Notice

Test method for comparative evaluation of instrument detergents for manual reprocessing of surgical instruments on the basis of fibrin

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Working Group for Testing Detergents – AG RMT of the German Society for Hospital Hygiene (DGKH e.V.)

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The authors declare that they have no conflicts of interest. Members of the Working Group for Testing Detergents (designated in the following by its German acronym *AG RMT*) made test products and information available in a non-discriminatory manner.

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Abstract

Currently, there are neither in Germany nor at international level any generally accepted methods for verification of the efficacy of the detergents used for reprocessing of instruments. The requirements addressed to an appropriate test method are as follows:

i) Selection of a test soil that is difficult to remove and of practical relevance,
ii) Defined application of the test soil to a relevant surface,
iii) Quantitative elution of residual soils as well as
iv) Definition of a representative analyte (lead substance) and appropriate methods for quantification of the analyte.

The Working Group for Testing Detergents (*AG RMT*) of the German Society for Hospital Hygiene (DGKH) e.V.), which was set up in 2011, devised and developed a new test method for comparative evaluation of detergents for instrument reprocessing.

Using the high molecular weight and water-insoluble test soil fibrin, this paper now reports on a method based on a fibrin process challenge device (F-PCD) that for the first time permits comparative testing of the efficacy of different detergents in an immersion model (no mechanical action) and is able to differentiate various instrument detergents in terms of their efficacy. The special feature of this method is the use of water-insoluble fibrin as test soil of practical relevance. Fibrin is rated as a critical protein soil component, the most difficult to remove from surgical instruments, and to date it has not been possible to measure and evaluate fibrin by means of conventional elution and protein quantification methods. Based on the development of an innovative elution method, the water-insoluble fibrin soil on the PCDs can be converted into a solubilized form and measured using traditional protein quantification methods (OPA, BCA method). The reproducibility and comparability of the results of the newly devised method were verified and confirmed in several multi-centre trials with up to six participating laboratories. The investigations by the *AG RMT* have led to the development of a new test method and to an advanced understanding of comprehensive assessment of the residual protein content on reprocessed medical devices.

Keywords
- surgical instruments
- reprocessing
- fibrin
- cleaning
- instrument detergents

1. Introduction

Cleaning is the first and most important step in the medical device (MD) reprocessing process. The purpose of the

cleaning process is to remove as far as possible all soils from the MDs regardless of the chemical nature or volume of soils. Irrespective of the reprocessing method employed, manual or automated, or possibly partially automated, it is only when soils are fully removed, possibly through a combination of manual precleaning and automated cleaning, that the subsequent reprocessing steps of disinfection and sterilization can be effectively performed to meet the hygiene quality of reprocessed MDs as stipulated by national laws, in the case of Germany by the Act on Medical Devices [1] and the German Medical Devices Operator Regulation (*MPBetreibV*) [2]. To assess the cleaning efficacy the protein content is generally used as the key parameter for residual soils [3,4,5,6,7]. This is also the case when evaluating the cleaning efficacy in washer-disinfectors. Other parameters, e.g. the content of polysaccharides, lipids and oils, carbohydrates, endotoxins, haemoglobin (with regard to pseudoperoxidase activity), total organic carbon (TOC), total bound nitrogen (TNB), adenosine triphosphate (ATP) [8], can provide insights into specific types of residual soils. However, the significance of these parameters to give a comprehensive assessment of the cleaning efficacy is a subject of critical debate because of the relevance of the specific type of soil and its ease of removal [9].

The effect of the cleaning process is based on the four classic factors: i) Chemistry, ii) Time, iii) Temperature and iv) Mechanics, according to the Sinner's Circle concept [10]. To assure the Chemistry factor special instrument detergents, which are approved as medical devices, are used for cleaning medical devices. The instrument detergents have various important functions for the intended cleaning effect:

- Reduction of the water surface tension assures rapid and complete wetting of all instrument surfaces, permitting penetration of the cleaning solution into narrow gaps and crevices.
- Protein solubilization is underpinned and expedited at alkaline pH values.
- The amphiphilic nature of the detergent surfactants permits dispersion of hydrophobic soils by enclosing them in micelles.
- Micelle formation contributes to the high soil dispersion capacity of the cleaning solution and prevents redeposition of soils on surfaces.
- Other primary active components of detergents may include, e.g. enzymes, which because of their hydrolytic activity contribute to degradation of high- to low-molecular soil substances, thus enhancing the water solubility of the products.

Pursuant to 93/42/EEC [11] and 2007/47/EC [12], instrument detergents are class I medical devices. Unlike the approval process applied for disinfectants (class IIb medical devices), which are currently (July 2018) the subject of 30 EN standards and 23 pr-standards or "work items", for instrument detergents there are at present no such normative test procedures or other generally accepted test methods for evaluation of the efficacy. By contrast (to disinfectants), the efficacy of instrument detergents is usually demonstrated by non-disclosed tests carried out by the respective manufacturer as part of the approval procedure. Hence, there are at present no objective criteria in the form of published test methods to enable the users of instrument detergents to differentiate or evaluate products when choosing a detergent.

The test models featured in ISO/TS 15883-5 [13] for evaluation of the cleaning efficacy are intended for investigation of automated reprocessing processes in washer-disinfectors (WDs). While numerous different proposals have been published with regard to the choice of test soils, to date no consensus has been reached on an acceptable proposal. That is reflected in the large number of appendices to ISO/TS 15883-5. In automated cleaning processes the Mechanics and Temperature factors make a major contribution to the cleaning outcome. Therefore these test models are not suitable without modifications for evaluating the sole contribution made by the "detergent" chemistry factor in the context of Sinner's Circle.

To pave the way for objective and transparent assessment of the efficacy of instrument detergents in the future, the German Society for Hospital Hygiene (DGKH e.V.) set up a working group in 2011 with the aim of devising and developing suitable test methods for quantitative and reproducible evaluation of the efficacy of instrument detergents.

On 22 June 2011 the inaugural meeting of the Working Group for Testing Detergents (*AG RMT*) was held under the direction of Dr. Jürgen Gebel and the group immediately got down to work. The following goals (primary objectives) were defined:

- Development of a test method for comparative assessment of instrument detergents for manual reprocessing. The detergent solution would be prepared in water of standardized hardness (WSH) and the process temperature would be 25 °C → typical conditions for manual reprocessing.
- Detergent testing using an immersion method based on the investigations by the *ad hoc* group at DIN (NAMed 063-04-09) – published by Köhnlein *et al.* 2008 [14] and 2009 [15] → simple, reproducible setup, no mechanics / mechanical action.
- Process challenge devices (PCDs) consisting of austenitic stainless steel (1.4301) with surface structuring based on longitudinal polishing, granulation 80 → reference to metallic surgical instruments.
- Test soil based on coagulated blood, reference to blood soils on real surgical instruments → blood makes stringent demands on the cleaning processes, in particular following drying and/or denaturation due to high temperatures and/or the effect of chemical agents.
- Application of an surface-related amount of test soil → to assure greatest possible reproducibility when preparing PCDs. The standard method used was that described by Brill *et al.* 2014 [16].
- The test method must be able to demonstrate the efficacy of instrument detergents compared with that of water (discriminatory capacity of the method) → WSH as control.

Once a method was developed to achieve the primary objectives, the method would be adapted so that it could also be used for assessment of disinfecting detergents for manual instrument reprocessing as well as of the detergents used for automated reprocessing processes.

In this paper the *AG RMT* presents the findings of the working group obtained from 18 meetings and 10 multi-centre trials. The participating labo-

ratories were as follows (in alphabetical order):

- Dr. Brill + Partner GmbH Institut für Hygiene und Mikrobiologie, Hamburg
- HygCen GmbH, Schwerin
- Institut für Hygiene und Öffentliche Gesundheit (IHPH), Universitätsklinikum Bonn AöR, Bonn
- Schülke & Mayr GmbH, Norderstedt
- SMP GmbH, Tübingen
- wfk – Cleaning Technology Institute e.V., Krefeld

The order in which the laboratories are presented above is not the same as that used in the figures.

