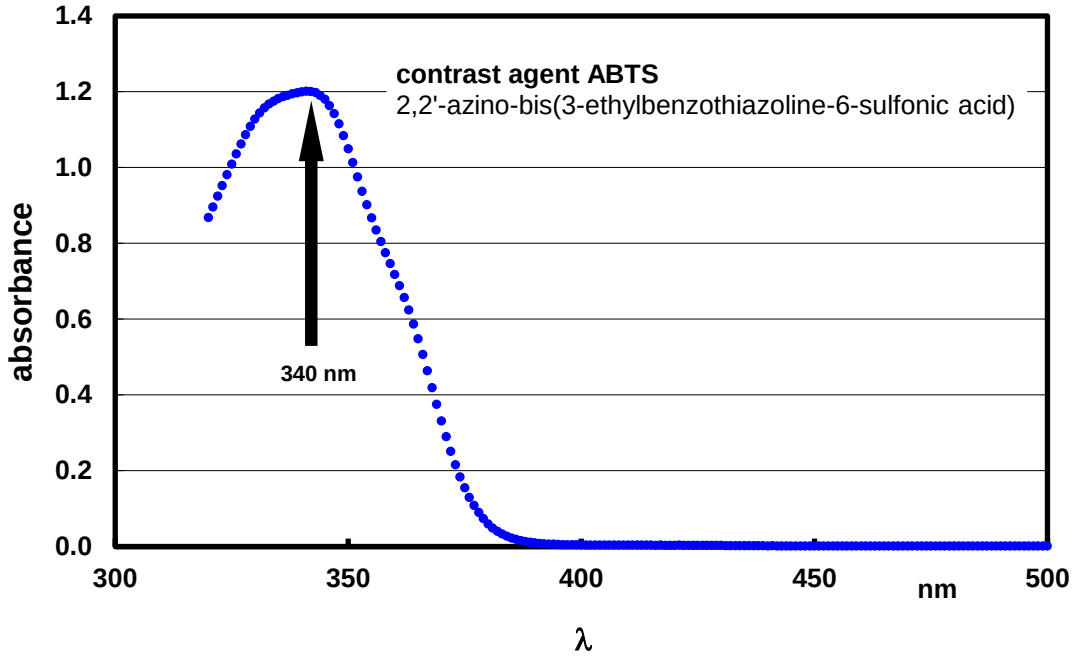


Fig. 41: Absorption spectrum of the contrast agent used (ABTS). The absorption maximum is 340 nm. This wavelength was used for the photometric investigations.

The linear area can be described by the numerical value equation $A = 1.7220 \cdot c$ where A is the absorbance, and c is the concentration of the contrast agent solution in %. The uncertainty of this indication, determined from the linear regression, amounts to $2.8 \cdot 10^{-3}$ at $c = 0$ and $4.4 \cdot 10^{-3}$ at $c = 1$ % (coverage factor $k = 2$). The minimum uncertainty is given with $2.0 \cdot 10^{-3}$ for $c = 0.32$ %. The corresponding values of the calibration curve for ABTS in serum are: numerical value equation: $A = 1.8212 \cdot c$, uncertainty: $3.2 \cdot 10^{-3}$ at $c = 0$ and $4.8 \cdot 10^{-3}$ at $c = 1$ %. The minimum uncertainty is given with $2.2 \cdot 10^{-3}$ for $c = 0.34$ %.

All volumes of liquids that were used to draw up the calibration lines were determined gravimetrically. Their uncertainties are negligible.

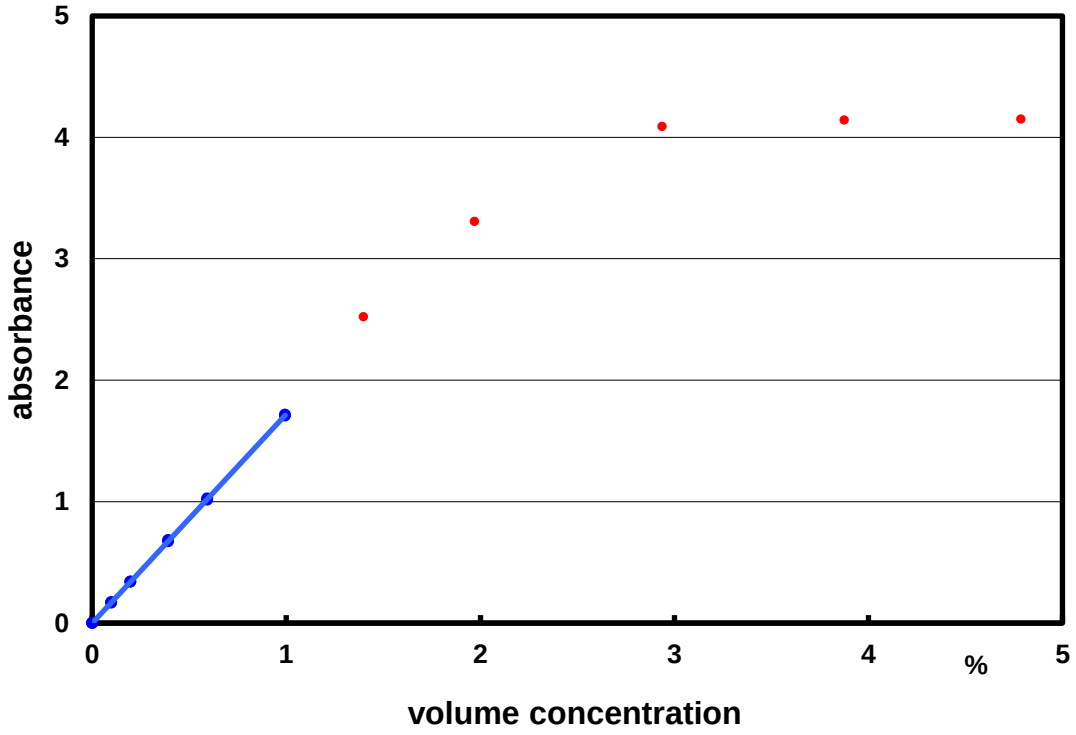


Fig. 42: Calibration curve used to verify Beer's law for a solution of ABTS in **water**. In the lower range, there is a linear correlation between absorbance and the concentration of the contrast agent solution.