2.Materials and Methodes

2.1 Solutions and equipment

- Heparinised sheep blood, with 10 IU heparin / ml. Sheep blood from the firm Acila GmbH was used. Package sizes of 10 ml were used.
- Protamine hydrochloride or protamine sulphate, quantity added 15 IU / ml blood; the product was sourced from blood suppliers or from local pharmacies.
- Saturated hygroscopic saline solutions composed of i) Potassium carbonate, K_2CO_3, ii) Sodium iodide NaI, iii) Sodium chloride, NaCl, iv) Ammonium sulphate, $(NH_4)_2SO_4$. To prepare these solutions, 1000 ml demineralized water was heated to 80 °C and, while stirring, the respective salt was added until there were insoluble residues on the bottom. Through cooling to room temperature (RT) the precipitated portion increased.
- Demineralized water, conductivity value ≤ 15 μS
- HPLC water (e.g. Carl Roth, ROTISOLV® HPLC gradient grade, Order No.: A511.3)
- WSH (water of standardized hardness) with hardness of 376 ppm, referenced to $CaCO_3$ [17]
- Test detergent 1 (TR-1): Alkaline detergent for manual cleaning of medical devices with alkali donors, chelating agents, dispersants, solubilizers, surface active substances and wetting agents, pH = 11.7 at 1 % concentration, 20 °C.
- Test detergent 2 (TR-2): Multi-enzymatic detergent for manual cleaning of medical devices with proteases, lipases and amylases, with 5 – 15 % non-ionic surfactants, < 5 % anionic surfactants, preservatives, pH = 8.4 – 8.6 at 0.1 – 3 % concentration, 20 °C.
- Steel plates: Steel plates in batch 1 measured 77 x 26 x 1 mm; steel plates in batch 2 measured 80 x 12 x 1 mm. The edges were rounded with a radius of 2 mm. The steel plates were composed of stainless steel 1.4301. The contaminated surface had a surface quality assured by longitudinal polishing, granulation 80 (available from e.g. Gerätetechnik Brieselang GmbH or Fa. JÜLEX Edelstähle / JÜLEX Maschinenbau Ing. Jürgen Lemke e.K. GmbH).
- Application template, adapted as per [16]: Application templates made of milled polycarbonate or nylon application templates generated through 3D printing (e.g. from 3D Activation GmbH) with dimensions suitable for fitting the batch 1 and batch 2 steel platelets were used. The application templates allowed contamination of a surface area of 3.0 cm^2 for batch 1 steel plates and of 6.0 cm^2 for batch 2 steel plates.
- Decon 90, Decon Laboratories Limited, Hove, East Sussex, UK
- Isopropanol, 99.95 % (e.g. Carl Roth, Order No.: AE73.2)
- Plastic boxes, tight closing (e.g. Lock & Lock, type HPL 834, 3.9 l, 295 x 230 x 84 mm)
- Desiccator (e.g. Lab Companion, Cubic Vacuum Desiccator, Model VDC-11)
- Beakers: 250 ml, low shape, height approx. 95 mm, external diameter approx. 70 mm (e.g. Carl Roth, Order No.: X691.1)
- Clamps for securing PCDs, corrosion-resistant (e.g. PutzKULT GmbH, Order No.: 607122)
- Magnetic stirring rod with a length of 35 mm and diameter of 6 mm (e.g. Carl Roth, Order No.: 1292)
- Magnetic stirrer with 350 rpm (different laboratory-specific models)
- Petri dishes, 92 mm diameter (e.g. Sarstedt, Order No.: 82.1473)
- Glass beads, 3 mm diameter (e.g. A. Hartenstein, Order No.: GP3)
- Horizontal shaker with 300 rpm (different laboratory-specific models)
- Glass screw-top test tubes, external diameter 16 mm, height 100 mm with screw cap (e.g. A. Hartenstein, Order No.: RG10)
- Screw-top test tubes, 15 ml with screw cap (e.g. Sarstedt, Order No.: 62.554.502)
- BCA kit (e.g. ThermoFisher Scientific, Pierce™ BCA Protein Assay Kit, Order No.: 23225).
- Chemicals for the OPA method, as per [18].
- Acrylamide gels (e.g. Carl Roth Roti® PAGE gradient 4 – 20 %, Order No.: 2843.2)
- Protein loading buffer (e.g. Carl Roth Roti®-Load 1, Order No.: K929.1)
- Protein size standard (e.g. Carl Roth Roti®-Mark PRESTAINED, Order No.: T852.1)

2.2 Methods

2.2.1 Precleaning materials

To thoroughly clean the steel plates visible soils were first removed under running water, if necessary using a soft brush. Then sets of around 30 steel plates were transferred to a beaker (500 ml), which was filled with 400 ml 5 % Decon 90 solution, and boiled for around 10 min. The Decon 90 solution was poured off and the steel plates were rinsed under running water until there was no longer any visible foam formation. Using tweezers, the steel platelets were individually rinsed by immersing them five times in 70 % isopropanol and then air-dried. The glass beads used for elution were cleaned in the same manner in Decon 90 solution, then transferred to a beaker with 250 ml isopropanol and incubated for around 2 min while swirling, and then decanted, collected in a sieve and dried.

2.2.2 Production of blood PCDs

Batch 1 steel plates were used to produce the blood PCDs (B-PCDs). Using an appropriate sized application template that permitted contamination of a surface area of 3.0 cm^2, the cleaned steel plates were contaminated with aliquots of 50 μl reactivated sheep blood. A detailed description of the application of surface-related amounts of test soil tailored to the specific surface area can be found in [16]. For one test batch 100 μl HPLC water and the specified amount of protamine were added to 900 μl heparinised sheep blood, which was then mixed and immediately

used to contaminate a maximum of 10 steel plates. The remainder of the blood was discarded. The contaminated steel plates were transferred immediately to a desiccator for conditioned drying. To generate different levels of defined humidity in the desiccator, at least 24 h before beginning conditioning of the contaminated steel plates the tray filled with around 300 ml (completely covering the tray) of the respective saturated saline solution was fitted at the bottom of the desiccator.

2.2.3 Production of fibrin PCDs

Batch 2 steel plates were used to produce the fibrin PCDs (F-PCDs). Using an appropriate sized application template that permitted contamination of a surface area of 6.0 cm^2, the cleaned steel plates were contaminated with aliquots of 100 μl reactivated undiluted sheep blood [16]. For one test batch the specified amount of protamine was added to 1 ml heparinised sheep blood, which was then mixed and immediately used to contaminate a maximum of 10 steel plates. The remainder of the blood was discarded. The contaminated steel plates were transferred immediately to a desiccator whose insert tray, at the bottom of the desiccator, had been filled with approx. 300 ml demineralized water to ensure that incubation was carried out in an environment with 100 % relative humidity. Incubation took place for 1 h to permit complete coagulation of the reactivated sheep blood. The steel plates with the moist blood soils were withdrawn and the haemoglobin was removed from the blood soils by means of "dehaemoglobinization treatment". To that effect, sets of three steel plates were transferred to a beaker and placed against the wall of the beaker such that the contaminated side faced the inside of the beaker. The beaker was filled with 250 ml demineralized water and this was mixed with a magnetic stirring rod at a rotational speed of 350 rpm. Dehaemoglobinization took place at 22 ± 2 °C, followed by incubation for 10 min, after which the demineralized water was discarded and fresh demineralized water added. This was followed by further wash steps with an incubation time of 10 min and 15 min, after which fresh demineralized water was added again; overnight incubation (17 – 20 h) while stirring continuously followed. The PCDs were withdrawn from the beaker, as much water as possible was removed by holding them vertically and they were then transferred to a plastic box whose bottom was covered with 300 g dried silica gel. Up to 40 PCDs were placed on the sieve-like false floor and the PCDs left to dry until the appearance of the fibrin layer changed from opaque to white, signalling drying. Drying took place at 22 ± 2 °C. For storage the prepared F-PCDs were removed and each transferred to a glass screw-top test tube and stored in a refrigerator at 2 – 8 °C for up to four weeks. The refrigerated F-PCDs were first brought to room temperature before the cleaning experiments and before opening the glass screw-top test tubes to prevent condensation effects on the test soil.

2.2.4 Conduct of cleaning experiments

The detergent solution (WSH, test detergent 1 or 2) was first brought to a temperature of 25 ± 2 °C in a glass bottle placed in a water bath. Depending on the measuring time points, three or four PCDs were placed against the wall of the beaker with the test soil facing the centre of the beaker, and secured to the edge of the beaker with a clamp such that the test soil was fully immersed in the detergent solution. The PCDs were positioned at equal distances apart in the beaker. One untreated PCD was included as a positive control in each cleaning test batch (beaker with PCDs). The test setup was placed on a heating/magnetic stirrer, the rotational speed set to 350 rpm and the temperature adjusted such as to assure a constant temperature of 25 ± 2 °C for the entire test period of up to 30 min. This was monitored throughout by means of a fitted thermometer. After adding 250 ml detergent solution the timer was started. After 5 min, 10 min, 20 min and 30 min one PCD was removed in each case and immersed ('dipped') for approx. 1 sec in demineralized water and then further prepared for elution of residual soils.

2.2.5 Elution of residual soils from blood PCDs

For elution of residues of reactivated sheep blood from B-PCDs, the PCDs were transferred to a Petri dish (D: approx. 90 mm) filled with 16 g cleaned glass beads and 10 ml 1 % SDS solution (pH = 11). The PCDs were positioned with the contaminated side resting on the glass beads and eluted for 20 min on a horizontal shaker at 300 rpm at room temperature. The eluate was transferred with a pipette to a 15 ml screw-top test tube for ensuing measurement of the residual protein content.

2.2.6 Elution of residual soils from fibrin PCDs

For elution of fibrin residues from F-PCDs, the PCDs were transferred individually to a glass screw-top test tube, to which 10 ml 1 % SDS solution (pH = 11) was pipetted and the test tube with loosely fitted cap was autoclaved, using a steam sterilization programme at 121 °C and a holding time of 20 min. To prevent superheating on cooling, a support pressure method of cooling was used whenever possible. After cooling to room temperature, the glass screw-top test tubes were closed tightly and mixed by manually shaking. This was followed by visual inspection to ensure there were no longer any residual test soils on the PCDs. In cases of doubt protein residues were stained with Amido Black [19].

2.2.7 Protein quantification

Quantification of solubilized proteins in the eluates can be performed with both the BCA and OPA methods. Guidance to using the BCA or OPA method can be found in Annex 8 of the Guideline compiled by the DGKH*, DGSV, DGVS, DEGEA and AKI [18] and DIN EN ISO 15883-1 [7]. Alternatively, modified methods or commercially available test kits can be used provided they are based on the same chemical detection reactions and have been demonstrated to be equally suited for protein quantification. If necessary, specific limits of quantification (LOQs) and interference factors should be taken into account. Method calibration was performed with defined amounts of bovine serum albumin (BSA), fraction V. For protein quantification of the multi-centre test results presented here the participating laboratories used the modified OPA method [20] throughout.

*DGKH: German Society for Hospital Hygiene
DGSV: German Society for Sterile Supply
DGVS: German Society for Gastroenterology, Digestive and Metabolic Diseases with the Section for Gastroenterological Endoscopy
DEGEA: German Society of Endoscopy Nurses and Assistants
AKI: Working Group Instrument Preparation

2.2.8 Gel electrophoresis for investigation of fibrin in eluates

To characterize the solubilized fibrin residues resulting from steam sterilization (121 °C, 20 min), fibrin coagula were produced by adding the prescribed amount of protamine to 250 µl sheep blood in a 1.5 ml reaction tube. Following coagulation (1 h) five fibrin coagula were dehaemoglobinized by washing in 500 ml demineralized water. The demineralized water was changed 3x within the first 8 h of dehaemoglobinization, and then changed daily over the entire four-day period. Two fibrin coagula were submersed in a glass screw-top test tube into 0.25 ml 1 % SDS solution (pH = 11) and converted to solubilized form (eluate) through steam sterilization. 10 µl aliquots of the eluate were mixed with 3.4 µl protein loading buffer, boiled for 10 min and loaded onto an acrylamide gel (4–20 % gradient gel) together with a protein size marker. Staining was performed with Coomassie stain [21].

2.2.9 Total organic carbon measurements

Total organic carbon (TOC) was measured with the system DIMA-TOC 2010-LC (DIMATEC Analysentechnik GmbH, Essen), a compact laboratory analyzer for measurement of carbon in liquids based on the thermal catalytic oxidation principle at 850 °C, followed by NDIR detection (non-dispersive infrared spectroscopy). Elution of residual soils from F-PCDs for these measurements was performed, as described in 2.2.6, by means of steam sterilization at 121 °C and with a 20 min holding time, however, using 0.001 N NaOH solution instead of 1 % SDS solution. No residues were found on the F-PCDs following staining with Amido Black. At direct measurement of TOC in the solution obtained, dissolved carbon dioxide, which had been converted to sodium (hydrogen) carbonate, was also included. Therefore, the solutions were acidified with hydrochloric acid, carbon dioxide-free carrier gas was purged through the solution for 5 min to degas the carbon dioxide and then one aliquot was subjected to TOC measurement. The mean measured values for this NPOC (non-purgeable organic carbon) measurement were then 22 µg carbon / ml lower than in the case of direct TOC measurement.

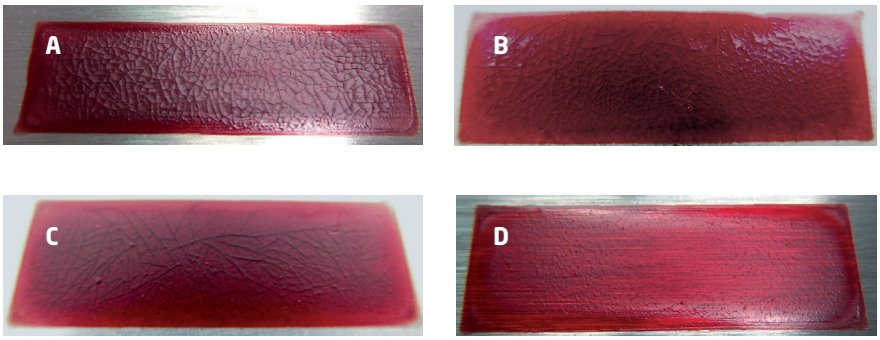

Fig. 1: Conditioning of blood PCDs (B-PCDs) at 22 – 24 °C and different relative humidity values over a period of 22 ± 2 h. Conditioning: A) 43 % rh over K_2CO_3 solution; B) 70 % rh over KI solution; C) 75 % rh over NaCl solution; D) 81 % rh over $(NH_4)_2SO_4$ solution

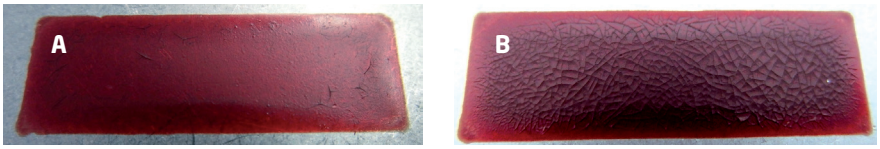

Fig. 2: B-PCDs were conditioned at 81 % rh over $(NH_4)_2SO_4$ solution, producing a homogeneously dried blood soil (A). Extensive fissure formation occurred within a short time (< 5 min) on incubation in room air (24 °C, 49 % rh) (B).

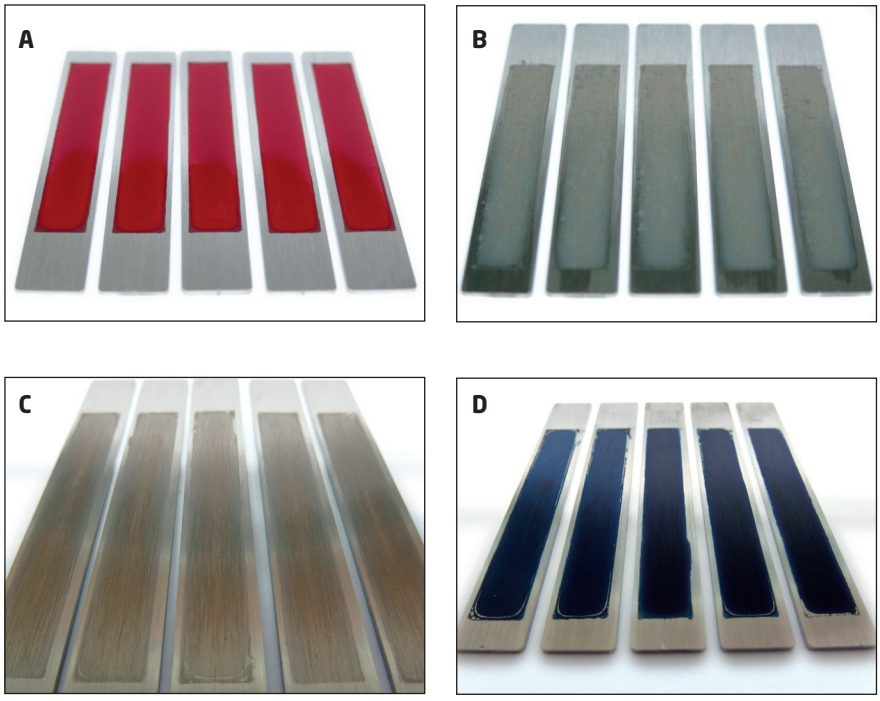

Fig. 3: Steps in production of F-PCDs: A) steel plates homogeneously coated with 100 µl reactivated undiluted blood (batch 2, dimensions: 80 x 12 x 1 mm, contaminated surface area 6 cm^2) after one-hour incubation at 100 % rh.; B) Wet F-PCDs immediately after the dehaemoglobinization step, the swollen fibrin hydrogel has an opaque appearance; C) F-PCDs after 24 h drying over silica gel; D) Dried F-PCDs after staining the fibrin layer with Amido Black.

3. Results

3.1 Production of homogeneous and reproducible test soils

Using a test soil composed of reactivated diluted sheep blood, PCDs with a blood soil (B-PCDs) were produced. The use of published methods for conditioned drying of blood soils [14,15] at 43 % relative humidity (rh) led to different results among the participating laboratories. Homogeneous, tightly adhering soils as well as greatly fissured soils were observed, with detachment from the PCD surface upon mechanical vibration. Experiments were carried out in several laboratories using different saturated saline solutions [22] to optimize the conditioning method. The comparative conditioning experiments were performed for 22 ± 2 h at 43 % rh over saturated K_2CO_3 solution, at 70 % rh over saturated KI solution, 75 % rh over saturated NaCl solution and at 81 % rh over saturated $(NH_4)_2SO_4$ solution. Despite multiply repeated experiments, the participating laboratories were unable under otherwise constant laboratory conditions (temperature, blood quantity and concentration, etc.) to define a uniform humidity conducive to the reproducible production of homogeneously dried and tightly adhering blood soils on steel surfaces. An exemplary set of images of various conditioned blood soils (laboratory A) is illustrated in Fig. 1.

Further experiments demonstrated that, to the extent that it was possible to produce a dried, homogeneous blood soil through conditioning (see Fig. 2A), subsequent, brief incubation (2-5 min) of PCDs in the laboratory ambient air (24 °C, 49 % rh) soon led to fissure formation of the soil surface and, in some cases, even detachment of the blood soil from the steel surface (see Fig. 2B). It is assumed that further drying of conditioned blood soils at 81 % rh caused shrinkage fissures. This instability of the blood soils hampered the reproducible use of B-PCDs.

Through development of F-PCDs it was possible to avoid the problems associated with conditioned drying of the applied blood soils. That was assured by means of blood coagulation in a saturated water-rich environment (100 % rh, incubation over demineralized water) and the wet, coagulated PCDs were immediately subjected to the subsequent dehaemoglobinization step to remove haemoglobin and other water-soluble protein constituents. Drying the test soil afterwards over silica gel produced a homogeneous and tightly adhering water-insoluble fibrin layer, see Fig. 3.