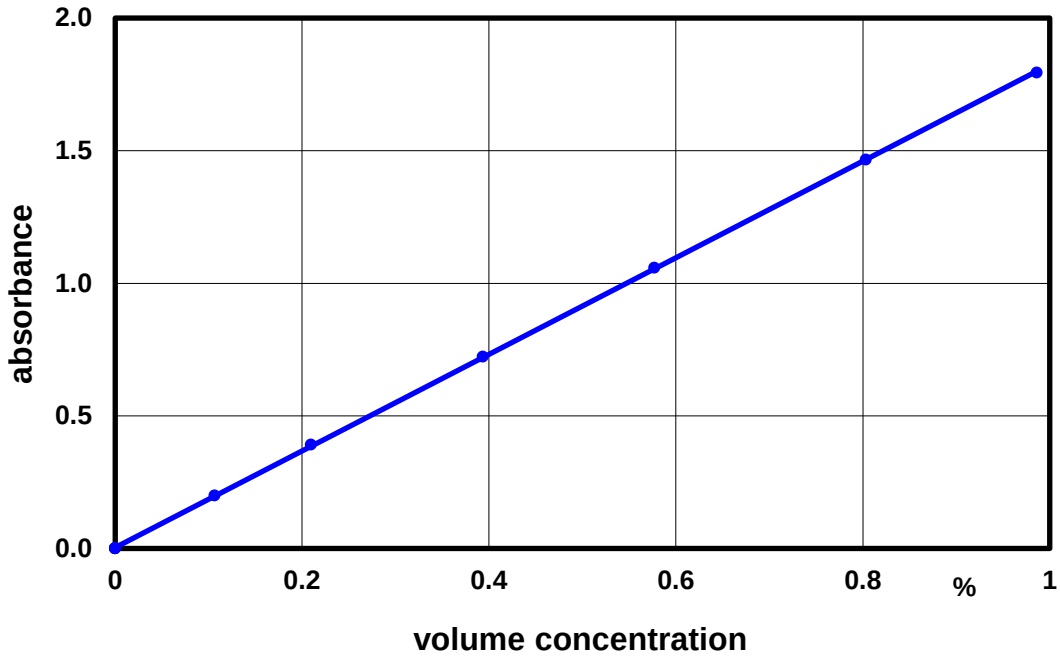


Fig. 43: Calibration curve used to verify Beer's law for a solution of ABTS in **serum**. The area represented is the linear area prevailing for volume concentrations $c < 1$ %.

12.3 Photometric calibration of two pipettes

To allow direct comparison between the gravimetric and the photometric procedures, calibrations were carried out simultaneously on two pipettes, i.e. using the same volume of liquid to be dispensed, both gravimetrically and photometrically. For this purpose, each dispensing operation was carried out with the contrast agent solution and expelled into a new vessel; the volume was then measured gravimetrically first. The measurements were carried out under similar conditions as the gravimetric measurements described in Section 4. The vessels used here, however, were 1.5 ml sealable plastic vials. Due this change, a weighing instrument with a larger maximum load was used. The volume of liquid already present in the vials was also determined gravimetrically.

Subsequently to the gravimetric measurement, the existing liquid in the vials and the added contrast agent solution added to it by dispensing were placed in a shaker for blending and then measured photometrically. Prior to the photometric measurement, the vials were stored next to

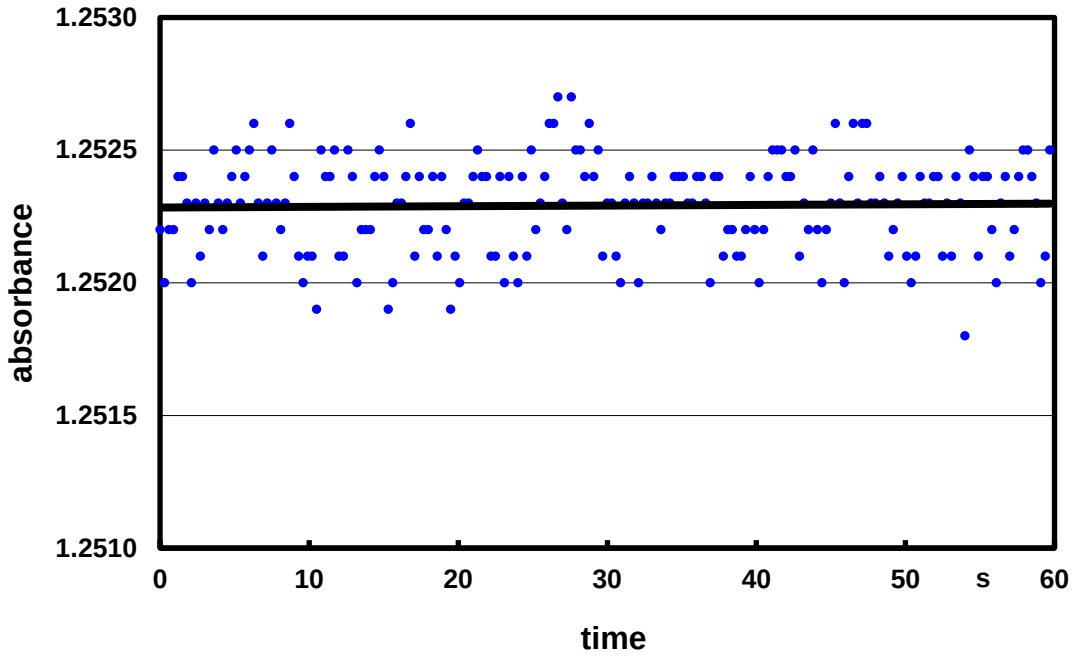


Fig. 44: Measured absorbance of a dispensing of 10 µl of contrast agent solution in 1.3 ml of water. The straight line plotted is fitted to the measurement data by means of a linear regression. It shows the time drift of absorbance from 1.252283 to 1.252298 after 60 s. The resolution of the photometer is 0.0001. The drift is therefore negligible within the limits of the measurement uncertainty.

the photometer for one hour for thermalization purposes. For the purposes of the photometric measurement, the liquid had to be filled into a photometric cell. The photometric cell was cleaned before being filled again for a new measurement. When transferring the liquid from the vial into the cell, attention was paid to keeping the heating due to the warmth of the operator's hands as low as possible. Since the temperature of the measuring chamber of the photometer was approx. 5 °C higher than the ambient temperature, the measurement was carried out immediately after inserting the photometric cell into the photometer. The measuring time in the photometer was 1 min. Control measurements carried out over a period of 2 min showed that the change in absorbance within this period is always smaller than 0.002. A measurement carried out over a period of 30 min showed a decrease in the measurement result by 0.01 due to the liquid's warming.

A photometric measurement is represented in Fig. 44. The measurement consists of 200 individual measurements that were performed at 0.3 s intervals. The relative standard deviation of the measured values for the measurement shown is $1.4 \cdot 10^{-4}$; the relative standard

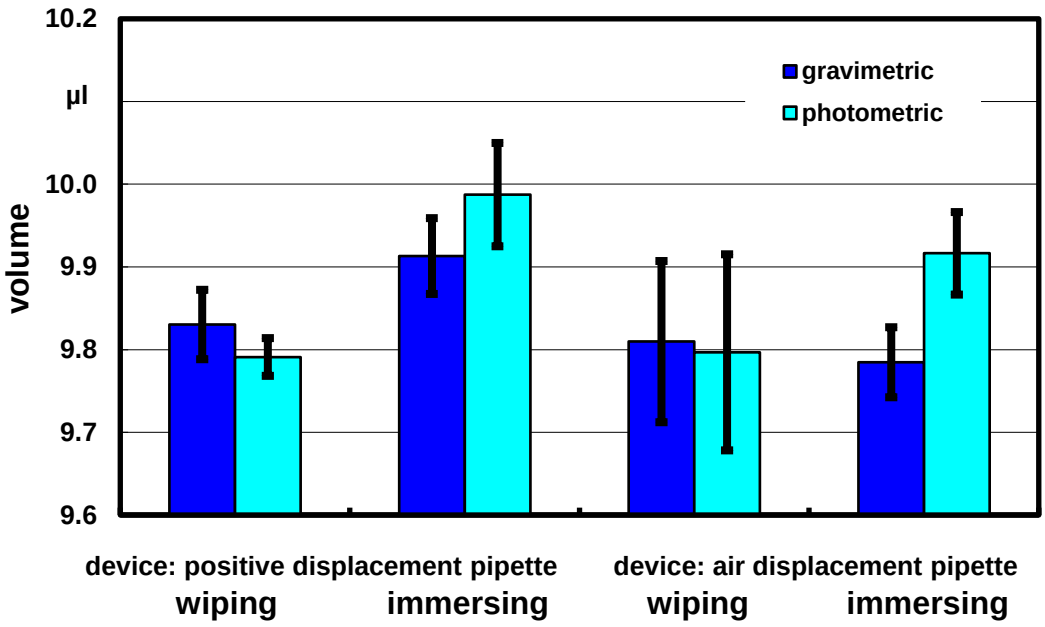


Fig. 45: Comparison of the dispensing results measured gravimetrically and photometrically. The liquid to be dispensed is **water** spiked with contrast agent; the test volume is **10 µl**. The results are values averaged from 10 individual measurements; the measurement uncertainty bars were determined from the repeatability standard deviation with the coverage factor $k = 2$. The dispensing operations represented here were obtained using a positive displacement pipette and an air displacement pipette for the two different dispensing methods described in the text.

deviation of the average value amounts to $1.0 \cdot 10^{-5}$. The time drift of the signal during the measurement time was determined by means of a linear regression. The relative change in absorbance amounts to $1.2 \cdot 10^{-5}$ after a measuring time of 60 s. The drift is therefore negligible within the limits of the measurement uncertainty.

From the absorbance values determined, the dispensed volumes were calculated according to the method described in Section 12.1. Similar to the gravimetric method, 10 individual measurements were carried out for one calibration.

The photometric measurements were performed using a positive displacement pipette and an air displacement pipette as examples. The dispensing liquids used were water spiked with contrast agent, and serum spiked with contrast agent. The dispensed test volumes were 10 µl and 2 µl. Moreover, two different dispensing procedures were investigated. The first procedure consisted in dispensing the liquid into a dry vessel. The pipette was wiped off against the wall of the vessel. The pre-existing volume of liquid was added only afterwards. This procedure

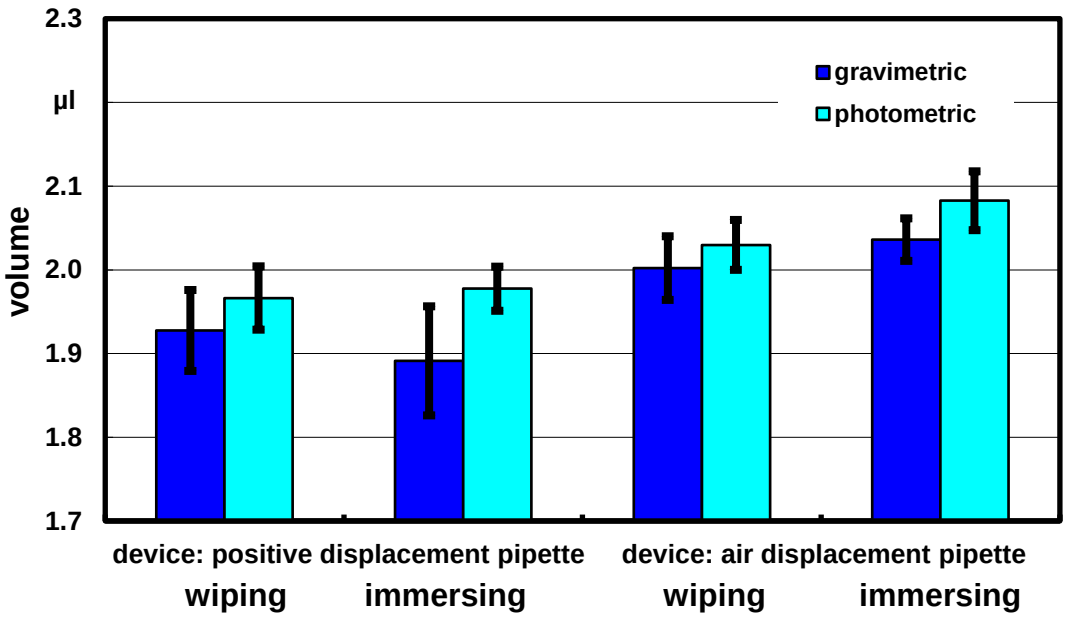


Fig. 46: Comparison of the dispensing results measured gravimetrically and photometrically. The liquid to be dispensed is **water** spiked with contrast agent; the test volume is **2 µl**. The results are values averaged from 10 individual measurements; the measurement uncertainty bars were determined from the repeatability standard deviation with the coverage factor $k = 2$. The dispensing operations represented here were obtained using a positive displacement pipette and an air displacement pipette for the two different dispensing methods described in the text.