3.2 Solubilization /elution of residual soils

On elution of the B-PCDs used in cleaning experiments, the participating laboratories unanimously observed that residual soils were removed from the PCDs through the purely mechanical action based on the spinning and shaking movements on the added glass beads and that insoluble residual soils were present in the elution medium as suspended flakes. Visualization of the suspension of insoluble residual soils is shown in Fig. 4 based on the example of elution of B-PCDs treated in preliminary experiments with a disinfecting detergent. The latter caused denaturation of blood soils, hence the enclosed haemoglobin was no longer water soluble, which is why no further experiments were undertaken with disinfecting detergents. In experiments with non-disinfecting detergents whitish/colourless residues, which proved to be stainable with Amido Black, were observed on the PCDs. Likewise, shaking on glass beads only resulted in these being suspended as insoluble flakes. The insolubility of soil constituents was deemed critical with regard to sampling of a homogeneous measuring solution as well as subsequent protein quantification.

For elution of the water-insoluble fibrin soil from F-PCDs a method was devised for quantitative solubilization by steam sterilization (121 °C / 20 min) and submersed incubation in 1 % SDS solution (pH = 11). That method assured complete removal of fibrin residues. Proof of complete elution was based on staining of any remaining fibrin residues with Amido Black, which demonstrated that no residues could be detected (level of detection < 20 ng / cm^2 [19]), see Fig. 5.

3.3 Influence of the thermal elution process on protein quantification

Since the steam sterilization process caused extreme heating of the fibrin residues in an alkaline medium (1 % SDS solution, pH = 11), avoidance of partial or total hydrolysis of fibrin residues had to be ensured as that would have led to a major change in the number of free α- and ε-amino groups as well as of the peptide bonds, to which OPA and BCA methods, respectively, are sensitive. Besides, no reaction of the amino groups should have taken place since it would no longer be possible to measure the latter with the OPA method. For verification purposes comparative tests were conducted with defined sheep blood solutions (5, 10, 25 µl blood / ml, blood protein concentration determined with the OPA method: 168.5 mg / ml) and BSA (type V) solutions of a defined concentration (50, 100, 200 µg / ml) in 1 % SDS solution (pH = 11). Direct protein quantification was performed with the OPA and BCA method for untreated solutions (25 °C). An aliquot of the same sheep blood and BSA solutions was subjected to the steam sterilization step (121 °C, 20 min) and the respec-

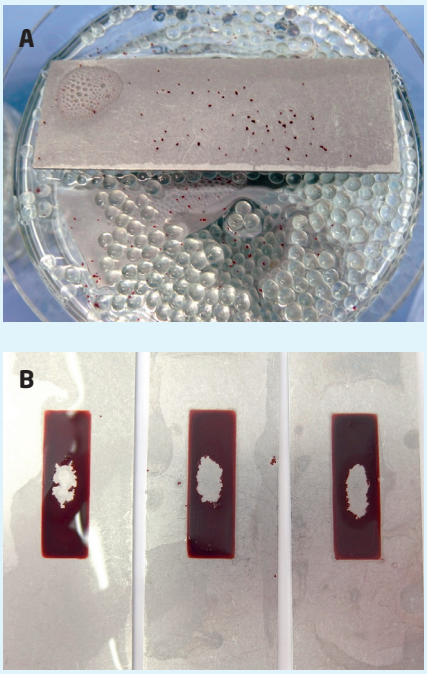

Fig. 4: Visualization of mechanical removal and suspension of water-insoluble residual soils from B-PCDs. Exposure to a disinfecting detergent led to denaturation of the blood soil, which had only been mechanically removed and suspended through submersed (1 % SDS solution (pH = 11)) agitation on glass beads (A). A limited region of detachment of the soil layer due to the mechanical agitation of the glass beads was observed on the surface of the treated PCDs (B).

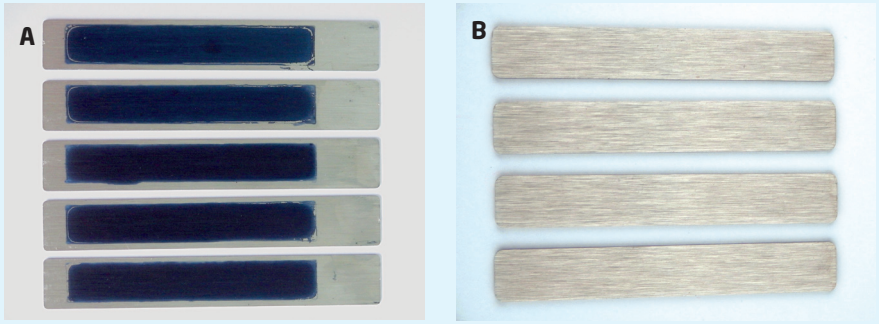

Fig. 5: F-PCDs after staining with Amido Black: A) Untreated F-PCDs not subjected to the elution step B) F-PCDs following thermal elution/solubilization of the fibrin layer with a steam sterilization step.

tive protein content was quantified with the OPA and BCA method (n = 1), see Fig. 6.

For the blood solutions of different concentrations differences were found in the protein content between those samples treated at 121 °C compared with those stored at 25 °C. Differences ranging from -10.0 % to +10.6 % were seen on using the OPA method and differences ranging from -2.7 % to +0.8 % were observed on using the BCA method. For the BSA solutions of different concentrations differences in the range 10.4 % to -6.8 % were found on using the OPA method and of -9.1 % to -7.1 % on using the BCA method. These rather minor differences were ascribed to the methodical precision of the respective protein quantification method.

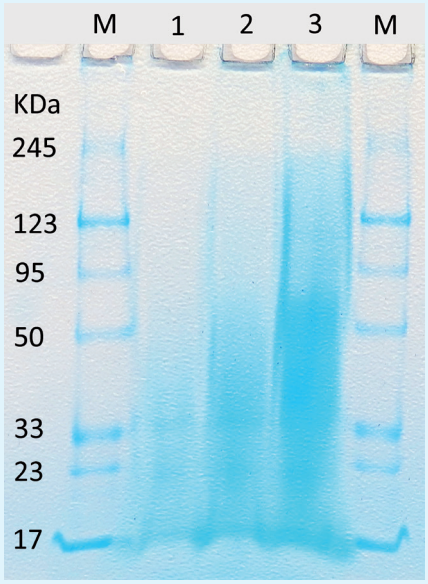

Fig. 7: Denaturing polyacrylamide gel electrophoresis (PAGE) of fibrin solubilized by a steam sterilization step (121 °C, 20 min). M: Molecular size marker 17-245 KDa, 1: 5 µl of a fibrin solubilizate (not quantified), 2: 10 µl of the fibrin solubilizate, 3: 20 µl of the fibrin solubilizate.

3.4 Qualitative characterization of solubilized fibrin soils

Polyacrylamide gel electrophoresis (PAGE) was carried out to gain an insight into the size of the fibrin fragments after elution, see Fig. 7. The size of the solubilized fibrin fragments generated by thermal treatment ranged across a broad spectrum between approx. 17 KDa and approx. 230 KDa. The majority of the fragments were detected in the range approx. 20–70 KDa. The thermal treatment during elution did not cause total hydrolysis of the fibrin. Protein fragments of various sizes were detected which indicated that partial hydrolysis had taken place. However, this di not have a relevant effect on protein quantification by the OPA and BCA method, see 3.3.

3.5 Constancy of the fibrin quantity on PCDs under repeatability and comparative conditions

To ascertain whether F-PCDs with comparable fibrin quantities could be generated in the various laboratories using a single original blood batch, five participating laboratories obtained sheep blood from a single blood collection batch. The protein content of the employed blood batch was measured by all laboratories with the OPA method and was 135 ± 5.56 mg / ml (n = 16, in each case, single or duplicate testing in the five laboratories). The first batch of F-PCDs (batch 1) was produced within 24 h of delivery or within 48 h of collecting the sheep blood. A second unopened bottle of sheep blood from the same blood collection batch was cooled in the respective laboratories (4–6 °C) while swirling daily. After storing for seven days a further batch of F-PCDs (batch 2) was produced. The protein content of the batch 1 and batch 2 F-PCDs was determined following solubilization with the OPA method; the results are illustrated in Table 1. For the F-PCD (n = 84) investigated in the five laboratories a mean protein content of 686 ± 156 µg / F-PCD was measured, with the maximum content being 984 µg / F-PCD and the minimum content 408 µg / F-PCD. No systematic increase or decrease in the measurable protein content was detected when storing the blood for one week before production of the F-PCDs. The fluctuations observed were ascribed to the precision of the protein quantification method applied in the respective laboratories.

3.6 Comparative evaluation of cleaning based on blood process challenge devices (B-PCDs)

Four laboratories (Lab. A-D) took part in a multi-centre trial for comparative evaluation of cleaning based on blood process challenge devices (B-PCD). After an exposure time of 5, 10 and 20 min, PCDs were withdrawn and inspected for their residual protein content. Laboratories A, B, C conducted two independent series of experiments with in each case n = 3 B-PCDs per measuring time point (total n = 6), while laboratory D carried out a single experimental series with n = 3 B-PCDs per measuring time point. Quantification of the residual protein content was always performed with the OPA method. It was not possible to present the standard deviations for the results of the various laboratories since only the arithmetic means of the residual protein content were reported by the laboratories.