corresponds to the dispensing procedure used for gravimetric calibration. The second procedure consisted in placing the pre-existing volume of liquid into the vessel before adding the contrast agent solution by dispensing. In this second procedure, the tip of the pipette was not wiped off against the wall of the vessel, but immersed in the pre-existing volume of liquid instead. No wiping off was carried out after the dispensing. This procedure reflects the usual handling in everyday analytical practice.

In Figs. 45 to 47, the measurement results of these two different methods are compared. These figures show the comparison between a positive displacement pipette and an air displacement pipette for the two above-described dispensing procedures ("wiping off" and "immersion"). These three figures also directly compare the gravimetric with the photometric results obtained from the same dispensing.

Fig. 45 shows the comparison of the results for the dispensing of 10 µl water. The results show a good agreement between the data obtained photometrically and those obtained gravimetrically. The values measured photometrically using the immersion procedure are approx. 1 % larger than those obtained gravimetrically. Here, there might be a transfer of at least part of the contrast agent from the liquid, which normally remains as a residue inside the pipette tip, into the pre-existing volume of liquid. This may be the result of either direct liquid exchange or diffusion. During the dispensing operation, the pipette tip is immersed in the liquid for approx. 3 s to 5 s, which is sufficient for exchange and diffusion processes. Such a process suggests an excessive dispensed volume.

The comparison of the measurement data obtained with a test volume of 2 µl is shown in Fig. 46. The results are also in very good agreement. The photometric values obtained with the "wiping-off" procedure are approx. 1 % to 2 % larger than the gravimetric values. The differences in the values measured with the immersion procedure are, however, twice as large.

The increase in the dispensed volume observed in the photometric measurement procedure obtained by immersion of the pipette tip compared to those obtained with the gravimetric procedure exhibits roughly the same order of magnitude as the measurement uncertainty determined from the repeatability standard deviation in all the measurements performed. However, this change in volume is always positive. The result must therefore be deemed significant.

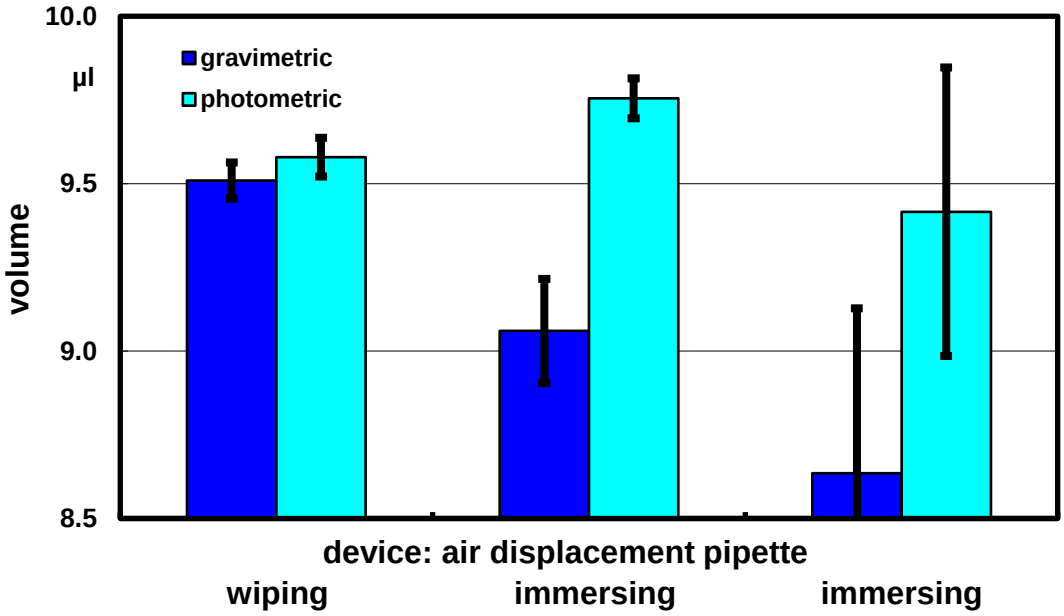


Fig. 47: Comparison of the dispensing results measured gravimetrically and photometrically. The liquid to be dispensed is **serum** spiked with contrast agent; the test volume is **10 µl**. The results are values averaged from 10 individual measurements; the measurement uncertainty bars were determined from the repeatability standard deviation with the coverage factor $k = 2$. The dispensing operations represented here were obtained using an air displacement pipette for the immersion method described in the text.

Fig. 47 shows three measurements performed with a test volume of 10 µl serum. The measurements were only carried out with the air displacement pipette that was also used for the measurements of the contrast agent solution in water. The results obtained using the wiping-off method show excellent agreement between the photometric and the gravimetric results. Both results confirm the underfeeding of 5 % already known from the investigations described in Section 8.

In contrast to this, the two measurements performed using the immersion method show clear differences. The first measurement performed according to the immersion method (the measurement in the middle in Fig. 47) confirms the photometric result obtained with the wiping-off method. The value is 1.8 % higher than the value obtained with the wiping-off method, as already shown by the measurements carried out using water. The gravimetric value, however, is clearly smaller than the photometric value and the values obtained using the wiping-

off method. Here, the difference amounts to 7.7 %. This discrepancy can no longer be explained by an exchange of liquid and diffusion of contrast agent.

The third measurement represented (on the right in Fig. 47) is a repeat measurement of the second measurement. It is less reliable since it contains a "faulty dispensing". This is expressed by the all in all lower values and by the very high measurement uncertainty. Nevertheless, nearly the same discrepancy (9 %) can also be observed in this measurement.

These two measurements performed using the immersion method show the problems raised by this procedure when using serum instead of water to determine volume gravimetrically. When filling the pipette, a thin film of serum forms on the outer surface of the pipette tip. This film dries (in part or fully) on the way from the reservoir to the dispensing vessel. When immersing the tip into the pre-existing volume of liquid, this dried serum film absorbs water from the afore-mentioned volume of liquid. This water is removed from the pre-existing volume of liquid when the pipette tip is removed, which leads to a reduction in the water quantity of the pre-existing volume in the dispensing vessel.

The removed volume of water is estimated by the difference to 0.7 µl shown in Fig. 47. This volume of water may only lead to a decrease in the pre-existing volume of liquid, but in the gravimetric measurement, it also leads to a corresponding significant decrease in the measured volume due to the fact that the gravimetric procedure merely detects the difference in the mass of the vessel before and after the dispensing operation as the measurement value for the volume. The same problem also occurs when a photometric measurement is performed, but here, the removed volume of water only affects the pre-existing volume of liquid. Since this volume of 1300 µl is very large compared to the removed volume of water, the change in the measured volume taking place is merely of a ratio of 0.7 µl to 1300 µl, which corresponds to a relative change by $5 \cdot 10^{-4}$. The volume measured in this case is too large by $5 \cdot 10^{-4}$ since the contrast agent becomes more concentrated due to the decrease in the pre-existing volume of liquid. The measurements performed do not allow the question as to whether the removed volume of water consists of pure water or of water spiked with contrast agent to be answered. We can assume that both water and contrast agent are removed.

A similarly problematic behaviour may also occur when using other liquids with good wetting properties. This is a case in which the gravimetric volume determination yields a result that is clearly underestimated due to reasons inherent to the system. Only when using water is this

effect negligible due to the large surface tension and the associated poor wetting of the pipette tip surface.

12.4 Advantages and disadvantages of the photometric calibration of volumetric dispensing devices

Photometric measurement for the calibration of volumetric dispensing devices is frequently deemed advantageous for small volumes compared to the gravimetric procedure. Arguments in favour of that are the insufficient resolution of the weighing instrument for small volumes and the strong influence of evaporation on the measurement result when using the gravimetric procedure.

The present results confirm the fundamental applicability of the photometric procedure. For small dispensing volumes, the measurement uncertainty of the photometric procedure is within the maximum permissible errors prescribed by standards. For volumes of more than 100 µl, this is, however, not always the case. For larger volumes, the gravimetric procedure should always be preferred.

Another disadvantage of the photometric procedure is that it does not allow pure liquids to be investigated. Liquids spiked with contrast agent have to be used instead. Adding contrast agent may modify substance parameters and thus the dispensing properties of a liquid. In the case of water, it is especially the surface tension that changes when foreign substances are added. This may lead to biased results und is not admissible for calibrations that must be carried out with a very small measurement uncertainty.

The short time requirement often praised as an advantage of the photometric procedure can only be exploited if the calibration liquid can be procured from an external source or if large quantities of this liquid are needed and can be produced by the laboratory performing the calibration, and if the laboratory does not have to measure calibration curves of its own. Furthermore, dispensing must be performed directly into the photometric cell of the photometer and subsequent blending must also be possible. When procuring the liquid from an external source, adequate traceability of the contrast agent solution must be ensured (as well as traceability of the photometer's calibration), since no comparison measurement is available in

this case. Since contrast agent solutions are often not stable in the long run, it is not possible to keep them in stock for a long time.

When calibrating with liquids such as serum, the calibration solution must be manufactured at short notice and be used up within a few days. Due to the limited stocks of serum, only small quantities of calibration liquid can be produced. The manufacturing procedure requires a gravimetric weighing-in quantity of liquid and contrast agent. Also, the solutions with different concentrations that are needed to measure the calibration straight lines must be verified gravimetrically to achieve the required accuracy. The volume of the pre-existing liquid quantity must also be determined gravimetrically, since volumetric determination with a volumetric dispensing device causes a considerable uncertainty contribution. The required gravimetric measurements and the required determination of the density of the contrast agent solution increase the time needed for a photometric measurement so much that it takes multiple longer than a gravimetric measurement.

The disadvantages of the gravimetric procedure mentioned at the beginning – namely the fact that the weighing instrument does not have sufficient resolution and sensitivity as well as the considerable influence of evaporation at small volumes – can be prevented by means of suitable measures. In the present work, it has been shown that with a suitable weighing instrument and applying a numerical correction for evaporation, very small volumes of liquid can be determined with an expanded measurement uncertainty of 2.4 nl. This was demonstrated experimentally by means of test volumes of 0.2 µl. The uncertainty calculations give reason to expect that volumes down to 50 nl can be measured with the conventional gravimetric procedure.

13 Summary

The present report describes investigations of the dispensing behaviour of piston-operated pipettes and microlitre syringes if the dispensing is performed with different liquids. The volumes dispensed were in the range between 0.2 µl and 50 µl; they were measured with a gravimetric measurement setup. The liquids used were water, ethanol, and nonane, and particular attention was paid to investigating the dispensing behaviour of human serum. In addition, the gravimetric procedure was experimentally compared with the photometric procedure within the scope of the investigations. To make gravimetric calibrations of volumetric dispensing devices with human serum possible in analytical laboratories, a simple density determination method for human serum was developed from the analytically determined common salt and protein concentrations in serum.

The present investigation has shown that the volumes of liquids dispensed with piston-operated pipettes and microlitre syringes may differ when different liquids are used. Calibration of such dispensing devices with water as a dispensing liquid, which is the usual procedure that is laid down in standards, may yield results that are not transferable to the dispensing of other liquids. The substance parameters that are responsible for these differences in dispensing behaviours are, in particular, the surface tension, the interface tension, and the vapour pressure of the liquids considered. If human serum is dispensed, its strong adherence to the pipette or microlitre syringe lead to additional deviations of the dispensed volumes.