The untreated control PCDs (0 min) had a protein content of between 8.430 and 13.682 µg / B-PCD. To standardize the results, the residual protein content measured at the different time points was compared to that on the untreated control PCDs (100 %) and the percentage of residual protein content was given. The results of the cleaning experiments obtained on using WSH, test detergent 1 and 2 (TR-1, TR-2) are shown in Fig. 8A, B, C. The results of the participating laboratories were summarized and the arithmetic mean of the residual protein content calculated for the respective measuring time points; the

results are presented as kinetic graphs in Fig. 8D.

Following 5 min treatment time a residual protein content of 11.5 ± 1.9 % was measured for WSH and 6.7 ± 4.3 and 6.8 ± 2.9 % for TR-1 and TR-2, respectively. After 20 min the residual protein content for treatment with WSH was 6.1 ± 2.9 % and for treatment with TR-1 and TR-2 it was 1.7 ± 0.8 % and 3.5 ± 1.8 %, respectively. It was not possible to distinguish between the cleaning efficacy of WSH, TR-1 and TR-2 because all three demonstrated comparative ability to remove the blood soils.

3.7 Comparative evaluation of cleaning based on fibrin process challenge devices (F-PCDs)

Six laboratories (laboratories A-F) took part in an initial multi-centre trial using the newly developed F-PCDs. The cleaning efficacy of WSH, TR-1 and TR-2 was compared. Due to the difficulty in effectively removing the fibrin soil the measuring time points were extended to 30 min. Laboratories A, B, E and F each carried out one experimental series with, in each case, n = 3 F-PCDs per measuring time point, laboratory D used n = 4 F-PCDs per measuring time point, laboratory C used for tests with WSH n = 2 F-PCDs /measuring time point and for the investigations with TR-1 and TR-2, in each case, n = 3 F-PCDs /measuring time point. The newly developed elution method with a steam sterilization step was used for elution of residual soils from the F-PCDs. As agreed with all laboratories, quantification of the residual protein was performed with the OPA method, and the measured residual protein content was referenced to the BSA standard protein, type V.

The untreated control PCDs (0 min, in total n = 56) exhibited a mean protein content of 625 ± 175 µg (minimum: 223 µg, maximum 962 µg) / F-PCD. To standardize the results, the residual protein content measured at the different time points was compared to that on the untreated control PCDs (100 %) and the percentage of residual protein content was given. The results of the cleaning experiments obtained on using WSH, test detergent 1 and 2 (TR-1, TR-2) are shown in Fig. (9A, B, C. The results of the participating laboratories were summarized and the arithmetic mean of the residual protein content calculated for the respective measuring time points; the re-

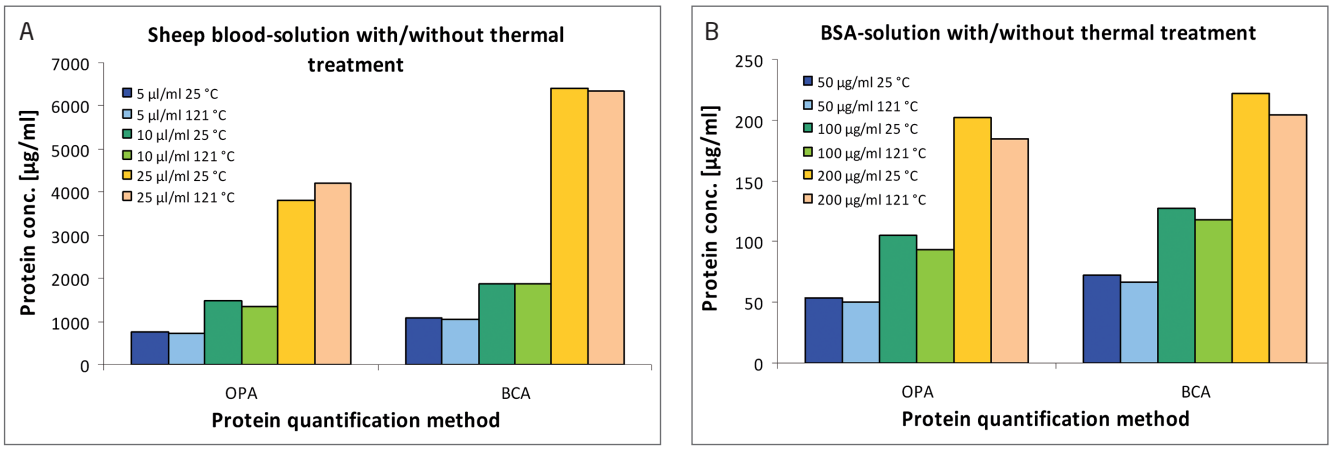

Fig. 6: Comparative investigation of the influence of thermal treatment (121 °C, 20 min) compared with Incubation at room temperature (25 °C) on protein concentrations measurable with the OPA and BCA method of different sheep blood solutions (A) and BSA solutions (B) (in each case, n = 1).

Laboratory	A		B		C		D		E	
Batch	1	2	1	2	1	2	1	2	1	2
n	9	9	9	9	9	9	9	9	6	6
Mean [µg / F-PCD]	884	856	520	567	624	844	726	722	492	495
SD [µg / F-PCD]	59.2	33.3	39.0	24.8	111	52.9	94.0	73.3	27.6	11.0
Percent SD [%]	6.69	3.89	7.49	4.38	17.8	6.27	12.9	10.1	5.61	2.22

Tab. 1: Investigation of the constancy of the fibrin quantity on F-PCDs. Laboratories A, B, C, D and E produced two F-PCD batches (batch 1 und 2), each comprising 6-9 PCDs. The protein content was quantified with the OPA method following solubilization of the fibrin soil. The number of F-PCDs (n) investigated, the arithmetic mean (mean), standard deviation (SD) and percent standard deviation (percent SD) were specified by each laboratory for the protein content measured on the various F-PCDs.

sults are presented as kinetic graphs in Fig. 9D.

Differences were identified in the cleaning efficacy between WSH, TR-1 and TR-2 as from a cleaning time of 10 min; here the percent residual protein content for WSH cleaning was 81.8 ± 8.9 % (n = 18), for TR-1 it was 59.9 ± 7.5 % and for TR-2 it was 26.7 ± 23.0 % (in each case n = 19). For TR-2 a further reduction of the residual protein content to 5.7 ± 5,8 % was observed for longer cleaning times (30 min), whereas when cleaning with WSH and TR-1 only a minor reduction was noted in the residual protein content, see Fig. 9D.

To further standardize the fibrin content on the F-PCDs and identify the influence exerted by different storage periods on the sheep blood test soil, the working group carried out one additional multi-centre trial. To that effect, laboratories A – F obtained heparinised sheep blood from a single blood collection batch. The first batch of F-PCDs was produced within 24 h of delivery of the sheep blood. A second unopened bottle of sheep blood from the same blood collection batch was cooled in the respective laboratories (4 – 6 °C) while swirling daily. After storing for seven days a further batch of F-PCDs was produced. The laboratories conducted comparative cleaning experiments with batch 1 and batch 2 F-PCDs with WSH, TR-1 and TR-2. For each measuring time point (0, 5, 10, 20, 30 min) and each PCD batch, n = 3 F-PCDs were investigated.

Since laboratories D and E used a batch of TR-2 beyond the use-by-date, these two laboratories repeated the cleaning experiments with F-PCDs that had been prepared from a different blood batch. The consistency of the results was assured by the use of controls (cleaning experiments with WSH) and concordant results.

The untreated control PCDs (0 min, in total n = 36 F-PCD) exhibited a protein content of between 487 and 915 µg / F-PCD when referenced to the respective production batch (arithmetic mean of n = 3 F-PCDs). The standardized results of cleaning experiments using WSH, TR-1 and TR-2 are presented in Fig. 10A, B, C. The results of the participating laboratories were summarized and the arithmetic mean of the residual protein content calculated for the respective measuring time points; the