Due to its high surface tension and interface tension, water wets the outer surface of pipettes and microlitre syringes only little. Liquids with low surface and interface tensions, in contrast, wet the outer surface both when filling and when emptying the device. The additional liquid on the outer surface can only be partially removed by wiping off the tip. The volume of liquid on the outer surfaces considerably depends on ridges and scratches at the tip of the devices as well as on the roughness of the surface. The deviation of the dispensing result is thus mainly influenced by the device's tip rather than by the device itself. As standard value for deviations of the dispensed volume due to liquid on the outer surface 1 % can be given for ethanol and nonane at test volumes between 2 µl and 50 µl. When dispensing water, this influence of the outer surface of the device tip will not show since water does not wet the tip sufficiently.

It has been known for some time that liquids with a high vapour pressure cannot be dispensed using air displacement pipettes because liquid vapour accumulates in the air interface whose size hereby increases. The present investigation has shown that underfeeding also occurs in the case of positive displacement piston-operated pipettes and microlitre syringes. The standard value for this underfeeding is 2 % for ethanol at test volumes between 2 µl and 50 µl. This is due to evaporation of liquid out of the device tip as well as from the outer surface of the device tip.

When dispensing human serum, these influences also occur; however, they do not cause deviations as large as those occurring with ethanol and nonane. With serum consisting of approx. 90 % water, the vapour pressure of serum is similar to that of water. With approx. 56 mN/m, the surface tension of serum is much higher than that of ethanol and nonane which amounts to approx. 22 mN/m. Calibration results obtained with water and positive displacement piston-operated pipettes are thus usually well transferable to the dispensing of serum. The problem with serum is especially the adherence of the liquid to the walls of the vessel. If the outer surface of the device tip is not conveniently designed, rather significant deviations of the dispensed volume may thus occur even when using positive displacement piston-operated pipettes and microlitre syringes. In principle, the measured deviations for test volumes larger than 2 µl are usually approx. 0.1 %, but may reach 1 % to 2 % in certain cases.

In the case of air displacement pipettes, the strong adherence to the walls of the tip considerably affects the expelling of the liquid. Proper expelling is often only possible pressing down to the final stop, which involves making use of an additional air interface. Yet, a significant quantity of liquid remains in the pipette. The standard value for the resulting underfeeding is 5 % for test volumes between 1 µl and 20 µl.

Air displacement pipettes should always be calibrated with the liquid to be dispensed. If during calibration, empirical repeatability standard deviations are observed that are much larger than when calibrating with water, using positive displacement pipettes should be considered.

In the case of positive displacement pipettes, the deviations of the dispensed volume when using different liquids are of the same order as the maximum permissible errors stated in standards. Calibrating the dispensing device with the liquid that will later be used for dispensing is therefore strongly recommended. For dispensing operations placing stricter requirements on accuracy, this is absolutely indispensable.

In general, microlitre syringes proved less vulnerable to changes in the dispensed liquid. Their longer displacement combined with a smaller tip diameter and a smaller outlet have a positive impact on the dispensing properties. Comparison with maximum permissible errors is not possible since there is no standardization available for microlitre syringes. Calibrating the dispensing device with the liquid that will later be used for dispensing is therefore also strongly recommended.

A major problem encountered when calibrating volumetric dispensing devices gravimetrically, namely the density of serum, which is not known well enough, has been solved. A simple equation was found which allows the serum density to be computed with good accuracy from analytical data for common salt and protein. Another calibration possibility was to replace serum with control material. Replacing human serum with this liquid also allows calibration results to be obtained that can be applied to the dispensing of serum.

Besides comparing the dispensing of different liquids, the present work has also investigated other influences that may affect the dispensing result. It has been shown that the temperature of the pipette tip considerably varies during the dispensing operation. The influence of liquid vapour on the air density – and thus on the air buoyancy correction – was estimated. The change in the quantity of liquid in the weighing vessel of the gravimetric setup due to evaporation was investigated. To compensate for this effect, a numerical procedure was successfully applied. The results obtained when calibrating volumetric dispensing devices are more often not reproducible than statistical previsions would have suggested. A possible explanation has been given. As a possible solution, increasing the number of individual measurements from 10 to 30 or even 50 (at least for calibrations meeting the highest standards) was suggested.

Photometric calibration was investigated as an alternative to gravimetric calibration. Photometric calibration has the disadvantage of detecting an excessive volume of liquid due to liquid exchange and contrast agent diffusion processes when the device tip is immersed directly in the pre-existing volume of liquid for dispensing. When wiping off the liquid against the vessel's wall (similar to the procedure used in the gravimetric procedure), photometric calibration yields measurement values that can be more readily compared to those obtained with the gravimetric procedure. The measurement uncertainty of the photometric procedure is greater than that of the gravimetric procedure, with the stability of the contrast agent solution and adsorption of the contrast agent on the walls of the vessel being difficult to detect. However,

the procedure may be used to calibrate dispensing devices if the volumes to be dispensed are smaller than 100 µl. As shown in the present paper, the gravimetric procedure is, however, also superior to the photometric one when measuring very small volumes. Even at test volumes of 100 nl, the absolute measurement of small volumes of liquid is still possible with an expanded measurement uncertainty of 2.4 % (k = 2).

List of Figures

Literature

[1] H. Koch
28. Sitzung des Beirats für Medizinische Meßtechnik
PTB-Mitteilungen **108** (1998) 31

[2] R. Belac, W. Gögge, H. Groß, W. Ludwig
Meßtechnische Untersuchungen an Kolbenhubpipetten
PTB-Mitteilungen **88** (1978) 93-98

[3] S. Mieke
Rechnergestützte gravimetrische Messung kleiner Flüssigkeitsvolumen (1 µl bis 50 µl) für die Prüfung von Volumenmeßgeräten
PTB-Mitteilungen **93** (1983) 75-79

[4] F. Michel, K. Sommer, F. Spieweck
Untersuchungen zur Ermittlung der Meßunsicherheit von Kolbenhubpipetten mit Volumina von 1 µl bis 50 µl
PTB-Mitteilungen **105** (1995) 437-444

[5] K.H. Lochner, T. Ballweg, H.-H. Fahrenkrog:
Untersuchungen zur Meßgenauigkeit von Kolbenhubpipetten mit Luftpolster
J Lab Med **20** (1996) 430-440