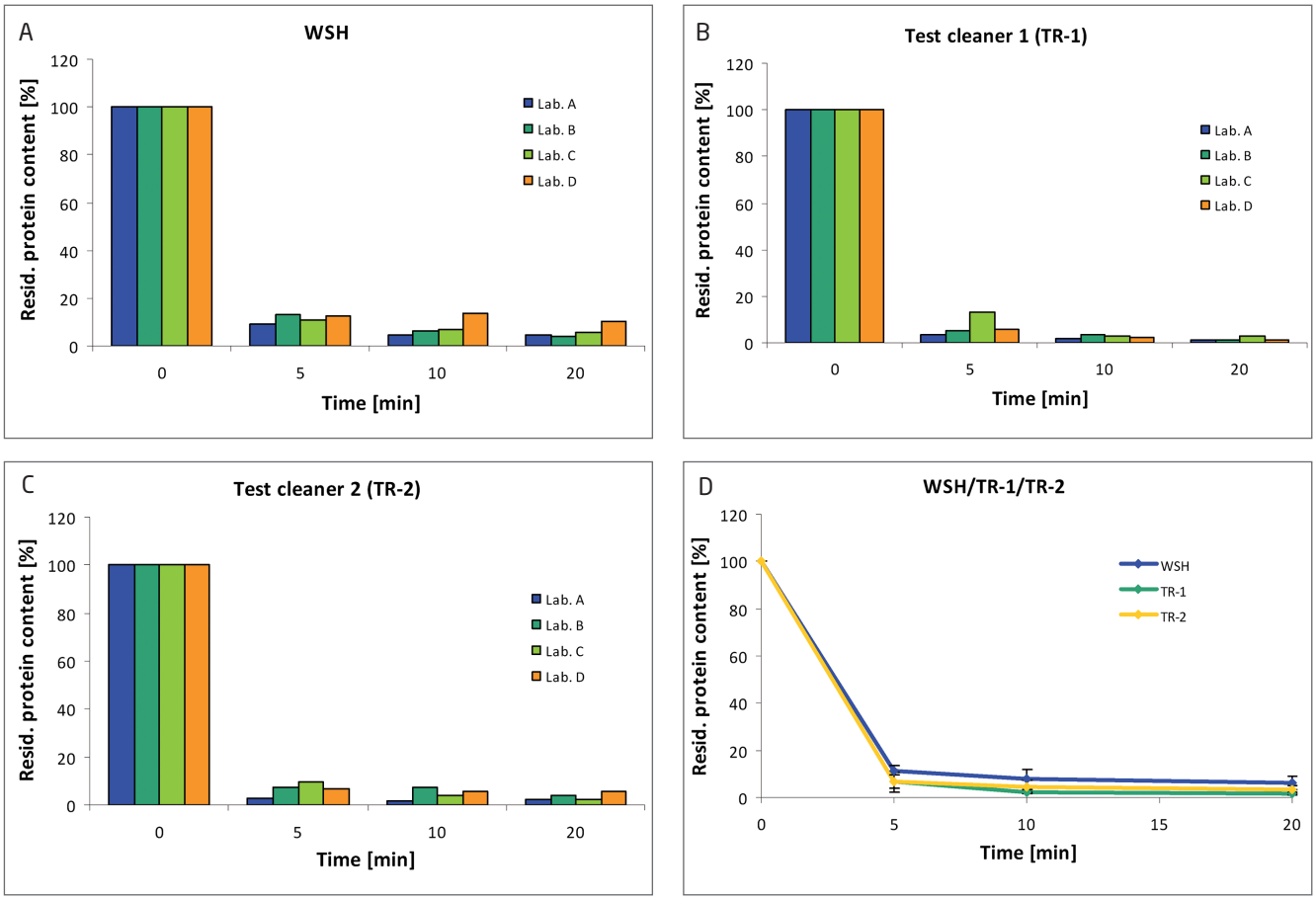

Fig. 8: Cleaning experiments in WSH and two test detergents using PCDs with reactivated blood soils (B-PCDs) in four laboratories (Lab. A-D). The PCDs were exposed to the detergent solution for different periods of time (5, 10, 20 min) and the residual protein content (blood residues) was measured with the OPA method after elution. Laboratories A-C carried out two series of experiments with, in each case, n = 3 B-PCDs / measuring time point, while laboratory D conducted one experimental series with, in each case, n = 3 B-PCD / measuring time point. The residual protein content is given as a percentage value and was referenced to the untreated controls (0 min). A: Cleaning in WSH; B: Cleaning in test detergent 1 (TR-1); C: Cleaning in test detergent 2 (TR-2); D: The residual protein content values reported by the laboratories were used to calculate the mean residual protein content (error bar: standard deviation, SD).

results are presented as kinetic graphs in Fig. 10D.

Showing good concordance with the previous multi-centre trial, already after a 10-min cleaning time clear differences were identified in the residual protein content measured for WSH, TR-1 and TR-2, albeit in this multi-centre trial, too, overlapping standard deviations were noted for the different products. For a cleaning time of 20 min an even more pronounced difference was observed between the three products compared with the shorter cleaning times. No differences were seen when using F-PCDs produced from fresh blood (batch 1) versus blood stored for one week (batch 2). Only on testing with WSH differences were discerned for the 10 min measuring time point but these fell within the standard deviation range of the other experimental series.

3.8 Comparative evaluation of the cleaning performance based on the TOC method

Exemplary investigations carried out in one laboratory which used the TOC method for quantification of fibrin residues revealed that untreated F-PCDs of the employed batch exhibited a carbon content of 782 µg (NPOC method). In cleaning experiments using TR-1 and a cleaning time of 30 min the mean residual carbon content (n = 3) was 446 µg / PCD or 57.0 %, thus showing good concordance with the residual protein content measured with the OPA method (57.4 % for batch 1 and 56.2 % for batch 2, Fig. 10).

4. Discussion

4.1 Blood test soil

The definition of suitable and generally accepted test soils for the evaluation of cleaning processes for medical devices is a challenge for which no solution has been found to date. For example, in DIN ISO/TS 15883-5:2006 [13] 19 different test soils and test methods are listed for the evaluation of automated reprocessing processes. Besides, there are many other commercially available test sys-

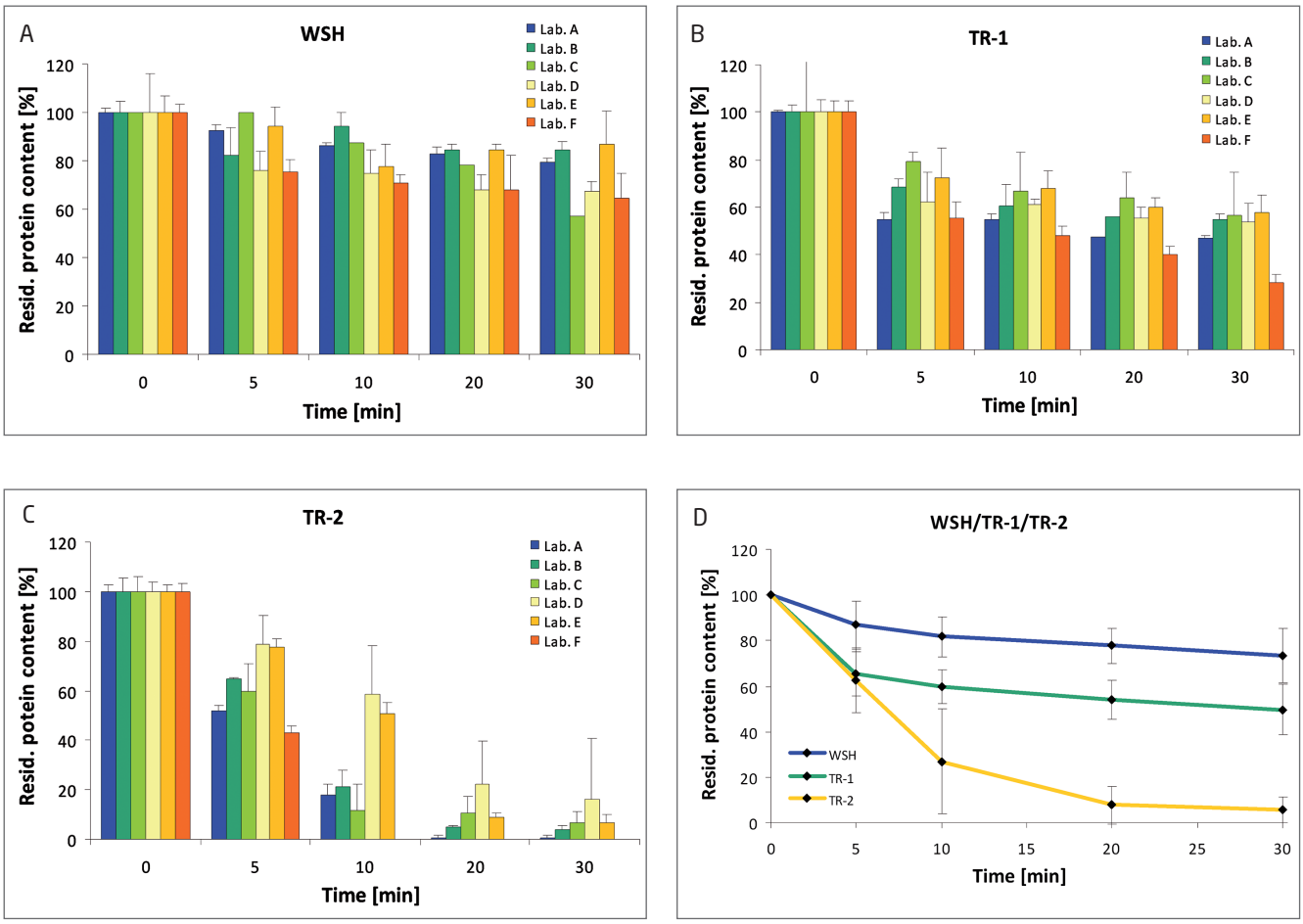

Fig. 9: Cleaning experiments with WSH and two test detergents using PCDs with fibrin soil (F-PCDs) in six laboratories (Lab. A – F). The PCDs were exposed for different periods of time to the detergent solution (5, 10, 20, 30 min) and the residual protein content (fibrin) was measured with the OPA method after elution. Laboratories A, B, E and F conducted one experimental series with, in each case, n = 3 F-PCDs / measuring time point, laboratory D used n = 4 F-PCDs / measuring time point, laboratory C used to test with WSH n = 2 F-PCDs / measuring time point, otherwise n = 3. The measured residual protein content was given as a percentage value and was referenced to untreated controls (0 min). A: Cleaning in WSH; B: Cleaning in test detergent 1 (TR-1); C: Cleaning in test detergent 2 (TR-2); D: The residual protein content values reported by the laboratories were used to calculate the mean residual protein content (error bar: standard deviation, SD).

tems based on other test soils reported to be more or less easy to remove during cleaning processes [23–25].

These test soils and methods are used for qualification of automated cleaning processes in which, as per Sinner's circle, the Mechanics factor is generated by the washer-disinfector, which plays a crucial role in the automated process outcome. Therefore, such test soils and methods do not lend themselves to comparative evaluation or qualification of the Chemistry factor, i.e. the efficacy of a detergent alone [26].