[6] DIN EN ISO 8655-2, Ausgabe:2000-05
Volumenmessgeräte mit Hubkolben - Teil 2: Kolbenhubpipetten
(ISO/DIS 8655-2:2000); Deutsche Fassung prEN ISO 8655-2:2000
(Norm-Entwurf)

[7] DIN 12650-2, Ausgabe:1981-10
Mechanische, physikalische und elektrische Laborgeräte; Volumenmeßgeräte mit Hubkolben; Kolbenhubpipetten

[8] DIN EN ISO 8655-6, Ausgabe:2000-05
Volumenmessgeräte mit Hubkolben - Teil 6: Gravimetrische Prüfverfahren
(ISO/DIS 8655-6:2000); Deutsche Fassung prEN ISO 8655-6:2000
(Norm-Entwurf)

[9] DIN EN ISO 8655-7
Piston-operated volumetric apparatus – Part 7: Non-gravimetric methods for the assessment of equipment performance (in process)

[10] EN ISO 8655-6:2002
Piston-operated volumetric apparatus – Part 6: Gravimetric methods for the determination of measurement error (ISO 8655-6:2002); German version
EN ISO 8655-6:2002

[11] DIN 12650-7, Ausgabe:1983-11
Mechanische, physikalische und elektrische Laborgeräte; Volumenmeßgeräte mit Hubkolben; Photometrische, titrimetrische und mit Meßgefäßen durchgeführte Prüfung der meßtechnischen Zuverlässigkeit (draft standard)

[12] F. Spieweck, H. Bettin
Methoden zur Bestimmung der Dichte von Festkörpern und Flüssigkeiten

PTB-Bericht **PTB-W-46**, Braunschweig, Oktober 1991, 2. Nachdruck Februar 1998, ISSN 0341-6739, ISBN 3-89429-132-X

[13] EAL Interlaboratory Comparisons, **EAL-P7**, Edition 1, March 1996, (new reference: EA 2-03)

[14] Das Blaue Heft, Hinweise für klinisch-chemische Untersuchungen
Herausgeber: Prof. Dr. Dr. J. Büttner, Medizinische Hochschule Hannover, Institut für Klinische Chemie I, 1994

[15] Guide to the Expression of Uncertainty in Measurement

[16] F.E. Jones, G.L. Harris: ITS-90 Density of Water Formulation for Volumetric Standards Calibration
Journal of Research of the National Institute of Standards and Technology **97** (1992) 335-340

Annex 1

Measurement uncertainty budget for a measurement of 20 µl water with the gravimetric device

Model equation:

Mass-to-volume conversion

$$V = m/\rho_W$$

Air buoyancy correction

$$m = W * (1 - \rho_L/\rho_G)/(1 - \rho_L/\rho_W)$$

Uncertainties in the determination of the weighing value

$$W = W_2 - W_1 + w_{Lin} + w_{evaporation}$$

$$W_1 = (W_{01} + w_{1Abl} + w_{1Rep}) * w_{cal} * w_{drift}$$

$$W_2 = (W_{02} + w_{2Abl} + w_{2Rep}) * w_{cal} * w_{drift}$$

$$w_{Drift} = 1 + t_{WD} * t_{Drift}$$

Formula for water density determination [16]

$$\rho_W = (a_0 + a_1 * t_W + a_2 * t_W^2 + a_3 * t_W^3 + a_4 * t_W^4) + \delta\rho_{approx}$$

Formula for air density determination [12]

$$\rho_L = (k_1 * p_A - \varphi * (k_2 * t_A - k_3))/(t_A + t_{A0})$$

List of the quantities:

Symbol	Unit	Quantity
W_{01}	kg	Weighing value for empty measurement (tare)
W_{02}	kg	Weighing value for measurement with load (gross)
$w_{1readout}$	kg	Reading accuracy (digital scale division) of the weighing
$w_{2readout}$	kg	Reading accuracy (digital scale division) of the weighing
w_{1rep}	kg	Reproducibility of the weighing instrument
w_{2Rep}	kg	Reproducibility of the weighing instrument
w_{Lin}	kg	Linearity uncertainty
w_{cal}		Calibration uncertainty of the weighing instrument
t_{WD}	°C	Temperature variation of the weighing instrument
t_{drift}	$°C^{-1}$	Drift constant of the weighing instrument
$w_{evaporation}$	kg	Uncertainty due to the correction for evaporation
t_W	°C	Liquid temperature
$\delta\rho_{approx}$	kg/m³	Unsicherheit in ρ_W durch die Approximationsformel
t_A	°C	Air temperature
p_A	hPa	Ambient pressure
φ	%	Relative humidity
ρ_G	kg/m³	Density of steel weights (constant: 8000 kg/m³)
k_1	kg °C/hPa m³	Constant k_1 for the air density formula
k_2	kg/m³	Constante k_2 for the air density formula
k_3	kg °C/m³	Constante k_3 for the air density formula
t_{A0}	°C	Constant t_{A0} for the air density formula
a_0	kg/m³	Constant a_0 for the water density formula
a_1	kg/m³/°C	Constant a_1 for the water density formula
a_2	$kg/m^3/°C^2$	Constant a_2 for the water density formula
a_3	$kg/m^3/°C^3$	Constant a_3 for the water density formula
a_4	$kg/m^3/°C^4$	Constant a_4 for the water density formula
Conversion	nl/m³	Scaling factor for conversion from m^3 to nl
Intermediate results		
m	kg	Mass of the liquid
ρ_W	kg/m³	Density of the liquid
ρ_A	kg/m³	Air density
w_{Drift}		Deviation of the weighing value due to the temperature drift
W_1	kg	Weighing value with uncertainties
W_2	kg	Weighing value with uncertainties
W	kg	Weighing value of the volume
Final result		
V	nl	Measured volume

Values of the quantities:

W_{01}:	Type B rectangular distribution	Value: 0 kg	Half-width of the limits: 0 kg
W_{02}:	Type B rectangular distribution	Value: $20.0149 \cdot 10^{-6}$ kg	Half-width of the limits: 0 kg
$w_{1readout}$:	Type B rectangular distribution	Value: 0 kg	Half-width of the limits: $5 \cdot 10^{-11}$ kg
$w_{2readout}$:	Type B rectangular distribution	Value: 0 kg	Half-width of the limits: $5 \cdot 10^{-11}$ kg
w_{1rep}:	Type B rectangular distribution	Value: 0 kg	Half-width of the limits: $1 \cdot 10^{-9}$ kg
w_{2Rep}:	Type B rectangular distribution	Value: 0 kg	Half-width of the limits: $1 \cdot 10^{-9}$ kg
w_{lin}:	Type B rectangular distribution	Value: 0 kg	Half-width of the limits: $9 \cdot 10^{-11}$ kg
w_{cal}:	Type B rectangular distribution	Value: 1	Half-width of the limits: $1 \cdot 10^{-6}$
t_{WD}:	Type B rectangular distribution	Value: 0 °C	Half-width of the limits: 0.5 °C
$w_{evaporation}$:	Type B rectangular distribution	Value: 0 kg	Half-width of the limits: $15 \cdot 10^{-10}$ kg
t_W:	Type B rectangular distribution	Value: 20 °C	Half-width of the limits: 0.1 °C
$\delta\rho_{approx}$:	Type B rectangular distribution	Value: 0 kg/m³	Half-width of the limits: 0.005 kg/m³
t_A:	Type B rectangular distribution	Value: 20 °C	Half-width of the limits: 0.1 °C
p_A:	Type B rectangular distribution	Value: 1013 hPa	Half-width of the limits: 2 hPa
φ:	Type B rectangular distribution	Value: 70 %	Half-width of the limits: 20 %
ρ_G:	constant	value: 8000 kg/m³	
k_1:	constant	value: 0.34844 kg °C/(hPa m³)	
k_2:	constant	value: 0.00252 kg/m³	
k_3:	constant	value: 0.020582 kg °C/m³	
t_{A0}:	constant	value: 273.15 °C	
a_0:	constant	value: 999.85308 kg/m³	
a_1:	constant	value: $6.32693 \cdot 10^{-2}$ kg/(m³ °C3)	
a_2:	constant	value: $-8.523829 \cdot 10^{-3}$ kg/(m³ °C²)	
a_3:	constant	value: $6.943248 \cdot 10^{-5}$ kg/(m³ °C³)	
a_4:	constant	value: $-3.821216 \cdot 10^{-7}$ kg/(m³ °C⁴)	
t_{Drift}:	constant	value: $1 \cdot 10^{-6}$ °C^{-1}	
m:	intermediate result		
ρ_W:	intermediate result		
ρ_L:	intermediate result		
w_{Drift}:	intermediate result		
$W1$:	intermediate result		
$W2$:	intermediate result		
W:	intermediate result		
V:	result		

Measurement uncertainty budget:

Quantity	Value	Standard measurement uncertainty	Degree of freedom	Sensitivity coefficient	Uncertainty contribution
W_{01}	0.0 kg	0.0 kg	∞	0.0	0.0 nl
W_{02}	$20.0149 \cdot 10^{-6}$ kg	0.0 kg	∞	0.0	0.0 nl
$w_{1readout}$	0.0 kg	$28.9 \cdot 10^{-12}$ kg	∞	$-1.0 \cdot 10^{9}$	$-29 \cdot 10^{-3}$ nl
$w_{2readout}$	0.0 kg	$28.9 \cdot 10^{-12}$ kg	∞	$1.0 \cdot 10^{9}$	$29 \cdot 10^{-3}$ nl
w_{1Rep}	0.0 kg	$577 \cdot 10^{-12}$ kg	∞	$-1.0 \cdot 10^{9}$	$-580 \cdot 10^{-3}$ nl
w_{2Rep}	0.0 kg	$577 \cdot 10^{-12}$ kg	∞	$1.0 \cdot 10^{9}$	$580 \cdot 10^{-3}$ nl
w_{Lin}	0.0 kg	$52 \cdot 10^{-12}$ kg	∞	$1.0 \cdot 10^{9}$	$52 \cdot 10^{-3}$ nl
w_{cal}	1	$577 \cdot 10^{-9}$	∞	$20 \cdot 10^{3}$	$12 \cdot 10^{-3}$ nl
t_{WD}	0.0 °C	$289 \cdot 10^{-3}$ °C	∞	$20 \cdot 10^{-3}$	$5.8 \cdot 10^{-3}$ nl
$w_{evaporation}$	0.0 kg	$866 \cdot 10^{-12}$ kg	∞	$1.0 \cdot 10^{9}$	$870 \cdot 10^{-3}$ nl
t_W	20.0 °C	$57.7 \cdot 10^{-3}$ °C	∞	4.2	$240 \cdot 10^{-3}$ nl
$\delta\rho_{Approx}$	0.0 kg/m³	$2.89 \cdot 10^{-3}$ kg/m³	∞	-20	$-58 \cdot 10^{-3}$ nl
t_A	20.0 °C	$57.7 \cdot 10^{-3}$ °C	∞	$-83 \cdot 10^{-3}$	$-4.8 \cdot 10^{-3}$ nl
p_A	1013 hPa	1.15 hPa	∞	$21 \cdot 10^{-3}$	$24 \cdot 10^{-3}$ nl
φ	70 %	11.5 %	∞	$-1.8 \cdot 10^{-3}$	$-21 \cdot 10^{-3}$ nl
V	$20.0720 \cdot 10^{3}$ nl	1.22 nl	∞		

Result:

Quantity: V

Value: $20.0720 \cdot 10^{3}$ nl; expanded measurement uncertainty: ±41 nl; coverage factor: 2

Bundesministerium
für Wirtschaft
und Energie

Die Physikalisch-Technische Bundesanstalt, das nationale Metrologieinstitut, ist eine wissenschaftlich-technische Bundesoberbehörde im Geschäftsbereich des Bundesministeriums für Wirtschaft und Energie.

Physikalisch-Technische Bundesanstalt
Braunschweig und Berlin
Nationales Metrologieinstitut

Bundesallee 100
38116 Braunschweig

Presse- und Öffentlichkeitsarbeit

Telefon: (0531) 592-93 21
Fax: (0531) 592-30 08
E-Mail: presse@ptb.de
www.ptb.de

Vertrieb:
Fachverlag NW in der
Carl Schünemann Verlag GmbH

Zweite Schlachtpforte 7
28195 Bremen

Telefon: (04 21) 369 03-0
Fax: (04 21) 369 03-63
www.schuenemann-verlag.de