The DIN *ad hoc* group (NAMed 063-04-09) published in 2008 [14] and 2009 [15] the results of their attempts at developing a test method for the evaluation of the cleaning efficacy of instrument detergents without, or with only negligible, mechanical action. To that effect, they used an experimental setup consisting of an immersion bath in which flow was generated by means of a magnetic stirrer (350 rpm) for macroscopic enhancement of diffusion processes but without any mechanics as understood in the context of Sinner's model. In multi-centre trials it was demonstrated that reproducible and comparable removal of reactivated diluted sheep blood test soil was possible [14, 15]

Reactivated, i.e. coagulated, sheep blood is used as test soil so that the test results can be directly applied to real instruments harbouring human blood, which is the most common soil on surgical instruments. Furthermore, blood is particularly difficult to remove since after exposure to heat (> 55–60 °C) or denaturing chemicals (e.g. disinfectant substances such as peracetic acid, aldehydes, quaternary ammonium compounds in combination with amines [27]), blood residues are extremely difficult to remove. That is why reactivated sheep or horse blood is specified as surrogate for human blood soils on surgical instruments and other medical devices in the corresponding guidelines as well as in DIN ISO/TS 15883-5, Annex A, E, I and M.

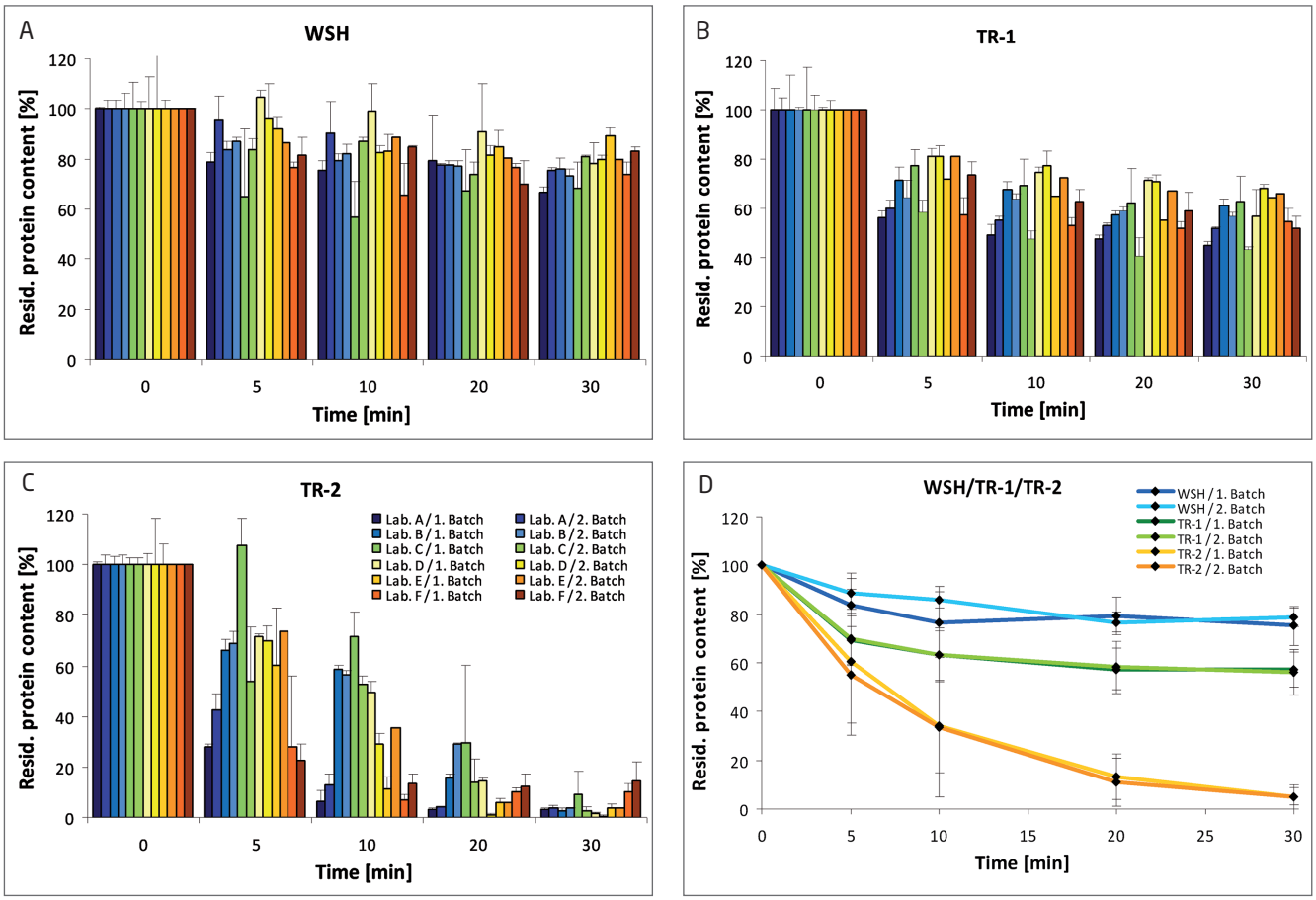

Fig. 10: Cleaning experiments in WSH and two test detergents using PCDs with fibrin soil (F-PCD) in six laboratories (Lab. A-F). Two F-PCD batches were produced; batch 1 was prepared with fresh sheep blood within 24 h of delivery, while batch 2 was made with sheep blood that had been stored for a week in a refrigerator. The PCDs were exposed to the detergent solution for different periods of time (5, 10, 20, 30 min) and the residual protein content (fibrin) was measured with the OPA method after elution. Laboratories A-F conducted one experimental series with, in each case, n = 3 F-PCDs / measuring time point for each PCD batch (1, 2). The residual protein content is given as a percentage value and was referenced to the untreated controls (0 min). A: Cleaning in WSH; B: Cleaning in test detergent 1 (TR-1); C: Cleaning in test detergent 2 (TR-2); D: The residual protein content values reported by the laboratories were used to calculate the mean residual protein content (error bar: standard deviation, SD)

4.2 Blood constituents

The sheep blood test soil consists of, inter alia, the corpuscular (cellular) constituents erythrocytes (red blood cells), leukocytes (white blood cells) and thrombocytes (platelets) with, based on the reference range, a haematocrit of 30 – 38 % (v / v). The number of erythrocytes is between 6.5 – 11.3 x 10^{12} / l; these are full of haemoglobin (red pigment). Based on the reference table [28], the haemoglobin content of sheep blood is 87 – 128 g / l. Based on the mean protein concentrations as per the reference range, haemoglobin accounts for 70 % of the entire blood protein content and is thus the major protein constituent of blood.

Drying of blood soils and incubation in demineralized water can cause destabilization of the erythrocyte membranes and bursting of the cells because of the changes in osmotic conditions versus the normal physiological conditions. Likewise, the effect of numerous surfactants leads to membrane destruction and release of haemoglobin from the erythrocytes. Haemoglobin solubility in water at 50 % [29] is extremely high and explains the rapid release of large amounts of protein from blood soils by aqueous media [14, 15, 23, 24, 25, 30, 31]. That effect was observed by all multi-centre trial participants. Virtually identical cleaning kinetics was observed regardless of whether an alkaline or enzymatic detergent or just WSH was used. Hence blood soils, and B-PCDs, proved unsuitable for distinguishing between the cleaning efficacy of water and detergents.

4.3 Fibrin

Apart from its corpuscular constituents, blood plasma contains other proteins (albumins, globulins, fibrinogen, coagulation factors), ions and transported substances (amino acids, carbohydrates, fats, etc.). The total protein content of sheep blood is composed of the haemoglobin content (87 – 128 g / l blood), the protein content of blood serum (55 – 75 g / l serum) and the fibrinogen content (1.8 – 7.2 g / l plasma) [32], amounting to a total protein content of 122 – 186 g / l for sheep blood. The fibrinogen content is 1.1 – 5.0 g / l sheep blood. The values are similar for human blood at 1.0 – 2.6 g / l blood.

Fibrinogen (human coagulation factor I) is a protein complex composed of three different fibrinogen subunits (α, β, γ). The entire human heterohexamer consists of approx. 3410 amino acids and has a mass of 340 KDa [33]. In the native protein complex two α, two β and two γ subunits are arranged such that the amino ends of the individual subunits are at the centre of the complex and are connected to each other by means of a disulphide ring [33,34,35]. This centre with the amino ends is known as the E-domain. The carboxyl ends of proteins face outwards forming the D-domain. The heterohexamer has 29 disulphide bridges (bonds), three high-affinity as well as ten low-affinity binding sites for Ca^{2+} [35].

Fibrinogen cleavage into fibrin, macroscopically visualized as blood coagulation, and covalent coupling of infinite numbers of fibrin units is triggered by a complex cascade of reactions involving the coagulation factors II to XIV [36]. In sum, this results in cleavage of four small peptides (in each case two fibrinopeptides A and B) from the E-domain, thus exposing four new amino ends via which intermolecular isopeptide bonds are formed with other fibrin molecules [35]. This in turn leads to cross-linking of high molecular fibrin now present as a gel-like coagulum and enclosing the corpuscular blood constituents (e.g. erythrocytes). This is the basis for the physiologic function underlying wound closure of damaged blood vessels.

Covalent bonding of fibrin units gives rise to a macromolecule of extremely high molecular weight which under physiological conditions is not soluble in water and thus makes high demands on cleaning processes [30, 31]. Furthermore, systematic studies of cleaned instruments and PCDs involving ^{99m}Tc labelled test soils revealed that high molecular weight fibrin residues cannot be completely solubilized by conventional elution methods and cannot at present be comprehensively quantified with established methods [31, 37].

4.4 Fibrin process challenge devices (F-PCDs)

When producing the F-PCDs all water-soluble components (haemoglobin, albumins, globulins, etc.) were removed by means of the dehaemoglobinization step provided that these were not found adhering to the surface of the fibrin fibrils. That left a hydrogel composed of swollen fibrin that is stable in demineralized water or WSH over more than four weeks with no further changes to the protein content (data not presented). A drying step was incorporated after dehaemoglobinization to facilitate storage of the fibrin PCDs without the risk of microbial contamination. That led to de-swelling ("dehydration)" of the fibrin hydrogel and, in turn, to compression (compaction) of the fibrin layer and stronger adhesion (data not presented) to the steel plate surface. De-swelling of the fibrin hydrogel is not fully reversible, i.e. even incubation of dried F-PCDs in demineralized water or WSH no longer causes the initial extent of swelling assumed to have been caused by the collapse of the microstructure following bursting of the enclosed erythrocytes and release of the cell constituents.

4.5 Fibrin content

The coagulation reaction leading to fibrin polymerization is a complex process triggered by a cascade of different factors (II to XIV). Besides, numerous other factors such, as e.g. the age of the blood, temperature and oxygen content, seem to play a role. In parallel experiments carried out by five laboratories for the production of fibrin PCDs based on sheep blood from a single original batch, it was possible to obtain within each laboratory PCDs with a reproducible fibrin content and homogeneous fibrin distribution. The mean standard deviation for the various PCD batches (n = 6 – 9) in the laboratories was 7.74 %, which was deemed acceptable. On producing a second PCD batch with the same blood that had been stored for one week most laboratories obtained similar fibrin contents. However, due to the different undefinable factors and influences coming into play during the coagulation reaction in the various laboratories, it was only possible to produce PCD batches with variable fibrin contents when comparing the laboratories with each other. The mean fibrin quantity was 686 µg / F-PCD (n = 84) with a standard deviation of 156 µg / F-PCD, corresponding to a percent standard deviation of 22.7 %. Based on these findings it was possible to stipulate that for the cleaning experiments PCDs from a single original batch had to be used and that a sufficient number of un-

treated controls had to be included in the experimental series. Furthermore, it was stipulated that the cleaning experiments should not be evaluated on the basis of the quantifiable fibrin residue amount but on the percentage referenced to the baseline value of the untreated controls.

4.6 Fibrin solubilization

For quantitative measurement of high molecular weight fibrin with conventional protein quantification methods (OPA, BCA method) it is imperative that the residues be converted to a dissolved form. Likewise, for cleavage of high molecular weight fibrin right down to the individual amino acids aggressive conditions, such as e.g. 26-hour incubation in 6 M HCl at 110 °C (excess pressure) [38], are needed. Experiments undertaken by the *AG RMT* participants demonstrated that incubation in alkaline solutions (e.g. 1 % SDS solution, pH = 11) at 80 °C for more than two hours assured at least partial dissolution of the fibrin network on the F-PCDs. However, that elution method did not prove to be sufficiently reproducible in the multi-centre trials, hence to expedite elution the incubation temperature was increased to 121 °C through the use of steam sterilization processes. Since this involved closed technical systems, evaporation of the water phase was minimized in the elution tests with no noticeable change in volume. Fibrin solubilization from the F-PCD was achieved in a quantitative and reproducible manner by all laboratories participating in the multi-centre trials, with no evidence of visible fibrin residues on the treated PCDs after staining with Amido Black.

The chemical processes taking place during thermal elution of high molecular weight fibrin are currently not fully understood. While already a long time ago it was reported that fibrin preparations [39] were solubilized by SDS as well as by the cationic surfactants CTAB, MTAB, HTAB and CPC at 37 °C or that fibrin was solubilized [40] at pH values above 9.5 in combination with 8 M urea and 1 M SDS at 45 °C, thought to be due to cleavage of the intermolecular disulphide brides, there are no conclusive findings regarding the mechanisms involved. Our own attempts by the *AG RMT* aimed at characterization of the solubilized fibrin by means of denaturing protein acrylamide gel electrophoresis (PAGE) demonstrated that thermal elution led to the release of a broad range of protein sizes, with a maximum of approx. 20 – 70 KDa. No distinctive protein bands indicative of released fibrin chains (α, β, γ) or fibrinopeptide A or B [41] were observed. The uneven size distribution may also be indicative for the presence of other soluble protein constituents from the plasma, since studies by Friedrich *et al*. [37] had already reported stabile adsorption of ^{99m}Tc-labelled human albumin macroaggregates to fibrin. Comparative investigations by the *AG RMT* aimed at studying the influence of thermal treatment on sheep blood as well as on bovine serum albumin (BSA) providing no evidence of significant hydrolysis, i.e. cleavage of the proteins present leading to a relevant change in the number of BCA-sensitive peptide bonds or free OPA-sensitive α- and ε-amino acids. Quantification of the solubilized fibrin can also be performed in principle with the TOC method. Quantitative sampling for TOC measurement is also possible using 0.001 N NaOH solution and a steam sterilization process. Valid results showing good concordance with those of the cleaning experiments conducted with the OPA method were also obtained on using the NPOC method for TOC measurement for evaluation of the cleaning efficacy.

4.7 Evaluation of instrument detergents

The newly developed test model based on the use of a dried and long-term stable fibrin layer as test soil permits for the first time evaluation of the performance of instrument detergents based on quantitative determination of fibrin residues that hitherto could only be qualitatively detected. For solubilization and quantification of the fibrin soil there must be no covalent cross-linking due to chemically reactive constituents in detergents. In any case, products with such protein-fixing effects would not be suitable for cleaning.

The cleaning experiments based on the use of WSH as control achieved a minor reduction by 23 % of the blood content after 30 min exposure. Here, it is thought that it was possible to remove other protein constituents of the blood soil adhering to the insoluble fibrin [37, 42], which could not be removed during the PCD production process (dehaemoglobinization in demineralized water) because of the different ionic composition and ionic strength. By contrast, a stronger reduction by 43 % of the protein content was achieved on using the cleaning experiments with the alkaline test detergent 1 (TR-1) at pH = 11.7 after 30 min. The enzymatic test detergent (TR-2) achieved an even greater reduction of the protein content by 95 %, see Table 2. Whereas already after 5 min treatment time it was possible to differentiate between WSH and detergents (TR-1/TR-2) based on reduction of the fibrin content (Fig. 9, 10), only after a 10 min treatment time or longer was it possible to distinguish be-

Detergent	Arithm. mean [%]	SD [%]	Minimum [%]	Maximum [%]
WSH	22.7	6.2	11.1	33.6
TR-1	43.2	8.0	32.1	56.8
TR-2	94.9	4.2	85.3	99.4

Tab. 2 : Reduction of the percent protein content after 30 min treatment of the F-PCDs (based on the findings of the multi-centre trial illustrated in Fig. 10, number of laboratories: 6, number of experimental series per laboratory: 2, number of F-PCDs per experimental series and per measuring time point: n = 3, in sum n = 36 F-PCDs per detergent).

tween the alkaline and enzymatic detergents.

Test detergent 1 (TR-1) was employed in the experiments as a representative of alkaline detergents recommended in the KRINKO/BfArM Recommendation*. This was not able to dissolve and remove the fibrin soil since under the given conditions cleavage of the isopeptide bonds and disulphide bridges/bonds was not possible. Enzymatic detergent formulations containing proteases are able to hydrolyze the high molecular weight fibrin network leading to complete removal through formation of low molecular weight and highly water-soluble peptides. However, depending on the reaction kinetics of the proteases contained in the detergent longer exposure times are needed. As demonstrated in multi-centre trials, the new test model permits not only evaluation of the efficacy of neutral to alkaline detergents but also evaluation of the proteolytic activity of enzymatic detergents. The results obtained by the up to six participating laboratories in several multi-centre trials show the very good comparability and reproducibility of the new test method.

4.8 Future studies

The current and future activities of the *AG RMT* are focused on investigation of the cleaning efficacy of a broader range of different commercially available detergents in order to, through definition of acceptance criteria, evaluate the suitability of instrument detergents. As commissioned, future work assignments will entail investigation and evaluation of the cleaning efficacy of disinfecting detergents or disinfectants with cleaning properties.

4.9 Implications for assessment of instrument reprocessing processes

The *AG RMT* studies have also helped to gain a better understanding of comprehensive measurement of the residual protein content on (instrument) surfaces following cleaning processes. For the first time it has been possible to develop a method for complete solubilisation of high molecular weight and water-insoluble fibrin residues as well as for reproducible quantification using the conventional OPA and BCA methods. Fibrin residues are known to persist as residues on instruments [24, 27, 30, 31] following inadequate reprocessing processes. Hitherto, the sampling methods needed for comprehensive quantification were not available, hence it must be assumed that when assessing the residual protein content on real instruments, the residual level of contamination might be underestimated.

*KRINKO/BfArM Recommendation: Hygiene requirements for processing medical devices, jointly compiled by the Commission for Hospital Hygiene and Infection Prevention at the Robert Koch Institute (KRINKO) and the Federal Institute for Drugs and Medical Devices (BfArM)

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